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Simultaneous Determination of Orysastrobin and Its Isomers in Rice Using HPLC-UV and LC-MS/MS

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ABSTRACT: Orysastrobin is a new strobilurin-type fungicide to control leaf and panicle blast and sheath blight in rice. An analytical method was developed to determine the residues of orysastrobin and its two isomers, the main metabolite F001 and the major impurity F033, in hulled rice by the use of high-performance liquid chromatography with ultraviolet photometry (HPLC-UV) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). All compounds were extracted with acetone from hulled rice samples. The extract was diluted with saline water, and an extraction step using dichloromethane/*n*-hexane partition was used to recover analytes from the aqueous phase. An *n*-hexane/acetonitrile partition and Florisil column chromatography were employed to further remove interfering coextractives prior to instrumental analysis. An octadecylsilyl column was successfully applied to identify orysastrobin and its isomers in sample extracts. Net recovery rates of orysastrobin, F001, and F033 from fortified samples ranged from 80.6 to 114.8% using HPLC-UV and LC-MS/MS. Relative standard deviations for the analytical methods were all <20%, and the quantification limits of the method were in the 0.002−0.02 mg/kg range. The proposed methods were reproducible and sufficiently accurate to evaluate the terminal residue of orysastrobin and its isomers in rice.

KEYWORDS: orysastrobin, strobilurins, fungicide, HPLC, LC/MS/MS

INTRODUCTION

Strobilurin fungicides have been identified as some of the most important agricultural fungicides, the discovery of which was inspired by a group of natural fungicidal derivatives of β-methoxyacrylic acid.[1] The strobilurins were first sold in 1996; by 2008 there were 12 commercially available strobilurins.[2]

These fungicides constituted the largest chemical group (21.7%) of pesticides in 2005, as a result of widespread use in cereal and soybean harvesting.[3] These chemicals act by inhibiting the cytochrome pathway (complex III) between cytochrome *b* and cytochrome *c*1, at the ubiquinol oxidizing (Qo) site.[4] The mode of action is preventative and curative, acting against spore germination and infection.

Orysastrobin, 2[(*E*)-methoxyimino]-2-[(3*E*,6*E*)-2-{5-[(*E*)-methoxyimino]-4,6-dimethyl-2,8-dioxa-3,7-diazanona-3,6-dienyl}-phenyl]-*N*-methylacetamide, is the common name of a new QoI fungicide discovered and developed by BASF Co.[4] Orysastrobin has two major isomers, F001 ((2*E*)-2(methoxyimino)-2-{2-[(3*E*,5*Z*,6*E*)-5-(methoxyimino)-4,6-dimethyl-2,8-dioxa-3,7-diazanona-3,6-dien-1-yl]phenyl}-*N*-methylacetamide) and F033 ((2*E*)-2-(methoxyimino)-2-{2-[(3*E*,5*E*,6*Z*)-5-(methoxyimino)-4,6-dimethyl-2,8-dioxa-3,7-diazanona-3,6-dien-1-yl]phenyl}-*N*-methylacetamide). The molecular structures of these compounds are shown in Figure 1. F001 is major metabolite in the crop, and F033 is an impurity comprising about 7% of the technical material.[5] Therefore, the levels of F001 and F033 must be controlled for safety assurance.

The fungicide controls most major diseases of rice, such as leaf and panicle blast (*Magnaporthe grisea*) and sheath blight (*Thanatephorus cucumeris*), at 1.75 g ai/seedling box (30 × 60 cm).[6] Orysastrobin is used in mixtures with carbosulfan, chlorantraniliprole, clothianidin, and thiamethoxam. Registered formulations are granule (GR) and wettable powders (WP) in which the active ingredient is 3.5−30% of the formulation.[7] Despite its being applied early in the cultivation process, orysastrobin and its metabolites persist in rice.[5] Therefore, risk assessment is necessary to confirm that the rice is safe for human consumption. Compared with other pesticides, the chronic toxicity of this fungicide, represented by the acceptable daily intake (ADI) value of 0.052 mg/kg bw (Japan), is fairly average. Due to the presence of orysastrobin and its metabolites in the final harvest, maximum residue limits (MRLs) have also been established as 0.3 mg/kg in Korea.[8] The persistent use of orysastrobin inevitably leads to its presence in the harvested crop. As rice is consumed in large quantities, the evaluation of the residue level of orysastrobin is of considerable importance to ensure food safety. Accordingly, the development of new methods is needed to routinely detect orysastrobin residues. Importantly, no appropriate method is currently available for orysastrobin detection.

