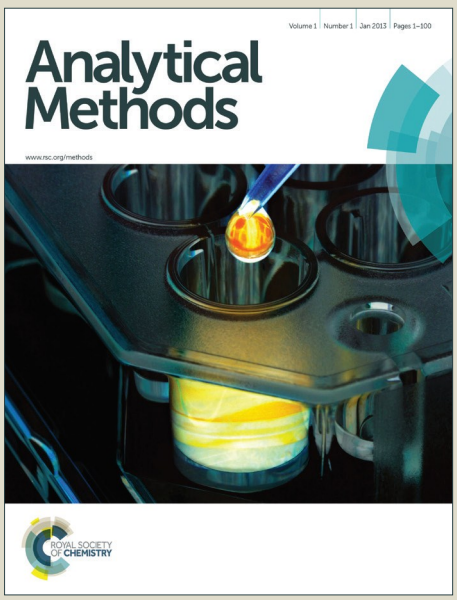
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

A trace analysis method based on HPLC-MS/MS for simultaneous determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil

He Zhu,[a] Fuyan Jia,[b] Qi Ding,[b] Chengtian Huang,[ab] Yang Yu,[a] Yuan Wang,[b] Weichang Xu[b] and Mingshan Ji*[a]

[a] *College of Plant Protection, Shenyang Agricultural University, Shenyang 110161, China*

[b] *Shenyang Research Institute of Chemical Industry, Shenyang 110021, China*

*Corresponding author. Address: Dongling Road 120, Shenhe District, Shenyang, 110161, China

E-mail: jimingshan@163.com

Abstract:

A simple, sensitive and accurate method for simultaneous determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil was established using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). All samples were extracted by acetonitrile and the tomato fruit samples were additionally dehydrated by adding anhydrous sodium sulfate (Na_2SO_4). The extract was then cleaned up by a GX-274 automated SPE system equipped with a Supelclean™ ENVI™-18 SPE tube. The separation was carried out on a XBridge™ C18 column using a mobile phase of methanol-0.1% aqueous formic acid solution at a flow rate of 0.6 mL min^{-1}. The quantification was achieved by MS/MS detection applying the multiple reaction monitoring (MRM) model while electrospray ionization (ESI) was operated in positive ion mode. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions from m/z 289.1 to 120.2 (for both two isomers of pyrisoxazole). The method showed satisfactory linearity for both isomers of pyrisoxazole in the concentration range of 10-500 μg L^{-1}, with correlation coefficients higher than 0.998 in all cases. For the two isomers of pyrisoxazole, the limits of detection (LODs) were

below 0.8 μg kg^{-1}, and the limits of quantification (LOQs) were below 2.8 μg kg^{-1}. According to the results of the recovery assay, the method presented satisfactory accuracy with mean recovery of 80.0–91.5% and satisfactory precision with all RSD values below 8.5% at the three concentration levels (10, 100 and 1000 μg kg^{-1}) for the two isomers of pyrisoxazole in the two matrices. With the established method, 30 real samples (15 samples for tomato fruit and 15 samples for soil) were analyzed. The two isomers of pyrisoxazole were not detected in all samples.

Introduction

Pyrisoxazole [3-[5-(4-chlorophenyl)-2,3-dimethyl-3-isoxazolidinyl] pyridine, previous development No. SYP-Z048] is a novel low-toxic fungicide developed by Shenyang Research Institute of Chemical Industry.[1,2] It has been described by Fungicide Resistance Action Committee (FRAC) as a DMI-fungicide whose mechanism is to inhibit fungal ergosterol biosynthesis.[3] Pyrisoxazole has high fungicidal activity due to its structural advantage that it has a pyridine ring and an isoxazolidine ring which both have fungicidal effect.[4] Pyrisoxazole is also a broad spectrum fungicide and exhibits good control efficacy on ascomycetes, basidiomycetes and deuteromycetes.[1,4] Its control efficacy is especially excellent on the ascomycete *Botrytis cinerea*.[2] Pyrisoxazole has been registered for control of tomato gray mold caused by *Botrytis cinerea* in China. (China Pesticide Information Network, http://www.chinapesticide.gov.cn) Since *B. cinerea* shows a high risk of resistance development[5, 6] and has already developed resistance to the various widely used fungicides,[7-18] as an effective alternative, pyrisoxazole has promising application prospect. However, the maximum residue limits (MRLs) of pyrisoxazole have not been set by any agencies yet. Previous studies[4, 19] have determined that pyrisoxazole is a mixture of two diastereomers, 3-[(3R,5R)-5-(4-chlorophenyl)-2,3-dimethyl-3-isoxazolidinyl] pyridine (3R, 5R-pyrisoxazole) and

