Development of an Analytical Method for Kasugamycin Residue in Herbal Medicine, *Achyranthes japonica* Nakai

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Abstract: *This study developed a suitable analytical method for kasugamycin residues in* Achyranthes japonica *using LC/MS/MS equipped with an amide column for polar substances. Extraction and cleanup processes were done at pH 4.5–5. Purification efficiency was assessed and confirmed step by step by selecting silica, hydrophilic-lipophilic balance (HLB), strong cation exchange (SCX), and double (HLB and SCX) cleanup SPE cartridges. The results indicated that silica SPE cartridge exhibited overloading tendency, while HLB SPE cartridge had low cleaning efficiency. Among SPE cartridges used, double cleanup and SCX were found sufficient with respective matrix effects of –15% and +14%, respectively. The LOD and LOQ were 0.008 ng and 0.04 mg/kg, respectively. The correlation coefficient (R^2) was higher than 0.99, recovery rate ranges were 86.3–97.2%, and the RSD was below 8.8%. All methods are consistent with the Codex guidelines criteria. This study developed an appropriate LC/MS/MS analytical method for kasugamycin residue analysis in A. japonica with optimized, efficient extraction and purification conditions using a single SCX SPE cartridge, which is simple and time-efficient. In addition, the HLB and SCX SPE cartridges of the double cleanup methods were identified as primary methods that can be applied for the cleanup of other medicinal herbs.*

Keywords: *LC-MS/MS analysis; Kasugamycin residues;* Achyranthes japonica*; SPE-cartridges*

■ INTRODUCTION

Herbal medicine uses medicinal crops' dried roots and leaves for various purposes such as health promotion and disease treatment [1]. The herbal medicine *Achyranthes japonica* Nakai (Japanese chaff flower) is a perennial herb belonging to the Amaranthaceae family. *A. japonica* contains saponin and a large amount of inflammation-relieving ingredients; thus, it is widely used for arthritis and as a diuretic and tonic [2]. However, in the fall, *A. japonica* is frequently affected by a powdery mildew disease caused by *Albugo achyranthis* (P. Henn.). This disease could be controlled using a fungicide of copper sulfate basic, copper oxychloride with metalaxyl-m, and copper oxychloride with kasugamycin [3]. Among these, kasugamycin of the aminoglycoside family is a subject of social interest because it can have a potentially adverse effect on the treatment of diseases in humans and livestock due to the occurrence of antibiotic-resistant bacteria [4].

Kasugamycin, developed as an agricultural fungicide, is a highly polar substance with a Log P_{ow} value of –5.75 and is a dissociative compound with carboxy and amino groups in the molecule [5]. The pKa of the carboxylic acid (pKa1), primary cyclic amine (pKa2), and secondary amine (pKa3) are 3.23, 7.73, and 11.0, respectively, which is characterized by ionization of certain areas in various pH ranges [6]. The solubility of

kasugamycin is 228 g/L in water (pH 7, 25 °C) and 7.44 mg/L in methanol (25 °C). However, it is hardly soluble in most organic solvents. Moreover, kasugamycin is non-volatile and is physically and chemically unstable in heat; thus, its analysis process is difficult due to its low sensitivity during analysis [5]. According to previous studies, biological potency assays (cup, standard strain *Pseudomonas fluorescens*) and capillary electrophoresis (CE) were performed, but there were disadvantages in that the analysis time was long, and there were poor precision and selectivity [7]. Also, in the case of HPLC and GLC, which are often used for residual analysis, HPLC/UV showed low absorbance because there was no chromophore for ultraviolet or fluorescence, so there was a sensitivity problem. In addition, it has been reported that the time required for pretreatment is long, and reproducibility is low [5-6,8]. In particular, it has been reported that aminoglycoside-based components are challenging to analyze using GLC without the derivatization of amino and hydroxyl groups due to the hydrophilicity and non-volatile nature of the molecule [9].

On the other hand, studies that analyze kasugamycin on agricultural samples using LC/MS/MS have been conducted steadily recently, with excellent selectivity and sensitivity. Several reports confirmed using various SPE methods to remove interfering substances from samples and improve the sensitivity of kasugamycin. Many authors reported the purification of peppers and soil extracts using an MCX SPE cartridge [7,10]. A study of simultaneous analysis of agricultural fungicides using an HLB SPE cartridge and MCX SPE cartridge was reported. The simultaneous analysis of kasugamycin and validamycin-A through a consecutive cleanup process combined HLB SPE cartridge and SCX SPE cartridge as described in previous studies [8], and simultaneous analysis of kasugamycin and streptomycin in five vegetables was reported [11].

Furthermore, research conducted by the Korean Ministry of Food and Drug Safety (MFDS) to extract kasugamycin with methanol (pH 13) for five representative agricultural products, namely brown rice, soybeans, peppers, potatoes, and tangerines, purify it with an HLB SPE cartridge, and conduct analysis using LC/MS/MS was reported [12]. As described above, there are various established methods for analyzing kasugamycin residues in agricultural products in the previous studies; however, there is no report of residual analysis for kasugamycin in herbal medicines containing complex active ingredients. Therefore, this study aims to establish a residual analysis method for the kasugamycin in herbal medicine for the first time in this field by selecting *A. japonica* Nakai among herbal medicines with various pharmacological components. In particular, to minimize the interference effect that may occur due to the complex matrix in herbal medicine (*A. japonica* Nakai); by systematically comparing and analyzing the purification efficiency of various SPE cartridges, including extraction and distribution processes. Furthermore, the analysis method also was intended to confirm whether it conforms to the analysis criteria of pesticide residues of the International Food Standards Commission (Codex Alimentarius Commission, CAC/GL 40) and the Guideline on Standard Procedure for Preparation of Test Methods for Food [13] of the Korean MFDS.

■ EXPERIMENTAL SECTION

Reagents and Instruments

The standard product for the analysis of kasugamycin was bought from Sigma-Aldrich (USA) and had a purity of 98.7% or higher. The HPLC grade solvents used in the analysis process were water, dichloromethane, acetonitrile, and methanol, purchased from Thermo Fisher Scientific (USA). Formic acid (98.0%) and ammonia solution were purchased from Sigma-Aldrich (USA) and Daejeonghwageum (Korea), respectively. Also, other organic solvents and reagents were purchased and used for residue analysis. For purification, SPE cartridges, silica ((55 μm, 70 Å), 1 g) and SCX ((55 μm, 70 Å), 1 g) obtained from Phenomenex (USA), and HLB ((60 μm, 80 Å), 500 mg) purchased from Waters (USA) were used. For uniform extraction, a sonicator (Jeio Tech. Co. Ltd., Korea) was used for sample extraction, and a centrifuge (Allegra X-1R, Beckman Coulter Life Sciences, USA) was used for

centrifugation. In the purification process, a vacuum manifold (Visiprep SPE vacuum manifold, Supelco, USA) and vacuum pump (DOA-P704-AC, Gast, USA) were used for the SPE cartridge. Kasugamycin is highly soluble in water or methanol and ionized in wide pH ranges. The physicochemical properties and chemical structural formulas of kasugamycin are shown in Table 1 [12,14].