In the present study, a new analytical method was devised for the simultaneous determination of orysastrobin and its isomers using high-performance liquid chromatography with ultraviolet photometry (HPLC-UV) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) in rice. The method was developed not only to achieve high reliability but also to yield high sensitivity and to offer a protocol for analytical operation.

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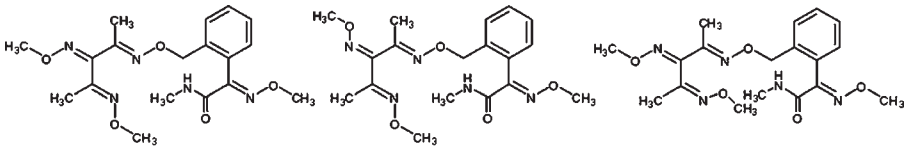

Figure 1. Molecular structures of orysastrobin and its isomers.

MATERIALS AND METHODS

Chemicals and Materials. Analytical standards orysastrobin (99.8% purity), F001 (96.2% purity), and F033 (99.0% purity) were supplied by BASF Agro Co. Korea. Each stock standard solution of 500 mg/L was prepared in acetonitrile. The stock solution was stable at 4 °C for at least 3 months. Acetonitrile and deionized water were of HPLC grade. Florisil (60−100 mesh, pesticide residue grade) was purchased from Aldrich Chemical and was activated at 130 °C for >5 h prior to use. All other solvents and reagents were of pesticide or reagent grade.

Rice Samples. The samples were purchased from local markets as organically labeled hulled rice and were prepared in compliance with the instructions in the *Korea Food code*.[7] Hulled rice was blended and sieved through a mesh. A representative sample was stored frozen at −20 °C until analyzed.

Extraction. A 25 g portion of hulled rice was weighed into a 50 mL homogenizer cup and was moistened with 20 mL of distilled water. After standing for 10 min, 100 mL of acetone was added. The mixture was macerated at 10000 rpm for 2 min in a high-speed homogenizer (Nihonseiki Kaisha, AM-8). The homogenate was suction-filtered through filter paper (Toyo no. 6) on a porcelain Büchner funnel. The cup and filter cake were washed with 40 mL of acetone, and the rinsate was combined with the previous filtrate. The filtrate was quantitatively transferred into a 1 L separatory funnel. Sequential addition of 100 mL of a dichloromethane/*n*-hexane mixture (20:80, v/v), 50 mL of saturated sodium chloride, and 450 mL of distilled water followed. After vigorous shaking for 1 min, the mixture was allowed to stand until two layers clearly separated. The lower aqueous phase was discarded. The organic phase was dehydrated over 20 g of anhydrous sodium sulfate layer, collected in a 250 mL distilling flask, and evaporated to dryness in vacuo at 40 °C.

The residue was dissolved in 30 mL of *n*-hexane saturated with acetonitrile and transferred into a 250 mL separatory funnel. The hexane phase was then vigorously extracted with two 30 mL portions of acetonitrile saturated with *n*-hexane. The lower acetonitrile phase was collected in a 125 mL distilling flask and was evaporated to dryness in vacuo at 40 °C. The residue was dissolved in 10 mL of dichloromethane and purified via Florisil column chromatography.

Florisil Column Chromatography. A chromatographic column (15 mm i.d. × 40 cm) was plugged with glass wool, dry packed with 10 g of activated Florisil, and topped with a 2 cm layer of anhydrous sodium sulfate. The column was prewashed by passing 50 mL of dichloromethane through it until the solvent level reached the top of the sodium sulfate layer. The dissolved solution from the *n*-hexane/acetonitrile partition step was poured into the column, and the column wall was rinsed twice with 2 mL portions of dichloromethane. Then the liquid drained to the sodium sulfate layer, and the column was washed with 100 mL of an ethyl acetate/dichloromethane mixture (3:97, v/v). The column was then eluted with 100 mL of an ethyl acetate/dichloromethane mixture (10:90, v/v), and the fractions were collected. The eluate was concentrated just to dryness, and the residue was reconstituted with 5 mL of acetonitrile. The final solution was diluted with 5 mL of deionized water for HPLC and LC-MS/MS.

