



Development and Interlaboratory Validation of an Analytical Method for the Determination of Saflufenacil in Agricultural Products by HPLC-UVD and LC-MS

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ABSTRACT - Saflufenacil is a low-volatile and uracil-based herbicide. This herbicide is used for pre- and post-emergence control of major broadleaf weeds. The objective of present study was to develop and validate an analytical method for saflufenacil determination in agricultural products for ensuring the food safety. The saflufenacil residues in samples were extracted with acetone, dichloromethane, and then purified with silica and graphitized carbon cartridge. The purified samples were analyzed by HPLC-UVD and confirmed with LC-MS. The linear range of saflufenacil was 0.1~5.0 $\mu\text{g mL}^{-1}$ with the correlation coefficient (r) = 0.999. Average recoveries of saflufenacil ranged from 80.5% to 110.2% at the spiked level of 0.02~0.5 mg kg^{-1} , while the relative standard deviation was 0.3~7.3%. In addition, the limit of detection and limit of quantification were 0.005 and 0.02 mg L^{-1} , respectively. Furthermore, an interlaboratory study among three labs was conducted to validate the method, and the results were satisfactory.

Key words : saflufenacil, herbicide, HPLC-UVD, LC-MS, interlaboratory validation

Saflufenacil, 2-chloro-5-[3,6-dihydro-3-methyl-2,6-dioxo-4-(trifluoromethyl)-1(2H)-pyrimidinyl]-4-fluoro-N-[[methyl (1-methylethyl)amino]sulfonyl]benzamide is a selective uracil and amide herbicide belonging to pyrimidinedione chemical class. It acts as an inhibitor of protoporphyrinogen IX oxidase, which is an enzyme involved in heme and chlorophyll biosynthesis^{1,2}. Saflufenacil is developed to control annual broadleaf weeds such as horseweed (*Coryza Canadensis*), kochia (*Kochia scoparia*), and Russian thistle (*Salsola iberica*) and is used in pre-plant and pre-emergence applications to field crops (cereals, oilseeds, legume vegetables), post-emergence under-canopy applications to fruit tree orchards, nut tree orchards, and vineyards, as well as in

the foliar treatments for desiccation and/or defoliation of green plant tissue before harvest (legumes, potatoes, sugar cane, coffee beans, sunflower, soybean, and cotton). The desiccation uses for soybean, peas and beans, and cotton, as well as for tropical fruits (bananas and mango) have been submitted to the United States Environmental Protection Agency (US EPA)³⁻⁵. The EPA has established a saflufenacil tolerance of 0.03 mg kg^{-1} for citrus fruit, stone fruit, and pome fruit, and their commodities, and of ~17.0 mg kg^{-1} for pea and hay (EPA 40 CFR 180.649)⁶. Saflufenacil was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2011 and a summary evaluation report was issued in January 2012⁷. The JMPR established maximum residue limits (MRLs) for saflufenacil residues in agricultural products of 0.01-0.1 mg kg^{-1} . In Japan, MRLs of 0.01-1.0 mg kg^{-1} for saflufenacil residues have been established for 63 food items⁸.

Saflufenacil is used widely for treatment of numerous crops in many countries around the world, so it is necessary to determine the levels of its residues in domestic and imported agricultural products with a very reliable and

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sensitive analytical method for food safety management. US EPA, EFSA, and FAO developed enforcement methods with liquid chromatography/mass spectroscopy/mass spectroscopy (LC-MS/MS) to enforce the MRL (tolerance) expression^{2,7,19}. Application of mass spectrometry in combination with modern HPLC leads often to good sensitivity as well as high selectivity. However, LC-MS/MS method is about 2-3 percent poorer precision against a well-established HPLC method. In addition, the initial costs for a sophisticated LC-MS/MS instrument are about 10 times higher than for conventional HPLC equipment, so it is hard to obtain in some food safety inspection agencies because of their lack of economic condition. Therefore, the objective of this study was to develop and validate an analytical method using conventional HPLC method for the determination of saflufenacil residues at the established new MRLs (0.03-0.7 mg kg⁻¹) for a wide range of agricultural products including rice, soybean, and orange in the Republic of Korea in 2012.