Procedure

Preparation of standard solution

A stock solution was prepared by accurately taking 10.13 mg of the standard compound (98.7%) of kasugamycin, placing it in a 100 mL volumetric flask, and then dissolving it in water to make 100 μg mL^{-1}. In the same way, the working solution was diluted stepwise to concentrations of 0.005, 0.008, 0.01, 0.05, 0.08, and 0.1 μg mL^{-1}. For the standard calibration curve for quantification, the matrix was included in a 1:1 ratio of the standard solution and the extraction solution to create a matrix-matched calibration curve. Since the target component is an aminoglycoside-based fungicide that has the property of adsorbing to glass, all containers used in the analysis were made of polypropylene [12].

Herbal medicine sample

The herbal medicine, *Achyranthes japonica* Nakai, was purchased from a pharmaceutical company (Iksan, Jeollabuk-do) that applied distribution according to the drug specifications and was used after verification and a sensory evaluation a herbal medicine expert. Since the roots are used as medicinal herbs, the dried roots were ground and passed through a 2 mm sieve, and then a 5 g sample was weighed and used for analysis.

Extraction and distribution

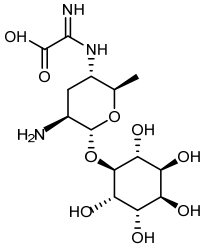
Ten mL of distilled water was added to 5 g of the dried roots sample, and the wetting process was performed for 1 h. The extraction solvent was used after adjusting the pH to 4.5–5 by adding 0.1% formic acid to methanol, methanol/water (5/5, v/v), and water. Sample extraction was performed by ultrasonic extraction using a sonicator for 30 min, followed by centrifugation for 10 min at 4 °C and 4,000 G. The liquid-liquid distribution process was carried out by taking 5 mL of the supernatant when using water in the extraction solvent and then adding 5 mL of dichloromethane.

Purification conditions using SPE cartridge

In this study, a comparative experiment was conducted using various SPE cartridges to establish the optimal purification conditions for kasugamycin in *A. japonica*. The SPE cartridges used during the experiment were silica, HLB, and SCX SPE cartridges. The purification conditions are as follows: In the SPE cartridge refining process, a vacuum manifold device equipped with a vacuum pump was used to allow elution at a flow rate of one to two drops per second to maintain a constant flow rate and shorten the time required.

Silica SPE cartridge purification conditions. After activating the filler, 3 mL of methanol and water was

Table 1. Physicochemical properties of kasugamycin

	Kasugamycin
IUPAC name	2-amino-2-[(2R,3S,5S,6R)-5-amino-2-methyl-6-[(2R,3S,5S,6S)-2,3,4,5,6-pentahydroxycyclohexyl]oxyoxan-3-yl]iminoacetic acid
Chemical structure	
Mol. wt.	379.4
V.p. (mPa)	$< 1.3 \times 10^{-4}$ mm Hg (25 °C)
K_{ow} logP	-5.75

added to the silica SPE cartridge (1 g), 1 mL of the extraction supernatant was adsorbed onto the cartridge. After that, 10 mL of water was sequentially discharged, and kasugamycin was eluted using 10 mL of methanol.

HLB SPE cartridge purification conditions. Hydrophilic lipophilic balance (HLB) was used after activating the filler with 3 mL of methanol and water in the SPE cartridge (500 mg). Then, 5 mL of the extraction supernatant was loaded into the cartridge; 5 mL of methanol was added and combined with the previously fractionated eluate to obtain a sample.

SCX SPE cartridge purification conditions. After activating the filler using 5 mL of water and methanol in the SCX SPE cartridge (1 g), 2 mL of the extracted supernatant was adsorbed to the cartridge. After that, 10 mL of water and 10 mL of methanol were sequentially discharged, and kasugamycin was eluted using 10 mL of methanol, to which 5% NH_4OH was added.

LC/MS/MS instrument analysis

The LC-20AXR series (Shimadzu, Japan) was used as an analytical instrument for HPLC, and the TSQ Quantum Ultra (Thermo Scientific, USA) was used for mass spectrometry. The columns used included an Imtakt C18 column (2 mm ID × 100 mm, 3.0 μm, USA), Waters Hydrophilic Interaction Liquid Chromatography (HILIC) column (3.0 mm i.d. × 100 mm, 3.5 μm, USA), and Xbridge amide column (3.0 mm i.d. × 100 mm, 3.5 μm, USA). The oven temperature was fixed at 35 °C. The mobile phase solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.4 mL/min, and the injection volume was 5.0 μL. The gradient conditions were set as the following: (1) 20% A and 80% B held for 1.0 min, (2) 60% A and 40% B for 4.0 min, (3) 90% A and 10% B for 2.0 min, (4) 20% A and 80% B for 0.5 min, and finally, (5) 20% A and 80% B for 2.5 min. The overall running time was 10.0 min. The MS/MS was analyzed by the multiple reactions monitoring (MRM) method using electrospray ionization (ESI) positive mode, a spray voltage of 4.0 kV, and capillary and vaporizer temperatures of 280 and 300 °C, respectively. The Ion sweep gas pressure was set to 1.5 Arb. Sheath gas pressure was 40 units, and Aux gas pressure was 30.0 units. The precursor and product ions were monitored (Table 2).

Test method validation

The established test method for kasugamycin was verified using the detection and quantitation limits of the device, the quantitative limit of the test method, linearity, accuracy, and repeatability. As for the instrument's detection limit, a concentration in which the signal-to-noise ratio (signal/noise, S/N) of the peak detected on the chromatogram was 3 or more, and the quantitative limit was 10 or more. The limit of quantification of the test method was calculated by taking into account the minimum detection amount, sample amount, test solution amount, and dilution ratio in instrument analysis (Eq. (1)). Linearity was evaluated by making a blank line using a matrix-matched standard solution and calculating the correlation of determination (R^2). The suitability, precision, and repeatability of the

Table 2. LC/MS/MS operating parameters for the analysis of kasugamycin

HPLC	LC-20AXR series (Shimadzu, Japan)		
Detector	TSQ Quantum Ultra (Thermo Science, USA)		
Column	① Imtakt C_{18} column (2 mm I.D. × 100 mm, 3.0 μm, USA) ② Waters HILIC column (3.0 mm I.D. × 100 mm, 3.5 μm, USA) ③ Xbridge amide column (3.0 mm I.D. × 100 mm, 3.5 μm, USA)		
Oven temp.	35 °C		
Scan events	Precursor ion	Product ions	CE
	380.093	111.993	32
		156.002	33
		199.963	33

above method were analyzed after adding a standard solution equivalent to the limit of quantification (0.04 mg/L) and the limit of quantitation 10 times (0.4 mg/L) to the untreated sample. The recovery rate and analysis error of three repetitions were verified.

$$A(ng)\times\frac{B(mL)}{C(g)}\times\frac{D(mL)}{E(mL)}\times\frac{F}{G(\mu L)}=MLQQ(mg/kg) \quad (1)$$

whereas A is the minimum amount of detection, B is the volume of extraction solvent, C is sample amount, D is the final volume, E is the supernatant amount taken from the extracted solvent, F is dilution factor, and G is injection quantity.