3-[(3R,5S)-5-(4-chlorophenyl)-2,3-dimethyl-3-isoxazolidinyl] pyridine (3R, 5S-pyrisoxazole), as shown in Fig. 1. Isomers of one fungicide usually vary in metabolism and degradation rate.[20, 21] The bioactivity difference of the two isomers of pyrisoxazole has been proved.[19] There is concern about food safety caused by pyrisoxazole residues in tomato fruit as well as environmental safety caused by potential exposure of pyrisoxazole to human and wildlife from its residues in soil. The published papers[22-25, 27] for determination of pyrisoxazole residues were for analysis of pyrosoxazole as a mixture of its two isomers but did not provide analytical methods for individual isomers, even though they have different biological fates and endpoints. It is essential to establish a simple and efficient analytical method for the two isomers of pyrisoxazole for residue detection, safety evaluation and further study of their degradation pattern and environmental behavior.

To date, only a few papers have been published for determination of pyrisoxazole. Han et al. first established a macro-analysis method for quantitative determination of pyrisoxazole residue in tomato plant via reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a UV-detector in 2006.[22] This method was later employed by Chen et al. to study the systemic properties of pyrisoxazole in tomato seedling.[23] Afterwards, Feng et al. described a trace analysis method for determination of pyrisoxazole residue in tomato fruit using HPLC-UV. The quantification limit (LOQ) of pyrisoxazole in tomato fruit was 0.01 mg kg^{-1}.[24, 25] These previous researches mainly focused on using UV-detector in assist of detection and quantification of pyrisoxazole after separation from liquid chromatography (LC). In recent years, the mass spectrometry (MS) provided the wide analytical scope and high selectivity and sensitivity for detection.[26] Liu et al. has set up a new trace analysis method based on HPLC-MS for study of the metabolic behavior of pyrisoxazole in *B. cinerea*, the pathogen of tomato gray mold.[27] Until now, no analytical method has been published for determination of the two

isomers of pyrisoxazole, and the existing papers have never reported determination of pyrisoxazole residue in soil samples. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) combining the highly selective separation of HPLC with the sensitivity and specificity of tandem MS detection has become a reliable and sensitive tool in trace analysis of residue.[28, 29, 30] The aim of the present study was to develop a HPLC-MS/MS method for simultaneous determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil at trace levels. The HPLC-MS/MS conditions were optimized based on the selection of column, the evaluation of different mobile phase combinations, and the MRM conditions. The extraction and cleanup procedure were optimized based on the selection of extraction solvent and the selection of SPE tube. The method was validated by a conventional validation procedure to demonstrate the specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, accuracy and precision. The method was finally applied to the analysis of real samples. To our knowledge, this is the first report of a trace analysis method for simultaneous determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil based on HPLC-MS/MS.

Materials and methods

Chemicals and reagents

3R, 5R-pyrisoxazole (99.1% purity) and 3R, 5S-pyrisoxazole (98.1% purity) were provided by Shenyang Sciencreat Chemicals Co. Ltd. (Shenyang, China). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Massachusetts, USA). HPLC-grade formic acid was obtained from Sigma-Aldrich (St. Louis, USA). Analytical grade anhydrous sodium sulfate (Na_2SO_4) was purchased from Sinopharm (Shanghai, China). Ultra-pure water was prepared with Milli-Q system (Millipore, Massachusetts, USA).

A mixed standard stock solution of 3R, 5R-pyrisoxazole and 3R, 5S-pyrisoxazole (both 2000 mg L^{-1}) were prepared by dissolving them in pure acetonitrile. The standard working solutions at 10, 20, 50, 100, 200 and 500 μg L^{-1} were obtained by serially diluting the stock solution with acetonitrile. All solutions were protected against light with aluminum foil and stored in a refrigerator at 4 °C before use. The working standard solutions underwent no degradation for 3 months.

Instrumentation and analytical conditions

The analysis was performed on a Shimadzu LC-20A series HPLC (Shimadzu, Japan) equipped with a DGU-20A_3 online degasser, a LC-20AD high-pressure pump, a SIL-20AC automatic sampler, and a CTO-20A column heater, and on an API 3200 triple quadrupole (TQD) mass spectrometer (MS) (Applied Biosystems/MDS SCIEX, Singapore) equipped with an electrospray ionization (ESI) source.