Instrumental Parameters. *HPLC.* HPLC was performed using a Waters (USA) 2695 HPLC system equipped with a 2489 UV−visible detector and Empower data acquisition and processing system. Capcell Pak (C18) UG120, 4.6 mm i.d. × 250 mm, 5 μm spherical (Shiseido, Japan) was used as analytical column. Operating parameters used for the determination of orysastrobin and its isomers were as follows: mobile phase, acetonitrile/deionized water (50:50, v/v), isocractic flow rate, 1.0 mL/min; column temperature, 40 °C; detection, UV absorption at 247 nm (0.01 AUFS); sample size, 20 μL. Under these conditions, the retention times of orysastrobin, F001, and F033 were 10.7, 12.8, and 9.4 min, respectively.

LC-MS/MS. LC-MS/MS was carried out using a Thermo Finnigan TSQ Quantum Ultra LC-MS/MS. Capcell Pak (C18) MGII 2.0 mm i.d. × 100 mm, 3 μm spherical (Shiseido, Japan). was used as analytical column. The composition of the mobile phase and the column temperature were the same as for HPLC analysis, except that the flow rate was 0.2 mL/min and the sample size was 5 μL. Analysis was performed in positive ion mode. The electrospray ionization (ESI) source values included the capillary voltage being at 4.5 kV, the sheath gas pressure at 40 psi, the auxiliary gas pressure at 15 psi, and the tube lens offset at 80 V. The mass spectrometer was operated in full scan and selected reaction monitoring (SRM) modes.

Validation of the Analytical Method. For linearity and quantitation of orysastrobin and its isomers, calibration curves have been produced. Stock standard solutions were diluted to obtain seven different concentrations: 0.05, 0.1, 0.2, 0.4, 0.8, 0.12, and 0.16 μg/mL and 0.005, 0.01, 0.02, 0.1, 0.2, 0.4, and 0.8 μg/mL for analysis by HPLC and LC-MS/MS, respectively. A recovery test was run on control hulled rice samples to validate the analytical method proposed for orysastrobin and its isomers. Prior to extraction, a series of control samples were fortified with compound standard solutions in acetonitrile at specified concentrations. After standing for 2 h, the analytical procedures mentioned above were carried out to generate quality assurance data.

RESULTS AND DISCUSSION

Instrumental Analysis. *HPLC Analysis.* Orysastrobin is known to be a moderate polar compound with a log *P* value of 2.36 (20 °C), but it exhibits low volatility (7×10^{-7} mPa, 20 °C),[9] as do other strobilurin fungicides. Therefore, the HPLC operation was conducted with the purpose of identifying the best instrumental conditions. Because orysastrobin and its isomers were not readily oxidized or reduced and had no fluorophore, the UV absorption detector was the only choice among common HPLC detectors.[10] UV absorption spectra were obtained from each 10 mg/L solution in an acetonitrile/water mixture (50:50, v/v) to select the optimum wavelength that would be suitable for all of the analytes, as shown in Figure 2. The absorption maxima were at 247 nm for orysastrobin, 243 nm for F001, and 245 nm for F033. The optimal measuring wavelength for simultaneous analysis was the longest wavelength, 247 nm. Extinction coefficients at 247 nm ranged from 1.90×10^4 to 2.21×10^4 cm^{-1} M^{-1}, which provided the necessary sensitivity for LC analysis. The instrumental detection limit (IDL) of each compound was calculated to be almost 1 ng on the basis of 3% full scale deflection.

LC-MS/MS Analysis. Mass spectrometry is commonly engaged as a confirmatory analytical tool, but also frequently employed

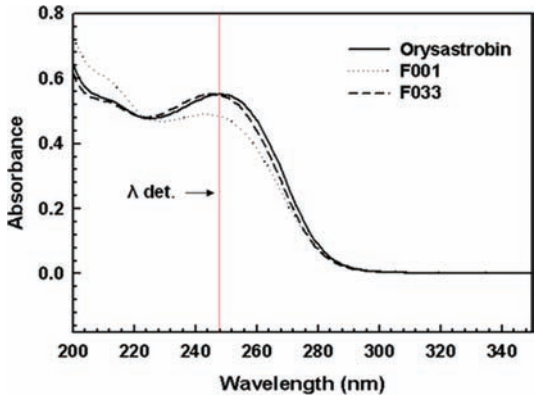

Figure 2. Ultraviolet absorption spectra of orysastrobin and its isomers in an acetonitrile/water mixture.