Matrix effect (ME) of the purification method

Matrix effect refers to suppressing or enhancing the component's ionization by affecting the analyte component's instrumental sensitivity due to the target sample [15]. Matrix effect calculation is determined by comparing the calibration curve of the standard solution with the calibration curve of the matrix-matched standard solution, and the formula is shown in Eq. (2). This study calculated the matrix effect after preparing a matrix-matched standard solution of 0.005, 0.008, 0.01, 0.05, 0.08, and 0.1 mg/kg using untreated samples extracted through each SPE purification process.

$$\text{Matric effect }(\%)=\left(\frac{\text{Slope of calibration curve matrix}}{\text{Slope of calibration curve solvent}}-1\right)\times 100 \quad (2)$$

■ RESULTS AND DISCUSSION

Establishment of LC/MS/MS Analysis Conditions

In this study, analysis conditions using LC/MS/MS with high sensitivity and high selectivity were established to analyze kasugamycin. For the LC/MS/MS instrument analysis, electrospray ionization (ESI) positive mode was selected, and precursor ion and collision energy were optimized using multiple reaction monitoring (MRM) mode. The parent compound was detected as $[M+H]^+$ in positive mode, and the precursor ion was selected as m/z 380.072. The product ion was selected as m/z 111.994, m/z 156.003, and m/z 199.964. The high intensity (m/z 156.003 and m/z 199.964) was selected for quantitation (Fig. S1).

Kasugamycin is a polar compound with a log P_{ow} value of –5.75 and high solubility in water or methanol. Besides that, this compound is ionized in a wide range of pH values [16-17]. Therefore, in this study, water and acetonitrile, commonly used to analyze aminoglycoside antibiotics, were selected as the mobile phase solvent, and 0.1% formic acid solution was added to improve the sensitivity and resolution efficiency of the target component [5,18]. Previous studies found that the sensitivity of the analyte is increased when a 0.1% formic acid solution is added to the mobile phase, while the sensitivity of the signal decreases when a concentration lower than 0.1% is added [8,19].

As a result of comparing and analyzing the resolution and sensitivity of kasugamycin through C18, HILIC, and amide columns for HPLC analysis, the C18 column was rapidly eluted without staying in the column due to the amino group of kasugamycin, and it was not easy to separate it from the interfering material, as shown in Fig. 1(a) [11,19]. The HILIC column acts effectively on polar basic compounds and aminoglycosides, but its retention time was short, and peak tailing was confirmed, which required improvement, as seen in Fig. 1(b) [20-21]. An amide column is a form in which polar amide groups are bound [7]. Nevertheless, it was reported that the retention time and sensitivity were excellent, and because of confirming this, sufficient retention time and sensitivity were secured, as shown in Fig. 1(c) [7,10,19]. Therefore, in this study, an amide column highly effective for kasugamycin was selected and analyzed.

Establishment of Extraction and Distribution Process

Dried samples showed low extraction efficiency in aqueous organic solvents, so the wetting process was essential. Thus, 10 mL of distilled water was added to the dried sample during analysis [22]. The extraction solvent was reported to increase the recovery rate when a small amount of acid was added during the extraction of kasugamycin; accordingly, the pH of the extraction solvent was maintained between about 4.5–5.5 using a formic acid solution [8]. Therefore, the recovery rates of

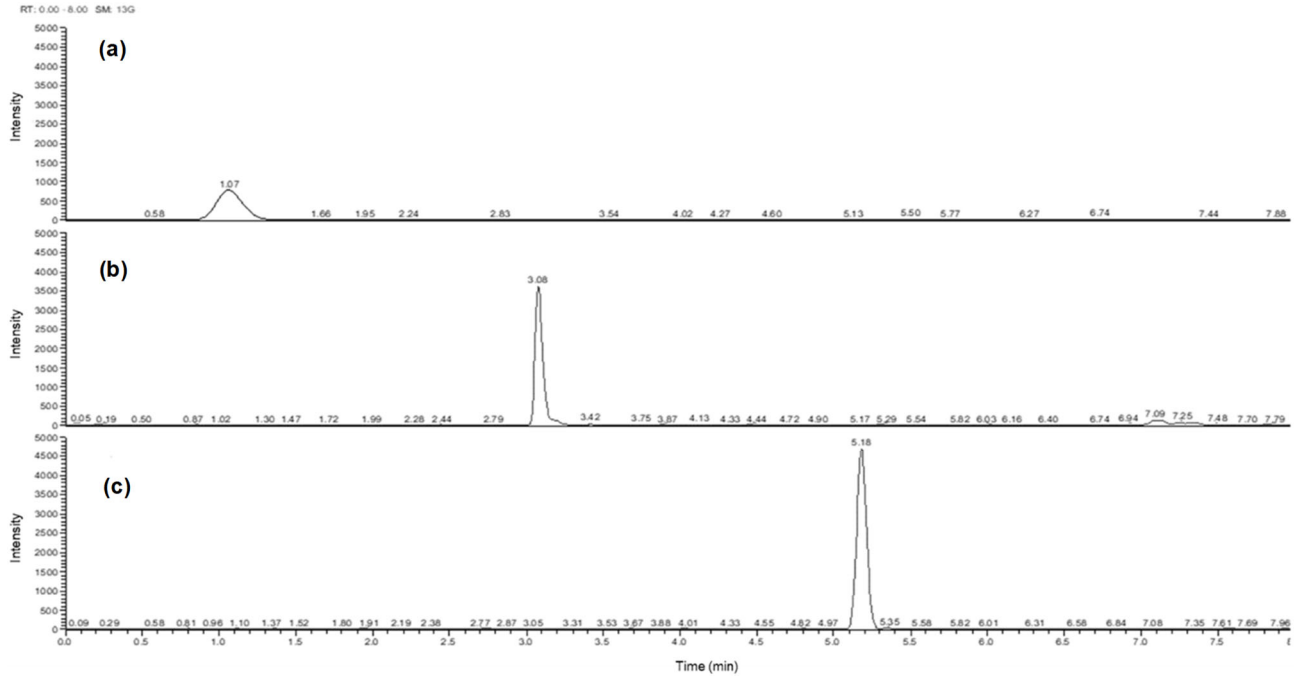


Fig 1. Chromatogram of 0.1 mg kg^{-1} kasugamycin standard solution compared by columns ((a): 0.1 mg kg^{-1} of kasugamycin using a C_{18} column, (b): 0.1 mg kg^{-1} of kasugamycin using HILIC column, (c): 0.1 mg kg^{-1} of kasugamycin using amide column)