Separation was achieved by HPLC with a XBridge™ C18 column (4.6×150 mm I.D., 5 μm, Waters, USA) as the analytical column and a SecurityGuard™ C18-ODS column (4 × 3.0 mm, Phenomenex, Torrance, USA) as the guard column. Mobile phase consisted of methanol (eluent A) and 0.1% (v/v) aqueous formic acid solution (eluent B). Isocratic elution was performed with a binary mixture of 70% eluent A and 30% eluent B at a flow rate of 0.6 mL min^{-1}. The injection volume for each sample was 10 μL and the total run-to-run time was 10 minutes. The column temperature was maintained at 30 °C and the temperature in the automatic sampler was set at 5 °C.

Quantification was achieved by MS/MS detection applying the multiple reaction monitoring (MRM) model while ESI was operated in positive ion mode. In order to obtain highest sensitivity and resolution, the monitoring conditions optimized for the target analytes were as follows: the ion source temperature was 400 °C, the ion spray voltage was 5,000 V, the declustering potential voltage was 30 V, the entrance potential voltage was 10 V, and the collision cell exit potential voltage was 4 V. Ion source

GAS1 and GAS2 were both nitrogen gas operated at a pressure of 50 psi. The curtain gas and collision gas were both nitrogen gas and respectively operated at a pressure of 10 and 3 psi. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions from m/z 289.1 to 120.2 (for both two isomers of pyrisoxazole) and the collision energy was 30 V. The Analyst 1.5.1 (Applied Biosystems, Singapore) software was employed to control the HPLC-MS/MS parameters and to acquire and process the data. The MRM mode was operated for the analytes for 100 ms. Under the described conditions, the retention time of 3R, 5S-pyrisoxazole and 3R, 5R-pyrisoxazole was respectively 5.5 min and 6.4 min.

Sample preparation

Tomato fruit samples. Blank tomato fruit samples were purchased from a local supermarket. Tomato fruit was cut into quartering. The diagonal two parts were picked, and then chopped and homogenized in an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany). The matrix was pre-checked to confirm the absence of the target analytes, and stored in the dark at -20 °C before analysis. An aliquot of 20 g thoroughly homogenized tomato fruit sample, was weighed and put into a 250 mL flask. Appropriate volumes of the standard working solutions were added to blank samples for the recovery studies, and the flasks containing the target samples were shaken by a digital orbital shaker (Changzhou Guohua Electrical Appliance Co. Ltd., Jiangsu Province, China) for 3 min and allowed to stand for 30 min at room temperature to distribute the pesticide evenly and to ensure complete interaction with the sample matrix. Then 30 mL acetonitrile was added. After shaking and extraction for 1 h, the sample was suction filtered by a Buchner funnel and then transferred into a 100 mL mixing cylinder with stopper. The flask, Buchner funnel and suction flask were rinsed twice with 5 mL acetonitrile each time. The rinse solutions were added into the 100 mL mixing cylinder which was later filled with acetonitrile

to 60 mL volume. Subsequently, 25 mL extracting solution was transferred into a 100 mL flask and 5 g anhydrous sodium sulfate (Na_2SO_4) was added to the flask. After 10 mins' standing, the water-free acetonitrile extracting solution was transferred into a 100 mL distillation bottle. The flask was rinsed with 15 mL acetonitrile, and the rinse solution was added into the distillation bottle. The extracting solution was vacuum distilled at 45 °C and -0.1 MPa to dry using a rotary evaporator (BUCHI Labortechnik AG, Switzerland). The remnant was dissolved in 1 mL acetonitrile and then added with 4 mL ultra-pure water. After well mixed, the sample solution was cleaned up by a GX-274 automated SPE system (Gilson, Middleton, WI, USA) equipped with a Supelclean™ ENVI™-18 SPE tube (3 mL/500 mg, Supelco, Bellefonte, PA). The SPE cleanup procedure was as follows: a) firstly, the tube was preconditioned with 5 mL acetonitrile and equilibrated with 5 mL ultra-pure water at a flow rate of 1 mL min^{-1}; b) then the sample was loaded and allowed to pass through the tube at a flow rate of 1 mL min^{-1}; c) afterwards, the tube was washed using 5 mL of ultra-pure water to remove any retained impurities; d) finally, elution was made using 5 mL 80% aqueous acetonitrile solution. The eluate was collected for the subsequent HPLC-MS/MS analysis.