Material and Methods

Reagents, Chemicals, and Samples

A certificated analytical standard of saflufenacil (99.6%) was provided by BASF (BASF Asia Pacific, Seoul, Korea). Pesticide-grade acetone, hexane, methanol, and dichloromethane were purchased from Merck (Darmstadt, Germany). Liquid chromatography (LC)-grade water was obtained by purifying distilled water in a high-performance reverse osmosis (RO) system (Saritorius Stedim Biotech, Göttingen, Germany). Other reagents such as sodium chloride and sodium sulfate anhydrous were purchased from Wako (Osaka, Japan). Silica solid-phase extraction (SPE) cartridges (6cc, 1 g) and carbon SPE cartridges (500 mg, 6 mL) were purchased from Waters (Milford, MA, USA) and Agilent Technologies (Santa Clara, CA, USA), respectively.

To test the applicability of the method for a variety of agricultural products, mandarin (fruit), hulled rice (cereal grains), pepper (vegetables), potato (potatoes), and soybean (beans) were selected as representative crops. Agricultural products used in this experiment were purchased from the nation's major retailers. Each sample was homogenized and stored in a deep freezer (-50°C) before use.

Stock and Working Standard Solution

A standard stock solution of saflufenacil was prepared in methanol at 500 µg mL⁻¹ and stored in a freezer at -18°C. The stock standard solutions prepared were used within 3 months of preparation. Suitable concentrations of the working standards were prepared from the stock solutions by serial dilution using 50% methanol, immediately before sample preparation.

Extraction Procedure

Crop samples (20 ± 0.1 g) were placed into a 400 mL capped beaker. The samples were extracted with 100 mL of acetone for 5 min. The homogenized samples were subjected to vacuum filtration, and then transferred into a 500 mL round bottom flask for evaporation in a rotary evaporator at 40°C. Concentrated samples were transferred into a 500 mL separation funnel and 100 mL of dichloromethane, 100 mL of distilled water, and 30 mL of saturated NaCl solution were added. This mixture was then vigorously shaken for 5 min. and the eluent (dichloromethane layer) was passed through anhydrous sodium sulfate for dehydration and collected into a round bottom flask. Dichloromethane (100 mL) was added into the separation funnel in which an aqueous layer remained and then this partitioning procedure was repeated. The dichloromethane layer collected in the round bottom flask was evaporated to near dryness by rotary evaporation at 40°C. Finally, 2 mL of hexane was added to make a test solution.

Purification Procedure

For conditioning silica and carbon SPE cartridges connected in series, 10 mL of hexane was introduced and discarded. The extracted solution in 2 mL of hexane was introduced into the silica-carbon cartridge and the eluate was discarded. In addition, 10 mL of hexane/acetone (90/10, v/v) and 5 mL of hexane/acetone (70/30, v/v) were introduced and discarded. The analytes were then eluted with 15 mL of hexane/acetone (40/60, v/v). The eluate was evaporated to dryness by rotary evaporation and the concentrated residue was reconstituted in 2 mL of 50% methanol. This solution was filtered through a 0.45 µm membrane filter before injection into a chromatograph.

Chromatographic Separation Parameters

High-performance liquid chromatography (HPLC, Table 1) was performed to separate the constituents of the samples. Recording of chromatograms and quantitative measurements of the peak areas were recorded using Waters HPLC Empower software (Waters, MA, USA). The absorption spectrum of the detected peak was confirmed by comparison with a saflufenacil standard.

For confirming the presence of saflufenacil in the agriculture products, a Quattro Premier XE LC-mass spectrometer (LC-MS, Waters, MA, USA) equipped with a HPLC Capcell pak C₁₈ UG120 column (3 µm, 2.0 mm I.D. × 150 mm, Shiseido, Tokyo, Japan) was used. The LC-MS (Table 1) was operated by in the electrospray ionization (ESI) selected ion monitoring (SIM) positive-ion mode. The selected mass was 523 m/z for the analysis of saflufenacil. The MS was optimized by the infusion of standard solutions of each in-

Table 1. Analytical and confirmative conditions for the determination of saflufenacil residues

HPLC-UVD			
Instrument	HPLC-UVD (Alliance 2695, Waters, MA, USA)		
Column	Capcell pak UG 120 C ₁₈ (5 μm, 4.6 mm I.D. × 250 mm, Shiseido, Tokyo, Japan)		
Column temperature	30°C		
Mobile phase	0.2% acetic acid in water /acetonitrile (50/50, v/v), isocratic		
Flow	1.0 mL/min		
Detection	Absorption (270 nm)		
Injection volume	40 μL		
LC-MS			
Instrument	Quattro Premier XE, (Waters, MA, USA)		
Column	Capcell Pak UG 120 C ₁₈ (3 μm, 2.0 mm I.D. × 150 mm, Shiseido, Tokyo, Japan)		
	Time (min)	0.01% formic acid in water (%)	0.01% formic acid in acetonitrile (%)
	0	80	20
	1.0	80	20
Mobile phase	1.5	50	50
	5.0	50	50
	7.0	5	95
	7.5	80	20
	15.0	80	20
Ionization mode	ESI positive-ion mode		
Cone voltage	75 V		
Injection volume	10 μL		
Flow	0.2 mL/min		

dividual compound, at a concentration of 1 μg mL⁻¹, in acetonitrile at a flow rate of 20 μL min⁻¹.