methanol, methanol/water (5:5, v/v), and water were compared to select the optimal extraction solvent when the pH was maintained at 4.5–5. The recovery rates were 58.4%, 90.2%, and 96.3%, respectively. Water was selected as the final extraction solvent in% with the highest recovery rate (Table S1). The extraction of kasugamycin from dried samples using methanol and water was previously reported, and the recovery rate found increased as the water content increased [7]. Therefore, in this study, 10 mL of distilled water was added to the sample (5 g), followed by a wetting process for 1 h, and then extracted with 40 mL of water adjusted to pH 4.5-5 by adding 0.1% formic acid solution. After 30 min of ultrasonic extraction using a sonicator, centrifugation was performed at 4 °C and 4,000 G for 10 min. Even after the extraction process, a liquid-liquid distribution process using dichloromethane, an organic solvent with high specific gravity, was added to remove the non-polar interfering substances contained in the sample extracts. It was visually confirmed that suspended matter or precipitates in the actual extract were removed. In addition, since dichloromethane effectively removes a large amount of non-polar substances and substances of fat components, the above analysis method was determined to be effective in removing non-polar substances and interference substances fat components [7]. However, the results in Fig. 2 show that the effect of removing interference substances on the chromatogram was insufficient, so various types of SPE purification methods were attempted for further purification.

Solid-Phase Extraction (SPE) Cartridge Purification

Interfering substances continued to appear on the chromatogram even after the extraction and distribution process, and based on previous studies, a suitable SPE cartridge of kasugamycin was selected and compared. In this study, a silica SPE cartridge representing the adsorption principle as a normal cartridge was selected and analyzed for the smooth separation and analysis of the large amount of pharmacologically active substances contained in *A. japonica* and kasugamycin, and the HLB SPE cartridge analyzed for agricultural products by the

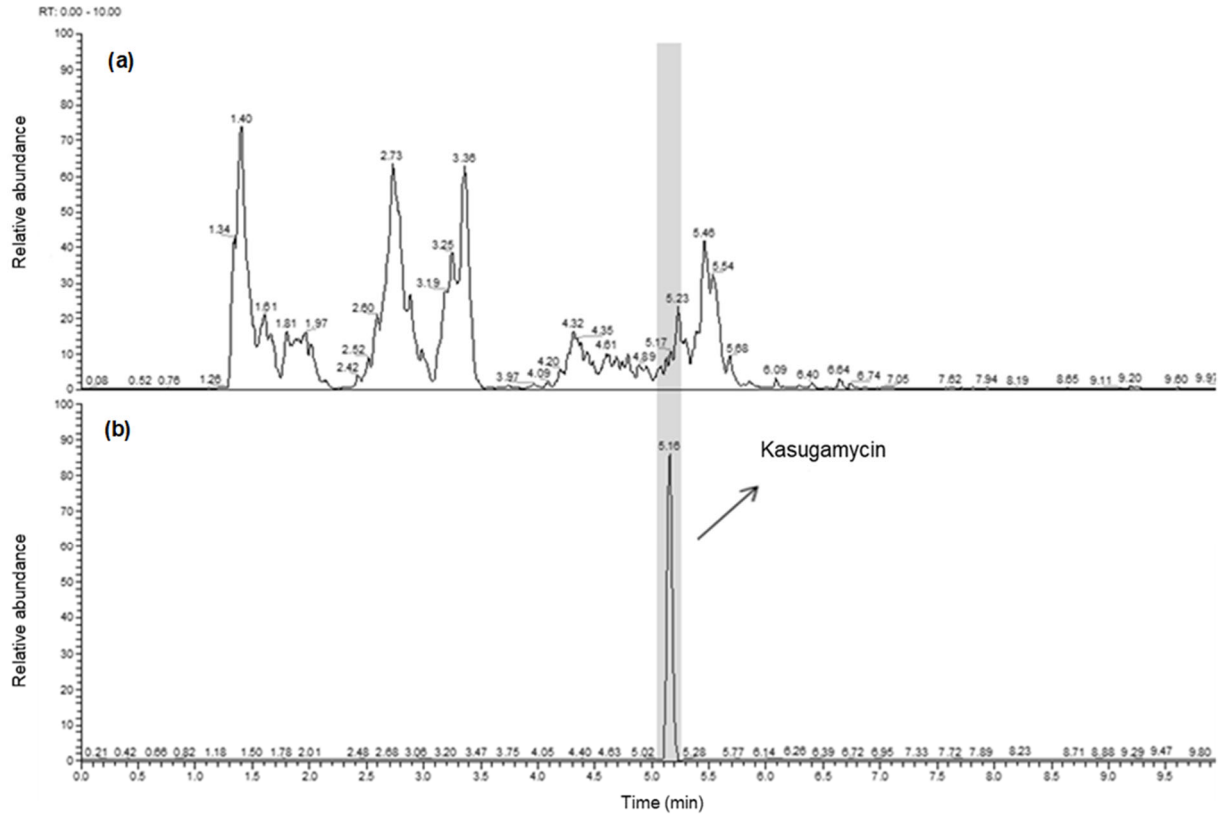


Fig 2. Chromatogram of liquid-liquid partitioning of kasugamycin in *Achyranthes japonica* Nakai and kasugamycin standard ((a): control, (b): kasugamycin 0.1 mg kg^{-1})

MFDS. As a result, it was confirmed that it could be applied to the herbal medicine *A. japonica* [12]. In addition, when analyzing kasugamycin, the SCX and SCE SPE cartridges were selected and analyzed as recommended by Zhang et al. [8].