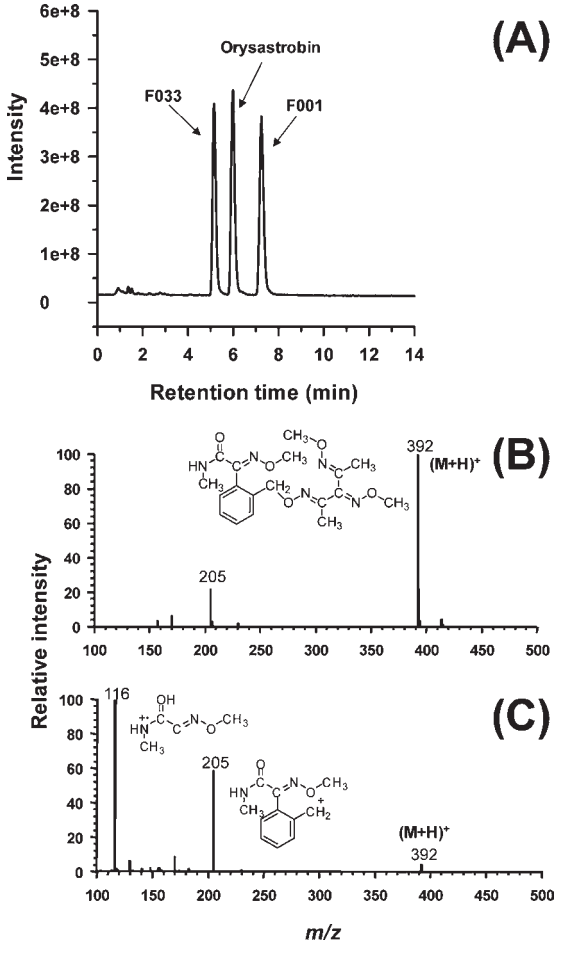

Figure 3. Total ion current chromatogram and mass spectra of orysastrobin and isomers: (A) TIC chromatogram; (B) full scan mass; (C) product ion spectrum of m/z 392 (protonated orysastrobin).

for quantification by using either single or tandem MS. We explored this instrumentation for our analyses. The mass spectra of the three compounds obtained in full scan mode and the product ion spectra of the three protonated orysastrobin isomer molecules are shown in Figure 3. All compounds exhibited identical product ion spectra. Both the full scan spectra and the product ion spectra displayed $(M + H)^+$ precursor ion m/z 392 and its fragment m/z 205. The product ion spectra also contained the dominant m/z 115 fragment, which is not present in the full scan spectra. The more stable transition signal in the SRM mode, m/z 392 → 205, was used for quantification, and the transition m/z 392 → 116 was used for confirmation. The IDLs in SRM mode were found to be 0.025, 0.022, and 0.03 ng for orysastrobin, F001, and F003, respectively.

Table 1. HPLC and LC-MS/MS Chromatographic Behavior of Orysastrobin and Its Isomers

		retention time (min) (capacity factor[a])					
column	compound	60/40[b]	IDL[c] (ng)	50/50[b]	IDL[c] (ng)	40/60[b]	IDL[c] (ng)
I[d]	orysastrobin	6.3 (2.7)	0.6	10.7 (5.3)	1.0	24.4 (13.4)	2.0
	F001	7.1 (3.2)	0.6	12.8 (6.5)	0.9	30.1 (16.7)	1.8
	F033	5.8 (2.4)	0.8	9.4 (4.5)	1.2	20.2 (10.9)	2.9
II[e]	orysastrobin			5.9 (6.4)	0.025		
	F001			7.1 (7.9)	0.022		
	F033			5.1 (5.4)	0.03		

[a] $k = (t_R - t_0)/t_0$. [b] Acetonitrile/water mixture. [c] Instrument detection limit, signal to noise ratio ≥ 10. [d] HPLC column, 4.6 mm i.d. × 250 mm, 5 μm, t_0 = 1.7 min. [e] LC-MS/MS column, 2.0 mm i.d. × 100 mm, 3 μm, t_0 = 0.8 min.

Separation of Analytes. When reverse-phase HPLC was employed using an octadecylsilyl column, orysastrobin and its isomers appeared as sharp symmetrical peaks under the mobile phase of the acetonitrile/water mixture. In the range of 40−60% acetonitrile contents in water, their capacity factor increased almost 2-fold as the acetonitrile content decreased by 10%; this indicated that all of the analytes existed in their neutral forms in the mobile phase of the acetonitrile/water mixture. Because hulled rice has many coextractives, the mobile phase was selected as an acetonitrile/water mixture (50:50) for quantitation, as it had high sensitivity and solvent efficiency without associated gradient parameters. The chromatographic behavior of analytes under isocratic conditions is summarized in Table 1.