Soil samples. Blank soil samples were collected from our experimental plots with no previous exposure to pyrisoxazole located in Liaoning Province of China and was ensured not containing the target analytes. Soil samples were passed through a 2.00 mm sieve and stored in the dark at -20 °C before analysis. The sample preparation procedure for soil samples was similar to that for tomato fruit samples. An aliquot of 20 g soil sample was weighed and put into a 250 mL flask. Blank samples were spiked with different concentrations of the standard working solutions for the recovery studies, and then the flasks containing the target samples were shaken by a digital orbital shaker (Changzhou Guohua Electrical Appliance Co. Ltd., Jiangsu Province, China) for 3 min and allowed to stand for 30

min at room temperature to distribute the pesticide evenly and to ensure complete interaction with the sample matrix. Then 30 mL acetonitrile was added. After shaking and extraction for 1 h, the sample was suction filtered by a Buchner funnel and then the filtrate was transferred into a 250 mL distillation bottle. The flask, the Buchner funnel and the suction filter were rinsed twice with 5 mL acetonitrile each time. The rinse solutions were also added into the 250 mL distillation bottle. The mixed solution was vacuum distilled at 45 °C and -0.1 MPa to dry using a rotary evaporator (BUCHI Labortechnik AG, Switzerland). The remnant was dissolved in 1 mL acetonitrile and then added with 4 mL ultra-pure water. After well mixed, the sample solution was further cleaned up by a SPE experiment whose procedure was the same as described before. The eluate was collected for the subsequent HPLC-MS/MS analysis.

Method validation

The method was validated by a conventional validation procedure to demonstrate the specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, accuracy and precision. Blank samples (tomato fruit and soil) were extracted and analyzed to make sure no interfering peaks around the retention time of the analytes under the same conditions. The linearity of the method was determined by least-squares linear regression analysis. The standard solutions and the matrix-matched standard solutions (tomato fruit and soil), spiked with the analytes at six concentrations (10, 20, 50, 100, 200 and 500 μg L^{-1}) were analyzed in triplicate by HPLC-MS/MS. The parameters of the linear regression equations including slope, intercept and the correlation coefficient (R) were calculated by Analyst 1.5.1 software. The LOD and LOQ for the analytes were defined as the concentration that produced a signal-to-noise (S/N) ratio of 3 and 10 respectively, and were estimated from the chromatogram corresponding to the lowest concentration. The matrix effect on the MS/MS detector

was calculated as follows: matrix effect (%) = (slope of calibration curves in matrix − slope of calibration curves in solvent)/ slope of calibration curves in solvent×100%.[31] There could be matrix-induced signal suppression if the value is negative and a matrix-induced signal enhancement if the value was positive. It was considered that the matrix-induced signal suppression/enhancement was mild when the value was in the range of ±20%, and medium when the value was between −50% and −20% or +20% and +50%, and strong when the value was lower than −50% or higher than +50%.[32]

A Recovery assay was carried out to investigate the accuracy and precision of the method. Five replicates of the blank samples (tomato fruit and soil) spiked at three concentration levels (10, 100 and 1000 μg kg^{1}) were prepared on three different days. The analytes were extracted and purified according to the above-mentioned procedure. The accuracy was expressed by the recovery of spiked samples. The precision were expressed as the intra-day and inter-day relative standard deviation (respectively RSD_r and RSD_R) for repeatability and reproducibility.

Results and discussion

Optimization of HPLC-MS/MS conditions

The selection of appropriate column plays an important role in improving the peak shape and the limit of quantification. In the present study, three Waters XBridge C18 columns of different length (respectively 100×4.6 mm I.D., 5 μm; 150×4.6 mm I.D., 5 μm; and 250×4.6 mm I.D., 5 μm) were used to obtain improved separation and peak shape. Relatively short columns were proved in favor of quick analysis which saves time and lessens the use of solvent. Relatively long columns were proved beneficial to the separation of samples. Taking the two aspects into consideration, a Waters XBridge C18 column (150×4.6 mm I.D., 5 μm) was selected as the separation column to achieve complete separation of the two analytes and to save the analysis time and solvent consumption.

In order to improve both LC separation and ionization efficiency, modification of the mobile phase with additives should be performed.[26] In this study, four different mobile phase combinations including methanol : water (70 : 30, v/v), methanol : 0.1% aqueous formic acid (70 : 30, v/v)，acetonitrile : water (70 : 30, v/v) and acetonitrile : 0.1% aqueous formic acid (70 : 30, v/v) were assayed with a 0.6 mL min^{-1} flow rate for optimization of the separation. The results showed that the analytes were well separated using methanol-water and methanol-0.1% aqueous formic acid. When having methanol-0.1% aqueous formic acid as the mobile phase, the best separation was achieved with a satisfactory peak shape and peak width. Therefore, chromatographic separation of the target analytes was finally performed with methanol- 0.1% aqueous formic acid. As shown in Fig. 2, the retention time of 3R, 5S-pyrisoxazole was 5.5 min and 3R, 5R-pyrisoxazole 6.4 min, and there were no interference peaks around the retention time of the analytes.