Method Validation

Validation of the method ensures credibility, and in this study various parameters, such as the linearity, specificity, accuracy, precision, and the limit of detection (LOD) and limit of quantification (LOQ), were considered.

Linearity was determined using different known concentrations (0.1, 0.2, 0.6, 1.0, and 5.0 μg mL⁻¹) prepared by diluting the stock solution with 50% methanol. The specificity of the method was evaluated by injecting a procedure blank, control samples, and control samples spiked at the lowest fortification level assayed (LOQ), i.e., 0.02 mg kg⁻¹.

The accuracy of the method was determined by means of a recovery test, using samples spiked at three concentration levels, 0.02, 0.03, and 0.3 mg kg⁻¹ for hulled rice and mandarin, and 0.02, 0.05, and 0.5 mg kg⁻¹ for soybean, potato, and pepper. Recovery experiments had three replicates at every concentration level. The precision of the method was evaluated within the same day at each recovery level and was calculated in terms of relative standard deviation (RSD) for three replicates.

The LOD (μg mL⁻¹) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The LOQ (μg mL⁻¹) was determined as the lowest concentration validated that gave acceptable recoveries (70%-120%) and precision (< 10%) or a response of 10 times the baseline noise.

Interlaboratory Validation

For interlaboratory validation to determine saflufenacil residues using the method developed in this study followed by concurrent analysis with similar equipment (HPLC and LC-MS), the 5 representative agricultural products were tested in 3 laboratories (Headquarter, Busan regional FDA, and Gyeongin regional FDA). The reported results are used to test the performance characteristics of the developed method. These characteristics are within-laboratory and between-laboratory precision, systematic error, recovery, sensitivity, limit of determination, and applicability.

Result and Discussions

Extraction Optimization

Acetone^{9,10}, acetonitrile^{11,12}, methanol^{13,14}, or ethyl acetate^{15,16} are commonly used as extraction solvents to optimize pesticide residue analysis. In this study, acetone was used as the extraction solvent because of its ability to dissolve many organic compounds. In addition, acetone is very volatile, making it easy to isolate any dissolved substance simply by evaporation of the solvent. The extraction process can be used either to obtain the desired substance from the raw material or to remove unwanted impurities, depending on the solubility of this compound.

Purification Optimization

Florisil-, aminopropyl-, or silica-SPE cartridges, which are widely used in pesticide residue analysis, were compared to regard the efficiency of purification. Saflufenacil was strongly absorbed and was not well recovered in florisil- or aminopropyl-SPE cartridges, whereas it was effectively separated from the silica-SPE cartridge. Therefore, silica-SPE cartridge was selected as the purification cartridge.

For selecting the composition of the purification solvent, various concentrations of hexane/acetone and dichloromethane/acetonitrile/hexane were tested. The composition of hexane/acetone is the more effective solution to isolate moderately polar saflufenacil ($\text{Log } P_{\text{OW}} = 2.6$) from other co-extracts than dichloromethane/acetonitrile/hexane mixture because acetone has a higher solvent strength (5.1) than dichloromethane (3.1). However, interferences from pigments or fat substances were observed after purification by a silica-SPE cartridge. Carbon-SPE cartridges can efficiently remove pigment or fat irrespective of the solvent polarity, so silica- or carbon-SPE cartridges connected in series were introduced in the purification process.

HPLC Optimization

HPLC allows the determination of low-volatile and thermolabile compounds. HPLC analysis was selected to determine saflufenacil residues because saflufenacil has a large molecular weight (500.9) and very low volatility with a vapor pressure of 4.5×10^{-12} . The HPLC-photo diode array technique was used to scan over a range of 210-400 nm in order to determine the optimum absorption wavelength for saflufenacil analysis. Saflufenacil can be measured at longer wavelengths due to molecular structures (C=O, C=C, and S=O) that act as strong electron withdrawing groups to the adjacent nitrogen. Measurements at longer wavelengths have the advantage of being less affected by the interference of impurities. The maximum absorbance of saflufenacil was measured at 270 nm.