Removal of Coextractives. A liquid−liquid partition step was employed to remove coextractives from rice extracts. Orysastrobin has a low solubility in water (80.6 mg/L), but it dissolves readily in acetone. Because hulled rice has a fat content of 2.68−2.92%,[11] acetone was selected as the extraction solvent. The acetone extract, diluted with saline, was completely recovered in the organic phase using three partition methods as described in Table 2. As all of the analytes were somewhat polar compounds, the *n*-hexane partition was not fully recovered. Liquid−liquid partitions using dichloromethane/*n*-hexane (20:80, v/v) and dichloromethane were recovered perfectly, whereas a single dichloromethane/*n*-hexane partition was employed as the liquid−liquid partition to reduce coextractives. As hulled rice includes substantial fat and nonpolar coextractives, the *n*-hexane/acetonitrile partition was added to remove nonpolar coextractives. The *n*-hexane/acetonitrile partition is a very efficient method for fat and nonpolar coextractive elimination.[12] The *n*-hexane/acetonitrile partition resulted in the effective removal of 86.6 ± 1.5% of the nonpolar coextractives from the hulled rice sample, based on weight.

Although liquid−liquid partition and *n*-hexane/acetonitrile partition methods were employed, concentrations of coextractives were left in the extract. Therefore, adsorption chromatography was

applied to further purify the extracts. Using an ethyl acetate/dichloromethane mixture (3:97, v/v), all analytes were not eluted. When the eluting solvent was changed to 100 mL of ethyl acetate/dichloromethane (10:90, v/v) after washing with 100 mL of ethyl acetate/dichloromethane (3:97, v/v), the three compounds were recovered at >90%, and the eluate could be sharply fractionated from the extract.

Method Validation. The linearity of the method was evaluated using peak area obtained after analysis of seven standard solutions with concentrations in the range of 0.05−0.16 and 0.005−0.8 μg/mL for analysis by HPLC and LC-MS/MS, respectively. Linear calibration curves were obtained, with a correlation coefficient $r > 0.998$. Typical HPLC and LC-MS/MS chromatograms for hulled rice extracts are shown in Figure 4. The proposed method produced very clean HPLC chromatograms for hulled rice sample. A recovery test was performed by analyzing a fortified hulled rice sample. This was fortified at three different concentration levels, 0.02, 0.2, and 1.0 mg/kg, in hulled rice samples. The outside recovery concentrations (0.2 and 1.0 mg/kg) above the calibration range were diluted prior to injection. The recovery data showed accuracy and precision, as presented in Table 3. Recovery rates averaged 83.9−92.3 and 80.6−114.8% using the proposed HPLC and LC-MS/MS methods. The coefficients of variation (CV) were <10% for the HPLC methods and <20% for LC-MS/MS. Although the proposed HPLC method showed better reproducibility than the LC-MS/MS method to analyze orysastrobin and its isomers, the LC-MS/MS method was more sensitive. The limits of quantitation (LOQ) of the proposed HPLC and LC-MS/MS approaches were 0.02 and 0.002 mg/kg, based on a 3% full scale deflection (signal-to-noise ratio ≥ 10). These sensitivities were sufficiently favorable to detect at least 1/15−1/150 of MRLs (0.3 mg/kg), as established for orysastrobin and its isomers in hulled rice. Therefore, the method's net detectability was also sufficient to allow for evaluation of terminal residues.

Table 2. Efficiency of Orysastrobin and Isomers in the Context of Different Partitions

extractant	recovery (%) orysastrobin	F001	F033
Liquid−Liquid Partition[a]			
100 mL of *n*-hexane	52	58	40
100 mL of CH_2Cl_2/*n*-hexane (20:80, v/v)	100	100	99
2 × 50 mL of CH_2Cl_2	103	103	102
n-Hexane/Acetonitrile Partition[b]			
2 × 30 mL of acetonitrile saturated with *n*-hexane	101	101	102

[a] Partition mixture: 140 mL of acetone + 1 mL of 1 mg/L orysastrobin, F001, and F033 standard mixture + 50 mL of saturated NaCl + 450 mL of distilled water. [b] Partition mixture: 30 mL of *n*-hexane saturated with acetonitrile + 1 mL of 1 mg/L orysastrobin, F001, and F033 standard mixture.