Silica SPE cartridge

The current study is intended to check whether the silica SPE cartridge normally used in the cleanup process during the analysis of pesticide residues can be used to clean kasugamycin, a bio-pesticide used in herbal medicine. The main problem in kasugamycin residue analysis in herbal medicine is the matrix's interference effect, continuously observed after extraction. Therefore, complete removal of *A. Japonica* roots matrix effect is important and required by the national MRLs [8]. The removal of the matrix effect during kasugamycin residue analysis in herbal medicine is determined using the elution conditions. Therefore, the current study focused on optimizing the elution conditions of kasugamycin residue analysis. The effect of the loading volume of the extract and the volume and type of the eluting solvent indicated that using 1 mL fortified elution volume with 10 mL methanol as an eluting solvent on silica SPE cartridge could result in a 93.2% dissolution rate of kasugamycin (Fig. S2). However, the dissolution rate of kasugamycin was increased to 95.5% when using 10 mL of pure water. The lost amount of kasugamycin residue in both cases is attributed to the interfering substances of the matrix causing the overloading of the cartridge and consequently the loss of the target compound (kasugamycin). Several trials were done to minimize the influence of the matrix of the *A. Japonica* roots of the purification method using a silica SPE cartridge without success. Therefore, another set of trials were done to replace the silica SPE cartridge with HLB SPE and SCX SPE cartridges which were reported to have better efficiency [5,23].

HLB SPE cartridge

The HLB SPE cartridge is known to have high purification efficiency in analyzing aminoglycoside antibiotics. Previous studies reported the analysis of kasugamycin among agricultural products by using an HLB SPE cartridge [12,5]. When using the kasugamycin standard solution, only the HLB SPE cartridge purification gave a recovery rate of 95.8%. When applying 5 mL of the sample extract to the standard condition, a 97.3% recovery rate of kasugamycin was obtained (Fig. S3). However, quantification of the minimum concentration was not possible due to the interference substances in the *A. japonica* roots extract co-eluted with kasugamycin on the chromatogram (Fig. 3). The current result is similar to the results reported by previous authors that the HLB SPE cartridge has low purification efficiency and high ion suppression [7-8]. Therefore, another purification condition was tested using the SCX SPE cartridge, a strong cationic SPE cartridge that exhibits an efficient purification effect for polar substances with many amino and hydroxy groups [8].

SCX SPE cartridge

The SCX SPE cartridge (1 g), a strong ion exchange cartridge, has been reported to effectively purify basic amine compounds from materials with complex matrices [24-25]. In particular, kasugamyacin has various pKa values, so the purification efficiency is higher when an ion exchange cartridge is used, rather than the florisil or silica SPE cartridge generally used [5]. In addition, the optimal purification method with SCX SPE cartridges has been widely reported. It can sufficiently separate the matrix interfering substance during kasugamycin and validamycin A analysis [18]. The purification conditions with the SCX SPE cartridge were tested using the standard solution, and the target component was not eluted in the loading and washing section. Kasugamycin showed a 98.1% dissolution rate when 10 mL of 5% NH_4OH in methanol was used. However, when the sample was applied, the recovery rate was 58.8%, which indicates a section lost before the elution section. Therefore, a recovery rate test was performed for each section to solve this problem by applying an *A. japonica* sample. As a result, about 40.5% was eluted in the loading and washing sections (Fig. S4).

The overloading phenomena may explain these results due to the matrix effect in the test cartridge as

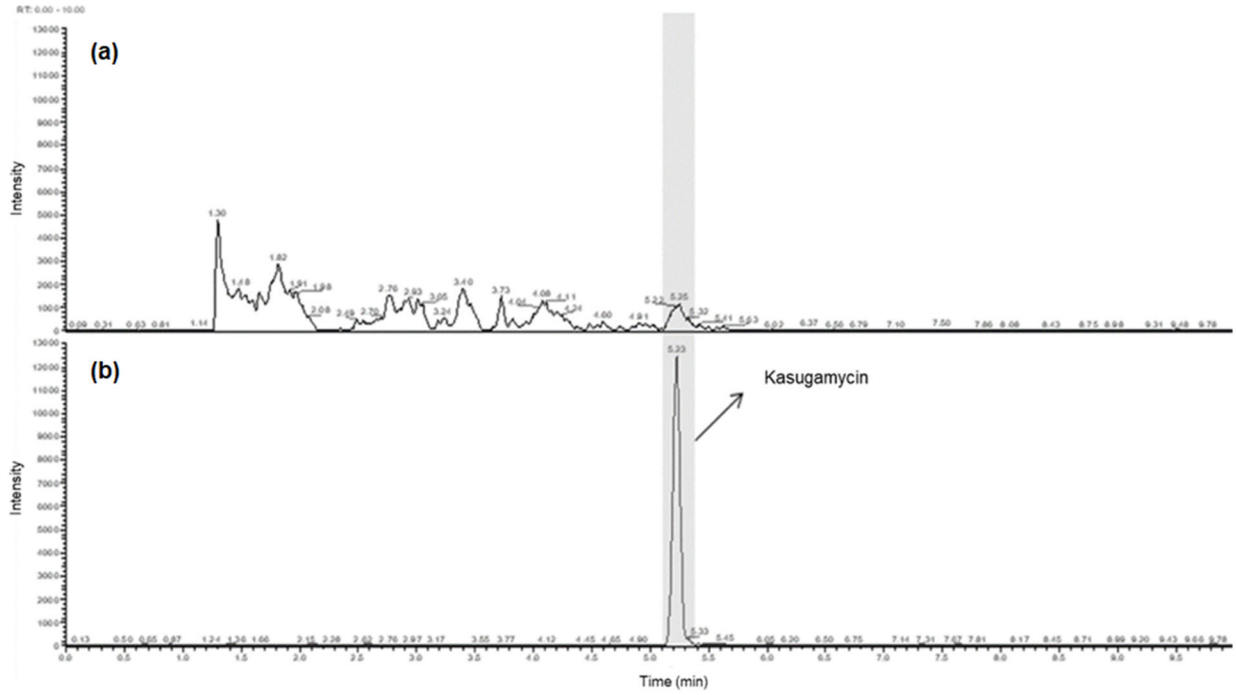


Fig 3. Chromatogram of HLB SPE cartridge test of kasugamycin in *Achyranthes japonica* Nakai and kasugamycin standard ((a): control, (b): kasugamycin 0.1 mg kg^{-1})

reported for the silica SPE cartridge [26]. Therefore, a test was conducted using a loading range of 1–4 mL sample extract. The results of the loading capacity experiment showed that the target component was not eluted in the loading and washing sections when 1–3 mL was used, and the entire amount was eluted in 10 mL of 5% NH_4OH in methanol from the elution section (Table S2). Based on this result, 2 mL was selected as the optimal loading capacity, and the elution solvent was established under purification conditions using 10 mL of 5% NH_4OH in methanol. The above purification conditions gave a recovery rate of 101.2%, and the interfering substances continuously appearing near the retention time of kasugamycin were removed, and quantification was possible even at the minimum concentration (Fig. 4). Further experiments were done using a consecutive cleanup process combining the HLB SPE cartridge and the SCX SPE cartridge, which was reported to have more efficiency [8].