LC-MS Optimization

Fig. 1 shows MS spectrum obtained using a mass spectrometry detector (MS) with an electrospray interface, in full scan mode for saflufenacil. The full scan spectrum (optimized at a cone voltage of 75 V) showed an abundant ion at m/z 523 corresponding to $[\text{M}+\text{Na}]^+$ (Fig. 1).

Linearity

Saflufenacil solutions with various known concentrations (0.1, 0.2, 0.6, 1.0, and 5.0 $\mu\text{g mL}^{-1}$) were prepared in 50% methanol by diluting the stock solution. Standard solutions were injected into the LC column and the peak area was measured for calculating saflufenacil residue. A calibration curve has been plotted for the concentration of the standards injected versus the observed peak area and the linearity of the method was evaluated by analyzing five working solutions. The peak area obtained from different concentrations of saflufenacil was used to calculate the residue concentrations ($r = 0.999$).

Specificity

The specificity (matrix effect) of the analytical method was judged from the absence of interfering peaks at the retention times of the analytes. The retention time of saflufenacil was constant at 11.4 ± 0.1 min, and there were no interfering peaks at the retention time of saflufenacil (Fig. 2. A-2 and B-2).

Accuracy and Precision

Recovery studies were carried out at 0.02, 0.03 and 0.3 mg

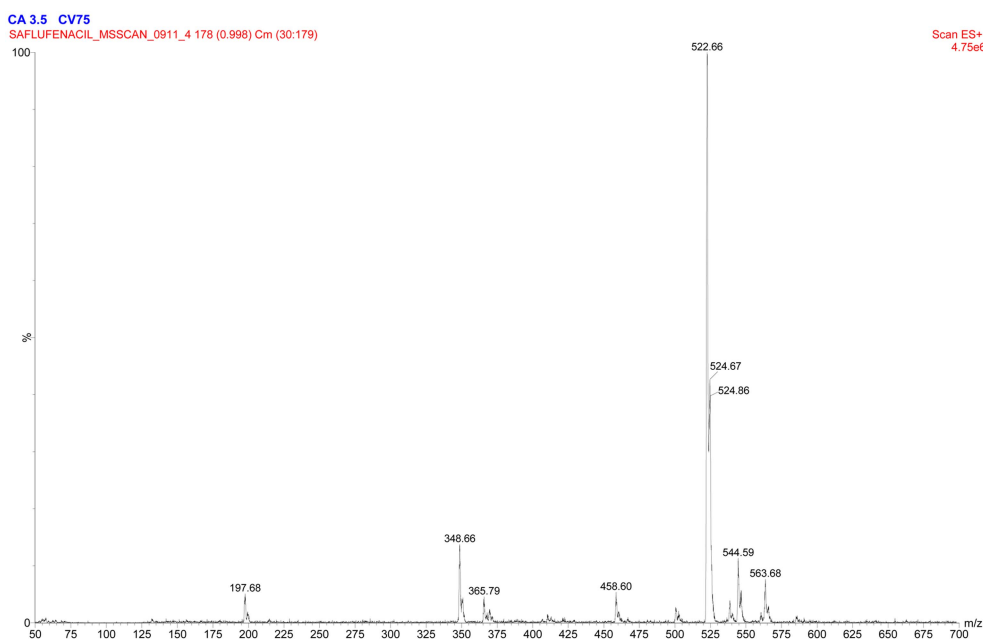


Fig. 1. Full scan mass spectrum of saflufenacil at 75 V.