For identification and quantification of the analytes at trace levels, syringe pump infusion experiment with tuning standard solution of the two isomers of pyrisoxazole (1 mg L^{-1}) prepared in acetonitrile was performed in MS. The signal intensities observed in the precursor and production mass spectra in full scan mode were investigated in both positive and negative ionization modes. The result showed that greater signal intensities were observed in the positive ionization mode with intense protonated molecular ion peaks, $[M+H]^+$, for both isomers of pyrisoxazole at m/z 289.1. The MS/MS fragmentation behaviors of the two isomers of pyrisoxazole were the same, as shown in their MS/MS spectra. For the analyts, m/z at 120.2, 80.1, and 210.0 were the major fragment ions of its $[M+H]^+$ m/z 289.1 in MS/MS spectra, and the most sensitive response was obtained for transitions from m/z 289.1 to 120.2 (Fig. 3). Furthermore, the ion source temperature, the ion spray, declustering potential, entrance potential and collision cell exit potential voltage, the ion sources GAS1 and GAS2, the curtain

gas and collision gas, and the collision energy were all optimized to increase instrument response (data mentioned above).

Optimization of the extraction and cleanup procedure

The selection of solvent was essential for efficient extraction. In this paper, acetonitrile, acetone and methanol were evaluated as extraction solvent by recovery assay using five replicates of the blank samples (tomato fruit and soil) spiked at 100 μg kg^{1}. 30 mL of each solvent was used in extraction procedure described above. The results showed that acetonitrile had the best extraction efficiency, followed by methanol and acetone (Table 1). Consequently, acetonitrile was selected as the extraction solvent. In consideration of the high water content in tomato fruit, anhydrous sodium sulfate (Na_2SO_4) was used for dehydration prior to distillation so that the extracting solution can be dried under a relative low temperature (45 °C).

In order to remove the possible matrix effects, an SPE cleanup procedure was undertaken for the extract samples prior to HPLC-MS/MS analysis. As reported previously[24]，a C18 SPE tube was found to show good retention for pyrisoxazole samples. In this study, a GX-274 automated SPE system (Gilson, Middleton, WI, USA) equipped with a Supelclean™ ENVI™-18 SPE tube (3 mL/500 mg, Supelco, Bellefonte, PA) was employed for cleanup procedure，in order to increase efficiency and minimize errors caused by manual operation. The tube was firstly washed with 5 mL acetonitrile to eliminate the impurities in the sorbent and then equilibrated with 5 mL water to create an environment appropriate for sample loading. The elution effect of aqueous acetonitrile solutions in different proportions was investigated. According to the elution curve (Fig. 4), the two isomers of pyrisoxazole remained in the sorbent and could not be eluted out of the tube with 5 mL water, which hence was adopted as the washing solution to remove the interferences in sample matrices. The two isomers of

pyrisoxazole could be completely eluted with 5 mL of 80% aqueous acetonitrile solution, which was used as the elution solution.

Method validation

Specificity. As shown in Fig. 2, there were no interfering peaks around the retention time of the two isomers of pyrisoxazole, which proved that the present assay is selective for the target analytes and suitable for their analysis in tomato fruit and soil.

Linearity, LOD and LOQ. A separate standard calibration curve for each pyrisoxazole isomer was constructed by plotting peak area (y) versus the corresponding concentration (x, μg L^{-1}) with a weighed factor ($1/x^2$). The linear range for the analytes was 10-500 μg L^{-1}. The regression equations and correlation coefficients (R) of the standard solution curves and the matrix-matched curves for the two analytes were shown in Table 2. Satisfactory linearities were observed for both isomers of pyrisoxazole ($R > 0.998$ in all cases). For the two isomers of pyrisoxazole, the limits of detection (LODs) were below 0.8 μg kg^{-1}, and the limits of quantification (LOQs) were below 2.8 μg kg^{-1}.

Matrix effect. The matrix effect values for the anaytes were all between 0% and -20% in the two matrices (Table 2), which indicated a mild matrix suppression effect. Therefore, the external matrix-matched calibration standards were used in order to eliminate the matrix effect and to obtain more realistic results in all samples in this study.

Accuracy and precision. The recovery results and the RSD values of the two isomers of pyrisoxazole in tomato fruit and soil are shown in Table 3. The proposed method presented satisfactory accuracy with mean recovery of 80.0–91.5% and satisfactory precision with all RSD values below 8.5% at the three concentration levels for the two isomers of pyrisoxazole in the two matrices. The mean recoveries for the two isomers of pyrisoxazole ranged from 80.0% to 89.6% with RSD_r of 2.1–6.5% and RSD_R of

4.2–5.4% in tomato fruit; and from 80.7% to 91.5% with RSD_r of 1.8–8.5% and RSD_R of 3.1–6.0 % in soil.