Double cleanup using HLB SPE cartridge + SCX SPE cartridge

Based on previous studies, the double cleanup method using the HLB and SCX SPE cartridge was performed. The result indicated a recovery rate in the elution section of 93.7% using the standard solution (Fig. S3). However, the recovery rate was reduced to 48.5%. Therefore, a second purification was performed using 5 mL of the sample extract. Overloading is reported to be caused by the difference in sample matrix type used and the cartridge capacity. Therefore, the overloading phenomenon observed in this study may be due to the overwhelming capacity limit of the SCX SPE cartridge by the *A. japonica* roots extract [27].

Unlike agricultural products, herbal extracts contain various and considerable pharmacological ingredients that saturate the cartridge's adsorption sites. As a result, the normal adsorption and desorption of the analyte components are impossible. Therefore, to overcome overloading from the sample, an additional cleanup process was performed by applying the improved SCX SPE cartridge purification method in which the volume of the extract was reduced from 5 to 2 mL. The result of the improved purification conditions gave a recovery rate in the elution section of 88.4%, and

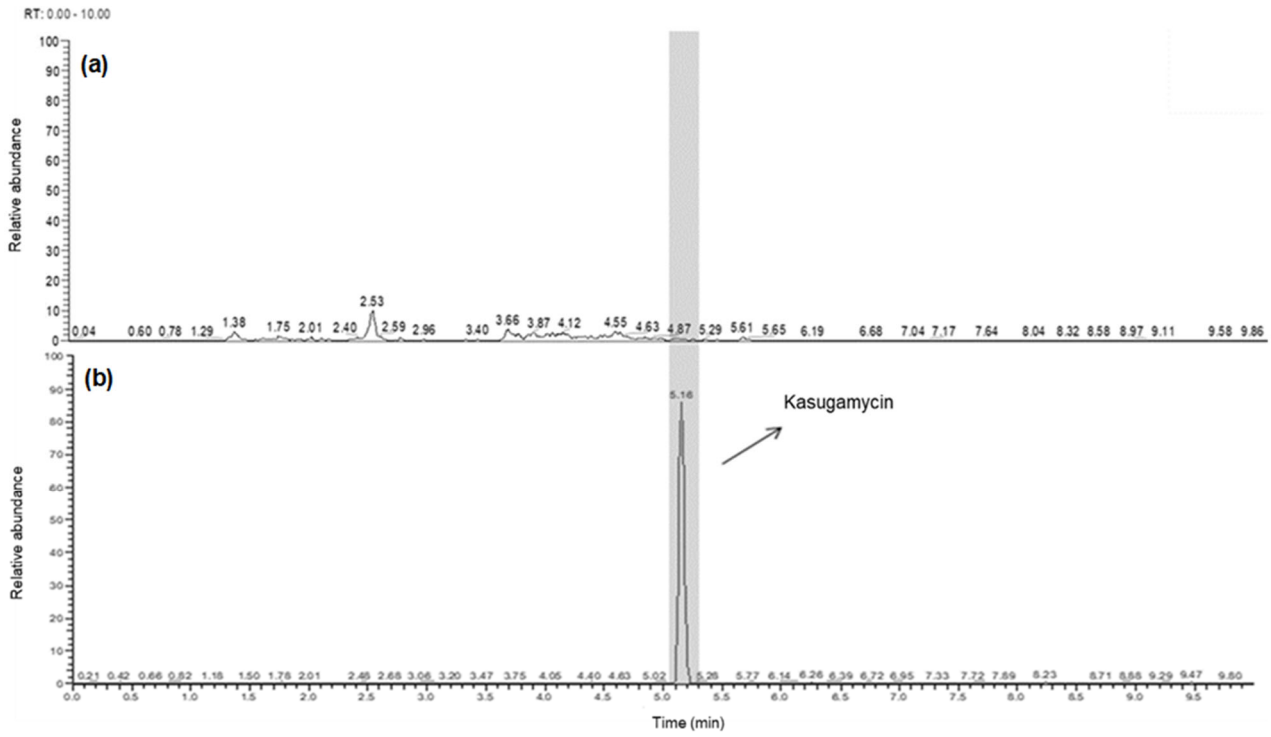


Fig 4. Chromatogram of SCX SPE cartridge test of kasugamycin in *Achyranthes japonica* Nakai and kasugamycin standard ((a): control, (b): kasugamycin 0.1 mg kg^{-1})

after double clean-up, the interfering substances eluted around the retention time of kasugamycin disappeared, and therefore that the minimum concentration could be quantified (Fig. 5). Thus, the current results indicated that the SCX SPE cartridge with the double clean-up method, and reduced loading to 2 mL, effectively prevents overloading and removes the herbal extract's interference effects. However, the reduction in loading volume could reduce the representativeness of the sample, therefore not recommended.

Comparison of Matrix Effect (ME) of the Purification Methods

The matrix effect of each purification method developed for kasugamycin analysis in *A. japonica* gave different results depending on the cartridge. The matrix effect of the HLB SPE cartridge was –36%, while those of the SCX SPE cartridge alone and the double clean up with HLB and SCX SPE cartridges were –15% and +14%, respectively (Table 3). The two analytical methods of the SCX SPE cartridge were matrix-matched in 1:1 ratio of the standard substance and the extract solution with the lowest possible effect of the interfering substance in the LC/MS/MS analysis at the lowest possible quantitative level. Since there were no differences in the matrix effect value from the presence or absence of the HLB SPE cartridge, it was assumed that the purification through a non-retentive filter was insufficient, and therefore most of the purification was performed in the SCX SPE cartridge. The consecutive cleanup process that directly connects HLB and SCX SPE cartridges gave excellent superior results. Nevertheless, since purification with a single SCX SPE cartridge gave sufficient purification efficiency and saved time, effort, and the method's simplicity, the current investigation recommends using a single SCX SPE cartridge for the purification of kasugamycin in herbal medicine [8].

Overview of the Method Established for Kasugamycin in Herbal Medicine

The sample should be wetted for one hour by adding 10 mL of water to the dry sample and adjusting the pH to 4.5 to 5.0 using 40 mL of 0.1% formic acid in the water extraction solvent. Then, ultrasonic extraction should be performed for 30 min. Then the extract should be centrifuged for 10 min, 5 mL of the supernatant should