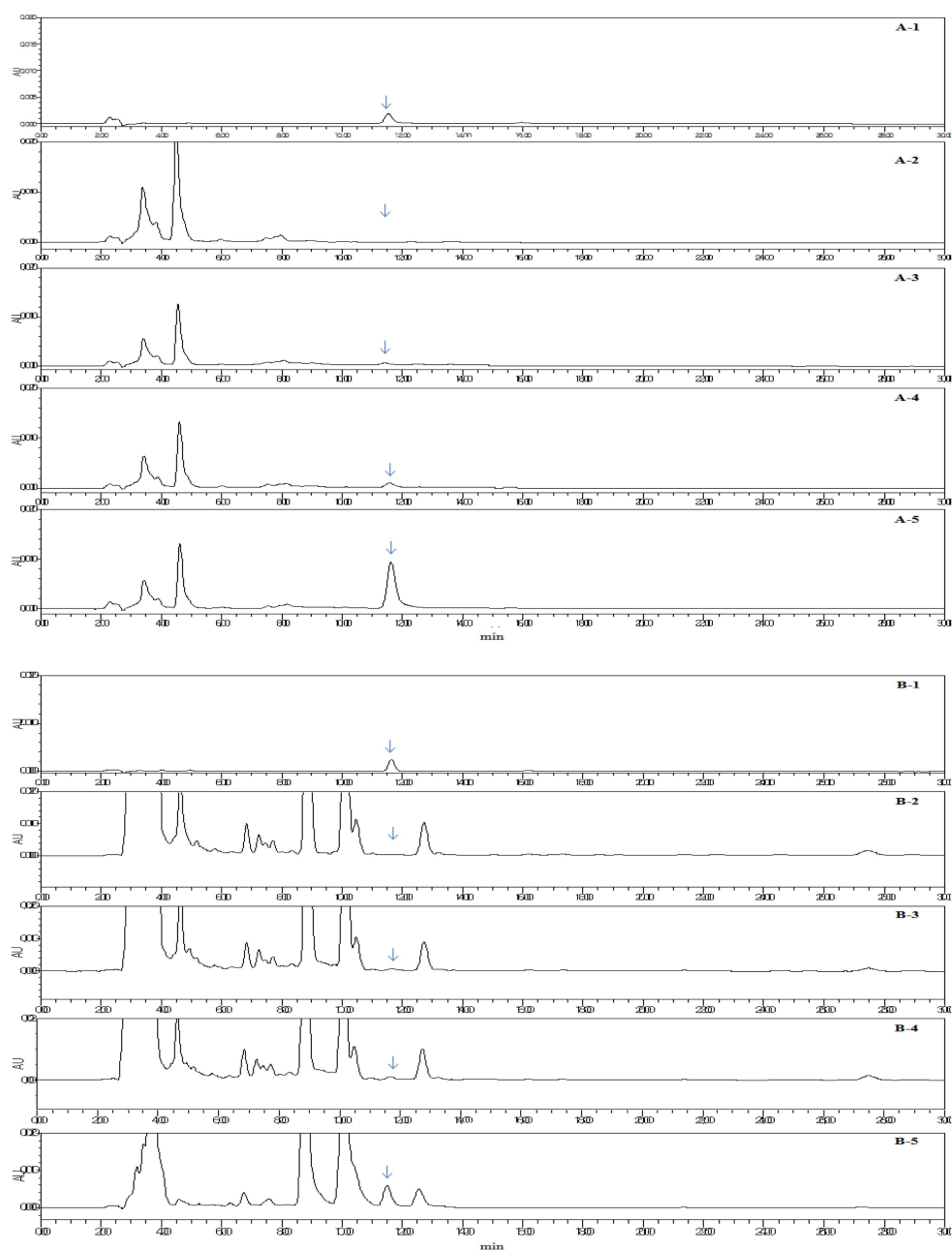


Fig. 2. HPLC-UV-D chromatograms corresponding to: A-1, saflufenacil standard at 1.0 $\mu\text{g/mL}$; A-2, control soybean; A-3, spiked at 0.02 mg/kg; A-4, spiked at 0.05 mg/kg; A-5, spiked at 0.5 mg/kg; B-1, saflufenacil standard at 1.0 $\mu\text{g/mL}$; B-2, control mandarin; B-3, spiked at 0.02 mg/kg; B-4, spiked at 0.03 mg/kg; and B-5, spiked at 0.3 mg/kg.

kg^{-1} fortification levels for saflufenacil in control hulled rice and mandarin samples and at 0.02, 0.05 and 0.5 mg kg^{-1} fortification levels in control soybean, potato, and pepper samples (Fig. 2). The recovery data and relative standard deviation (RSD) obtained by this method are summarized in Table 2. These numbers were calculated from six replicate analyses of given samples made by a single analyst on one day. The repeatability of method was satisfactory (RSD < 10%).