Conclusion. HPLC and LC-MS/MS methods were optimized to perform the simultaneous quantitation of orysastrobin and its isomers in hulled rice. Each sample was extracted with acetone, followed by liquid−liquid partition and Florisil adsorption chromatography to remove interfering coextractives. A C18 column was successfully used to determine the concentrations

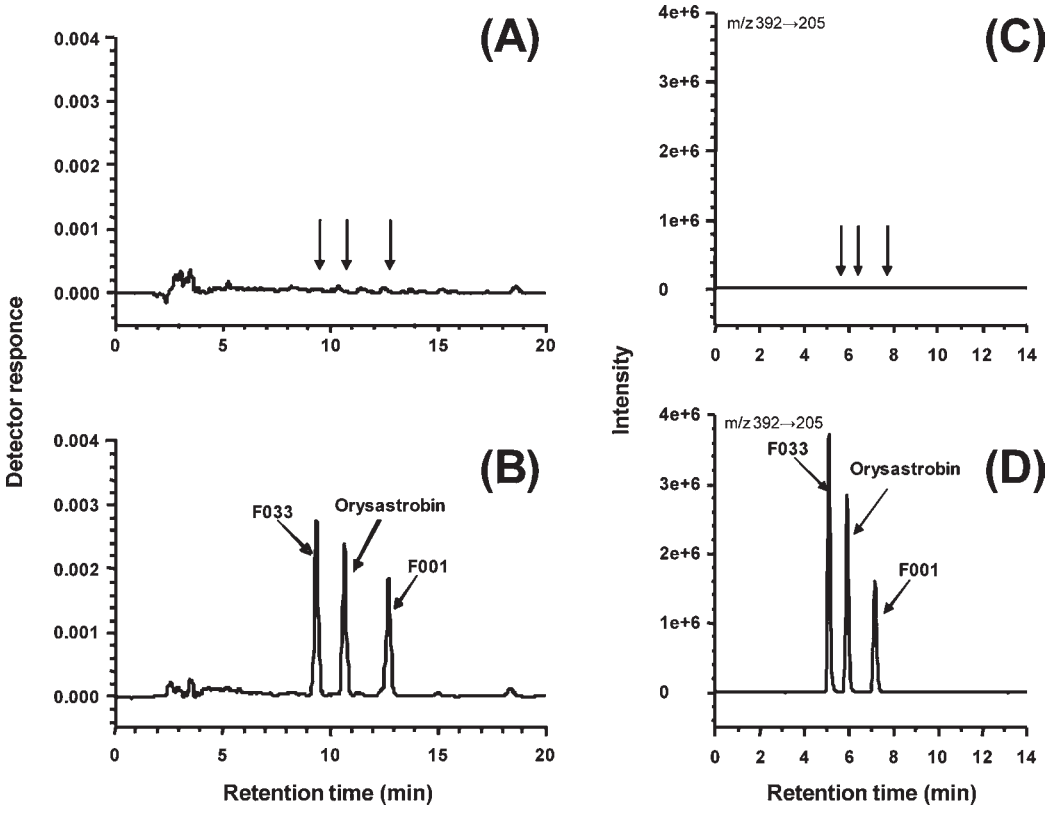

Figure 4. Typical chromatograms of the analytes in hulled rice extract detected by HPLC-UV (A, B) and LC-MS/MS (C, D): (A, C) control sample; (B) fortified with orysastrobin, F001, and F033 at 0.2 mg/kg, respectively; (D) fortified with orysastrobin, F001, and F033 at 0.02 mg/kg, respectively.

Table 3. Recovery and Limit of Quantitation (LOQ) of the Compounds

		recovery[a] (%)			
quantitation method	fortification (mg/kg)	orysastrobin	F001	F033	LOQ (mg/kg)
HPLC-UV	0.02	91.7 ± 1.6	89.5 ± 4.4	91.2 ± 3.3	0.02
	0.2	89.3 ± 1.4	83.9 ± 1.1	92.3 ± 1.1	
	1.0	89.0 ± 3.5	85.4 ± 3.8	90.8 ± 4.1	
LC-MS/MS	0.02	112.4 ± 4.5	114.8 ± 5.4	114.4 ± 5.7	0.002
	0.2	83.6 ± 6.4	86.9 ± 6.9	85.8 ± 5.4	
	1.0	81.0 ± 8.3	83.3 ± 8.2	80.6 ± 5.8	

[a] Mean values of triplicate samples with standard deviations.

of orysastrobin and its isomers in the sample extracts. The method developed shows satisfactory recovery, together with a favorable limit of quantitation and reproducibility. This proposed analytical procedure is simple and easy to perform, and it may be utilized for the regular monitoring of orysastrobin residues in rice samples.

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