Application to real sample. In this study, a new method for simultaneous determination of the two isomers of pyrisoxazole at trace level was established. The effectiveness and applicability of the proposed analytical method were evaluated by analyzing real samples (tomato samples purchased from local markets and soil samples collected from Liaoning Province of China). A total of 30 samples (15 samples for tomato fruit and 15 samples for soil) were analyzed, and the two isomers of pyrisoxazole were not detected in all the real samples.

Conclusions

In the present study, a simple, sensitive and accurate analytical method based on HPLC-MS/MS for determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil at trace levels was established and validated for the first time. Three extraction solvents were evaluated for the optimization of extraction procedure, and acetonitrile was chosen. Automated SPE system was employed in order to achieve better cleanup efficiency and less error caused by manual operation. Three analytical columns of different length and four different mobile phase combinations were compared for optimization of the HPLC conditions. The MRM conditions for MS/MS were also optimized. Satisfactory selectivity, linearity, accuracy, precision and repeatability were obtained. The LODs and LOQs were sufficiently low for determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil samples at trace levels. The application of this method on real samples validated its reliability and efficacy for routine simultaneous determination of residues of the two isomers of pyrisoxazole in tomato fruit and soil samples. The proposed method can facilitate further studies in analyzing and comparing the bioactivities, toxicities, metabolisms and environmental behaviors of the

two isomers of pyrisoxazole, and finally help to minimize the risks to the environment and human health posed by pyrisoxazole.

References

1. N. G. Si, Z. J. Zhang, J. L. Liu, Z. N. Li, D. M. Zhan, L. Chen and L. Z. Wang, *Chin. J. Pestic.*, 2004, **43**, 61-63.
2. J. L. Liu, N. G. Si, L. Chen, D. M. Zhang and Z. J. Zhang, *Chin. J. Pestic.*, 2004, **43**, 103-105.
3. FRAC (Fungicide Resistance Action Committee), 2015, http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2015-finalC2AD7AA36764.pdf?sfvrsn=4.
4. F. P. Chen, P. Han, P. F. Liu, N. G. Si, J. L. Liu and X. L. Liu, *Sci. Rep.*, 2014, **4**, 6473.
5. FRAC (Fungicide Resistance Action Committee), 2013, http://www.frac.info/docs/default-source/publications/pathogen-risk/pathogen-risk-list.pdf?sfvrsn=8.
6. P. E. Russell, 2004, http://frac.sw.aa-g.de/docs/default-source/publications/monographs/monograph-3.pdf?sfvrsn=8.
7. P. Leroux, in: *Botrytis: Biology, Pathology and Control*, Springer, 2007, pp. 195-222.
8. S. Topolovec-Pintarić, in: *Fungicides - Beneficial and Harmful Aspects*, 2011, pp. 19-44.
9. C. K. Myresiotis, G. S. Karaoglanidis and K. Tzavella-Klonari, *Plant Dis.*, 2007, **91**, 407-413.
10. C. Q. Zhang, J. W. Zhu, F. L. Wei, S. Y. Liu and G. N. Zhu, *Phytoparasitica*, 2007, **35**, 300-313.
11. M. Esterio, C. Ramos, A. S. Walker, S. Fillinger, P. Leroux and J. Auger, *Phytopathol. Mediterr.*, 2011, **50**, 414−420.
12. H. Ishii, J. Fountaine, W. H. Chung, M. Kansako, K. Nishimura, K. Takahashi and M. Oshima,

Pest Manage. Sci., 2009, **65**, 916–922.