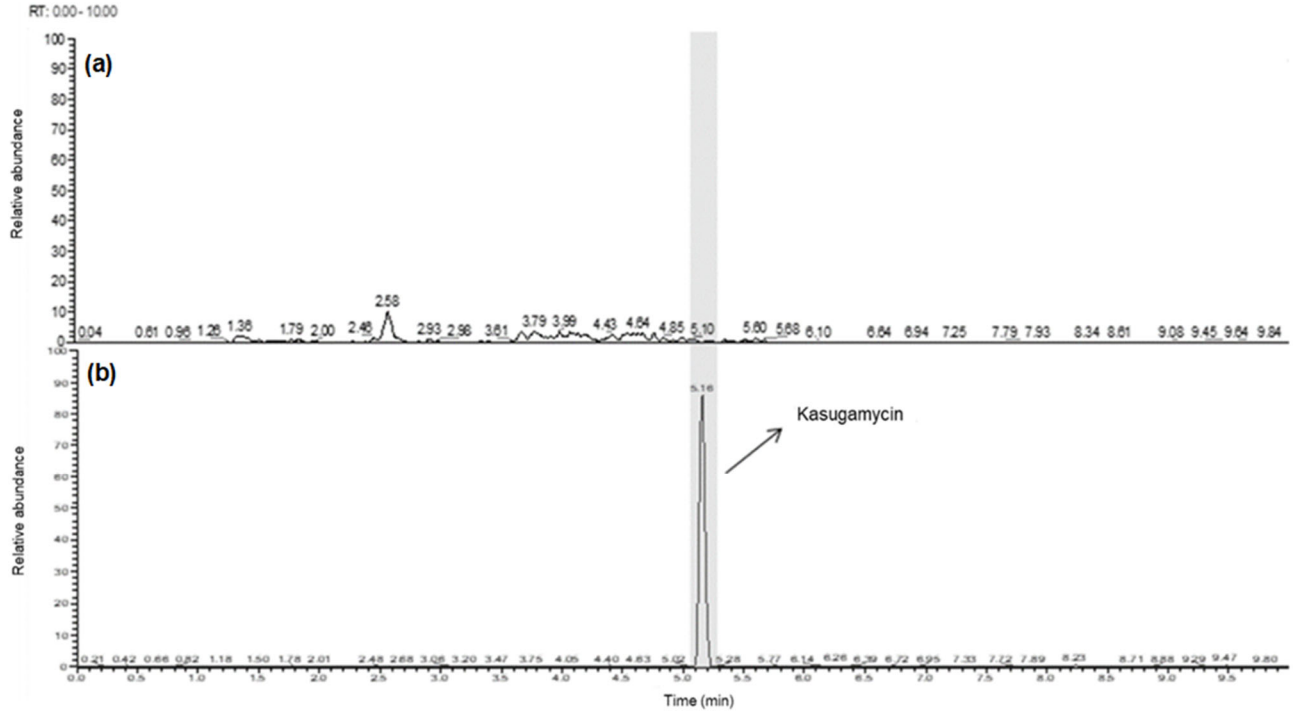


Fig 5. Chromatogram of HLB & SCX SPE cartridge test of kasugamycin in *Achyranthes japonica* Nakai and kasugamycin standard ((a): control, (b): kasugamycin 0.1 mg kg^{-1})

Table 3. Results of matrix effect (%) of kasugamycin in *Achyranthes japonica* Nakai

Sorbent	Regression equation	Linearity (r^2)	Matrix effect (%)
Solvent	y = 265136.035x - 473.3349723	0.9964	-
HLB	y = 168547.8359x + 6578.965386	0.9951	-36
SCX	y = 225862.6871x - 36.41891167	0.9968	-15
HLB + SCX	y = 301291.8221x + 422.1827966	0.9923	+14

be taken and centrifuged after liquid-liquid partitioning at the same ratio as dichloromethane, then 2 mL of the supernatant should be used as a loading sample for purification. Purification conditions should be done on the SCX SPE cartridge (1 g), a strong ion exchange cartridge. First, the filter should be activated by adding 5 mL of water and methanol, then loaded with 2 mL of the sample extract, followed by washing with 10 mL of water and methanol, and eluting with 10 mL of 5% NH_4OH in methanol. Next, the elution solution should be concentrated completely under a gentle stream of nitrogen, then reconstituted in 1 mL of water, filtered with a 0.2 µm membrane filter, and subject to LC/MS/MS analysis (Fig. 6).

Test Method Validation

The established method eliminated the effect of the interfering substance co-eluting with kasugamycin and therefore allowed accurate estimation of levels close to the quantification level. As a result of evaluating the selectivity of the test method, when the established analysis method was applied, the effect of interference near the kasugamycin retention time was minimized, and the quantitative limit level was quantifiable. Therefore, it could be said that it was a preferable analysis method. The reliability of the results of residue analysis in *A. japonica* was by a matrix-matched calibration curve done by diluting the standard solution

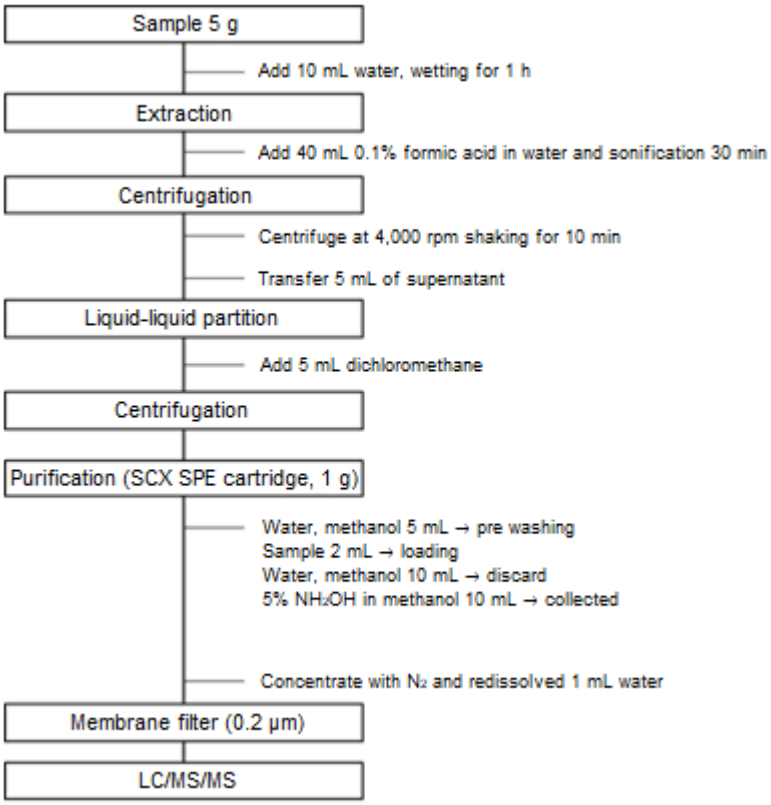


Fig 6. Improved analytical method of kasugamycin residue in *Achyranthes japonica* Nakai

of kasugamycin to 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L. The results gave a matrix-matched calibration curve of 0.996 or higher (Fig. 7 and Table S3).