Detection and Quantification Limits

The limit of quantification was determined to be 0.02 $\mu\text{g mL}^{-1}$, defined as the lowest fortification level evaluated at which acceptable average recoveries (80.5~110.2%, RSDs < 10%) were achieved. This quantification limit also reflects the fortification level at which an analyte peak is consistency generated at approximately 10 times the baseline noise level of the chromatogram. The limit of detection was determined to be 0.005 $\mu\text{g mL}^{-1}$ at a level of approximately three times

Table 2. Validation results of the analytical method for the determination of saflufenacil residues

Sample	Fortification (mg/kg)	Recovery ^a ± RSD ^b (%)	LOQ ^c (mg/kg)
Hulled rice	0.02	80.5 ± 5.2	0.02
	0.03	107.4 ± 5.6	
	0.3	94.0 ± 1.8	
Mandarin	0.02	97.7 ± 7.3	
	0.03	97.2 ± 1.8	
	0.3	85.1 ± 1.1	
Pepper	0.02	90.0 ± 1.8	
	0.05	81.5 ± 0.3	
	0.5	84.4 ± 1.9	
Potato	0.02	85.3 ± 1.8	
	0.05	83.6 ± 1.1	
	0.5	87.6 ± 1.6	
Soybean	0.02	110.2 ± 6.6	
	0.05	110.0 ± 4.5	
	0.5	105.8 ± 0.6	

^aMean values of triplicates with standard deviation.^bRSD, relative standard deviation^cLOQ, limit of quantitation

the background of control injection around the retention time of the peak of interest.

Interlaboratory validation

Interlaboratory validation was performed among 3 laboratories with the developed analytical method for the determination of saflufenacil. Average recoveries in each lab were 80.5%-110.2% (Headquarter), 73.8%-98.2% (Busan

regional FDA), and 80.9%-112.2% (Gyeongin regional FDA) with less than 10% RSD (Table 3). In this inter-laboratory validation, the RSDs among the three labs were less than 20% (Table 3), which is lower than the value of 32% set in the Codex guideline¹⁷⁾. These validation results showed that this analytical method is suitable for determining saflufenacil residues in agricultural products.

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요약

농산물 중에 있는 제초제 saflufenacil의 잔류량을 검사하기 위해 HPLC-UV와 LC-MS를 이용한 정확하고 감도가 좋은 분석방법을 개발하였다. Saflufenacil 잔류물은 acetone 추출, dichloromethane을 이용한 액-액 분배, silica와 carbon 카트리지를 정제를 거쳐 기기분석을 수행하였다. 검량선 작성을 위해 0.1~5.0 µg mL⁻¹ 범위로 표준품을 만들어 실험한 결과 상관계수(r^2)는 0.999로 높은 직선성을 보였다. 0.02~0.5 mg kg⁻¹ 처리수준으로 회수율을 실험한 결과는 80.5~110.2% 이었으며, 상대표준편차는 10% 미만이었다. 분석방법의 검출한계와 정량한계는 각각 0.005와 0.02 mg L⁻¹ 이었다. 확립된 시험법으로 본청, 부산지방식품의약품안전청과 경인지방식품의약품안전청에서 실험실간 검증을 실시한 결과 만족스런 결과를 얻었다. 이러한 결과들을 통해 확립된 시험법은 농산물 중 saflufenacil의 잔류량 분석에 적합함을 확인할 수 있었다.

Table 3. Interlaboratory validation results of the analytical method for the determination of saflufenacil residues in various agricultural samples

Sample	Fortification (mg/kg)	Recovery ± RSD _r ^a (%)			Ave. Recovery (%)	RSD _R ^b
		Headquarter	Busan FDA	Gyeongin FDA		
Hulled rice	0.02	80.5 ± 5.2	88.0 ± 8.6	112.2 ± 5.1	93.6	16.5
	0.03	107.4 ± 5.6	87.6 ± 3.1	107.7 ± 9.9	100.9	11.5
Mandarin	0.02	97.7 ± 7.3	92.1 ± 8.6	84.7 ± 4.9	91.5	9.1
	0.03	97.2 ± 1.8	78.6 ± 8.2	109.4 ± 4.9	95.1	15.0
Pepper	0.02	90.0 ± 1.8	93.7 ± 3.8	108.0 ± 0.7	97.2	8.7
	0.05	81.5 ± 0.3	98.0 ± 5.4	80.9 ± 8.8	86.8	11.4
Potato	0.02	85.3 ± 1.8	98.2 ± 3.0	99.3 ± 8.6	94.3	8.7
	0.05	83.6 ± 1.1	91.2 ± 2.8	101.8 ± 2.6	92.2	8.9
Soybean	0.02	110.2 ± 6.6	91.9 ± 5.9	103.6 ± 4.4	101.9	9.3
	0.05	110.0 ± 4.5	73.8 ± 1.6	91.4 ± 6.2	91.7	17.6

^aRSD_r: intralaboratory validation^bRSD_R: interlaboratory validation

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