13. Y. H. Huangfu, D. J. Dai, H. J. Shi, Z. H. Xu and C. Q. Zhang, *Chin. J. Pestic. Sci.*, 2013, **15**, 504-510.
14. Y. K. Kim and C. L. Xiao, *Plant Dis.*, 2010, **94**, 604-612.
15. G. A. Bardas, T. Veloukas, O. Koutita and G. S. Karaoglanidis, *Pest Manage. Sci.*, 2010, **66**, 967–973.
16. D. Fernández-Ortuño, F. P. Chen and G. Schnabel, *Plant Dis.*, 2012, **96**, 1198-1203.
17. A. Amiri, S. M. Heath and N. A. Peres, *Plant Dis.*, 2014, **98**, 532-539.
18. X. P. Li, D. Fernández-Ortuño, A. Grabke and G. Schnabel, *Phytopathology*, 2014,**104**, 724-732.
19. Y. L. Huang, M. Y. Ning, P. F. Liu, Z. Q. Huang, J. L. Liu and X. L. Liu, *Acta Phytopathologica Sinica*, 2013, **43**, 549-555.
20. T. A. Müller and H.-P. E. Kohler, *Appl. Microbiol. Biotechnol.*, 2004, **64**, 300-316.
21. D. L. Lewis, A. W. Garrison, K. E. Wommack, A. Whittemore, P. Steudler and J. Melillo, *Nature*, 1999, **401**, 898-901.
22. P. Han, P. F. Liu, N. G. Si and X. L. Liu, *Chin. J. Pestic. Sci.*, 2006, **8**, 288-290.
23. F. P. Chen, P. Han, J. L. Liu, N. G. Si, Y. H. Wang, P. F. Liu and X. L. Liu, *Chin. J. Pestic. Sci.*, 2014, **16**, 144-152.
24. D.L. feng, C. P. Zhang, M. F. Xu, C. S. Cheng, J. C. Zheng, H. Y. Qiu and Q. Wang, *Agrochemicals*, 2013, **52**, 587-589.
25. D.L. feng, J. C. Zheng, W. X. Chen, L. M. Cai, M. F. Xu, H. Y. Qiu and Q. Wang, *Food Sci.*, 2014, **35**, 224-228.
26. H. H. Zhao, J. Xu, F. S. Dong, X. G. Liu, Y. B. Wu, J. G. Zhang and Y. Q. Zheng, *Anal. Methods*,

2014, **6**, 4336.

27. P. F. Liu, Y. X. Liu, P. Han, J. Q. Li, J. L. Liu and X. L. Liu, *Chin. J. Anal. Chem.*, 2011, **39**, 317-322.

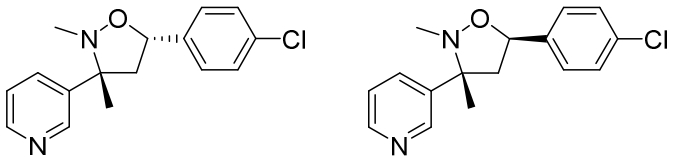
28. U. Koesukwiwat, S. J. Lehotay, S. Miao and N. Leepipatpiboon, *J. Chromatogr. A*, 2010, **1217**, 6692–6703.

29. F. Dong, L. Cheng, X. Liu, J. Xu, J. Li, Y. Li, Z. Kong, Q. Jian and Y. Zheng, *J. Agric. Food Chem.*, 2012, **60**, 1929–1936.

30. S. J. Lehotay, K. Son, H. Kwon, U. Koesukwiwat, W. Fu, K. Mastovska, E. Hoh and N. Leepipatpiboon, *J. Chromatogr. A*, 2010, **1217**, 2548–2560.

31. Y. P. Cheng, F. S. Dong, X. G. Liu, J. Xu, W. Meng, N. Liu, Z. L. Chen, Y. Tao and Y. Q. Zheng , *Anal. Methods*, 2014, **6**, 1788.

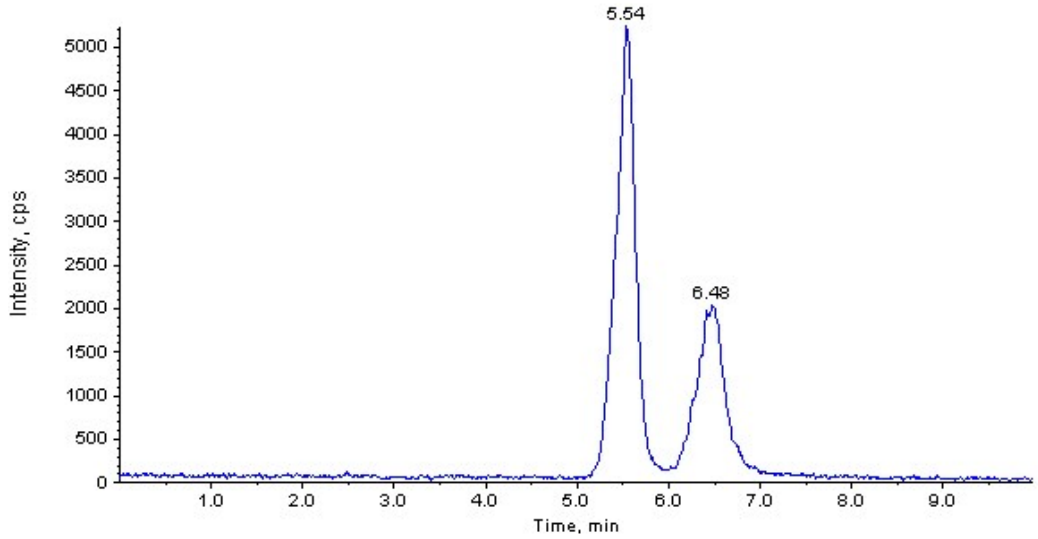
32. B. Kmellár, P. Fodor, L. Pareja, C. Ferrer, M. A. Martínez-Uroz, A. Valverde and A. R. Fernandez-Alba, *J. Chromatogr. A*, 2008, **1215**, 37-50.