The detection limit (LOD) of the device used in the current study was 0.008 ng, while the quantification limit (LOQ) of the test method was 0.04 mg/kg (Eq. (3)). The LOQ obtained by the current method satisfies the minimum quantification limit of less than 0.05 mg/kg or less or 1/2 of the residual tolerance standard, the pesticide residues analysis standard in the international standard Codex [28], and the Pesticide Residue Analysis Standard in the MFDA [21]. The analytical quantitation limit calculated in the current method was consistent with the minimum quantification limit of less than 0.05 mg/kg or less set by FAO codex Alimentarius or 1/2 of the residual tolerance standard set by MFDS [21,28]. To evaluate the accuracy, precision, and repeatability of the tested method standard solution containing the limit of quantitation (0.04 mg/L) and 10 times the limit of quantification (0.4 mg/L) was added to the untreated samples. The average recovery rates were 88.8–106.3% and 83.1–88.4%, and the RSD was generally good at a maximum of 8.8%, indicating that the current method is sensitive and selective. Furthermore, the ranges of the recovery rate and analysis error satisfy the 70–120% of the single component analysis method of the MFDS, and the result satisfies the value within 10% of the RSD (Table S4).

$$0.04(\text{ng}) \times \frac{50(\text{mL})}{5(\text{g})} \times \frac{1(\text{mL})}{2(\text{mL})} \times \frac{1}{5(\mu\text{L})} = 0.04(\text{mg/kg}) \quad (3)$$

The improved analysis method was used to analyze kasugamycin residues (Fig. 8) in *A. japonica* and agricultural products. The kasugamycin analysis method developed in this study is expected to be applied to the

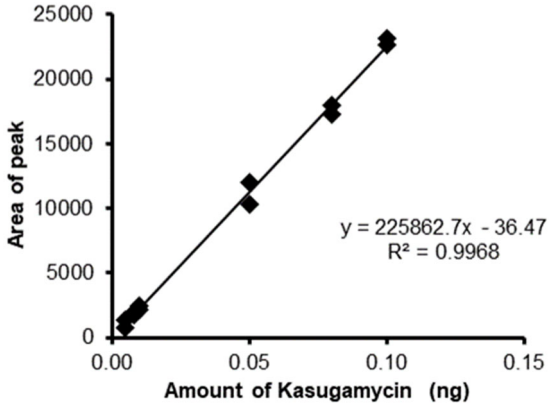


Fig 7. Calibration curve of kasugamycin matrix-matched standard by LC/MS/MS

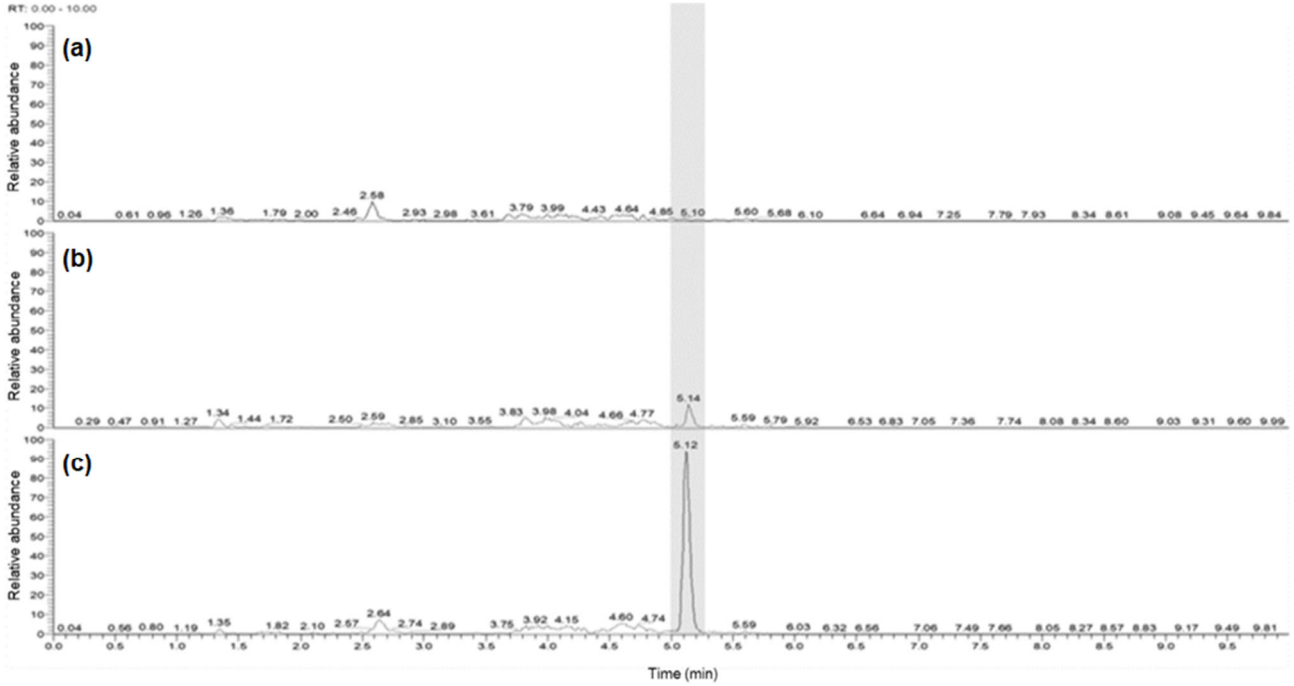


Fig 8. Chromatograms of kasugamycin residue analysis in *Achyranthes japonica* Nakai ((a): control, (b): 1 MLOQ (0.04 mg kg^{-1}), (c): 10 MLOQ (0.4 mg kg^{-1}))

aminoglycoside-based agricultural fungicides and bactericides in various herbal medicines. Furthermore, it could also be applied to analyze the residues of other fungicides and bactericides in agricultural, livestock, and aquatic products, which are major food sources in the Republic of Korea and other countries. Therefore, this method may contribute to human safety.

■ CONCLUSION

A suitable analytical method for the determination of the trace residue of kasugamycin in *A. japonica* was developed. The developed method involved using LC/MS/MS equipped with an amide column for polar substances with efficient extraction and purification processes optimized using a single SCX SPE cartridge, which is simple and time-efficient. The developed purification and cleanup method showed high efficiency in removing interferences with respective matrix effects while maintaining a high recovery rate of kasugamycin. The current results are consistent with the criteria ranges indicated in the guidelines of the Codex (CAC/GL 40-1993, 2003) and MFDS (2016). Furthermore, currently developed kasugamycin analysis could be applied to the aminoglycoside-based agricultural fungicides and bactericides and also the residues of other fungicides and bactericides. Therefore, this method may contribute to human safety.

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■ AUTHOR CONTRIBUTIONS

Jang Hyun is the team leader who proposed the work and designed the experiment. Jeong Yoon, Min-woo, and Hun Ju executed the experiments and analyzed the results. Jang Hyun and Abd Elaziz wrote the experiment. The first author (Jeong Yoon Choi) and the second author (Hun Ju Ham) have equal contributions. All authors read and approved the manuscript.

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