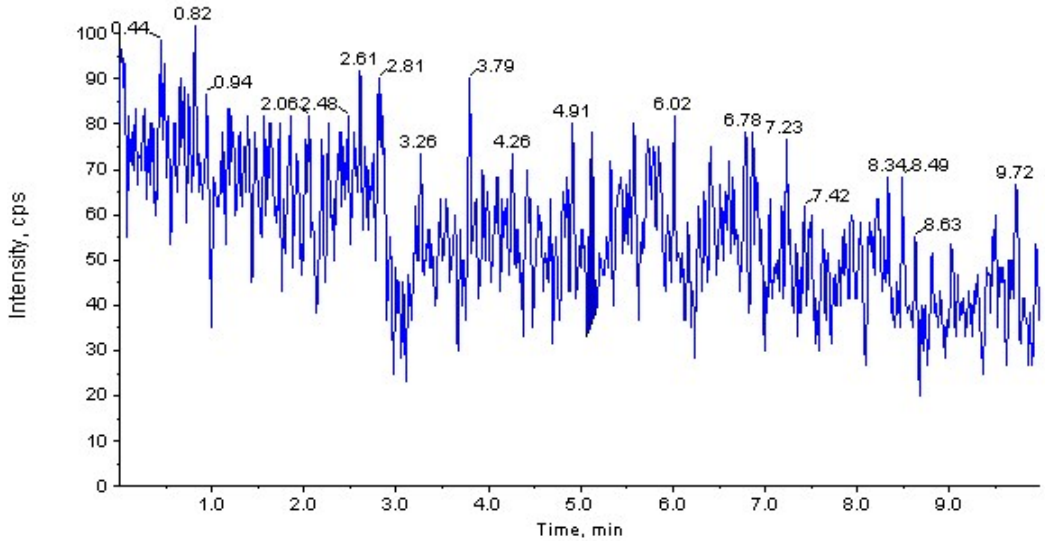
3R, 5S-pyrisoxazole 3R, 5R-pyrisoxazole

Fig.1 The chemical structures of the two diastereomers of pyrisoxazole

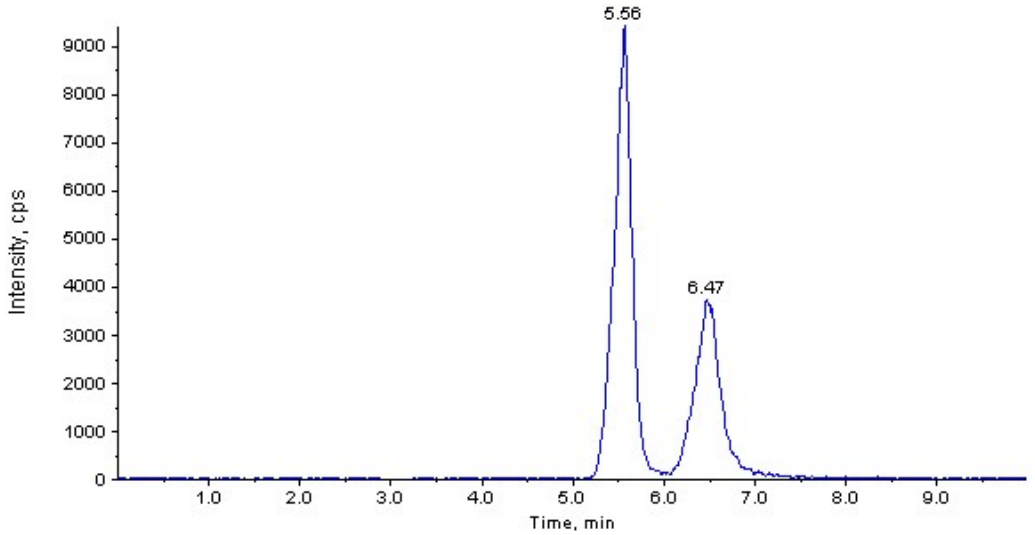
A



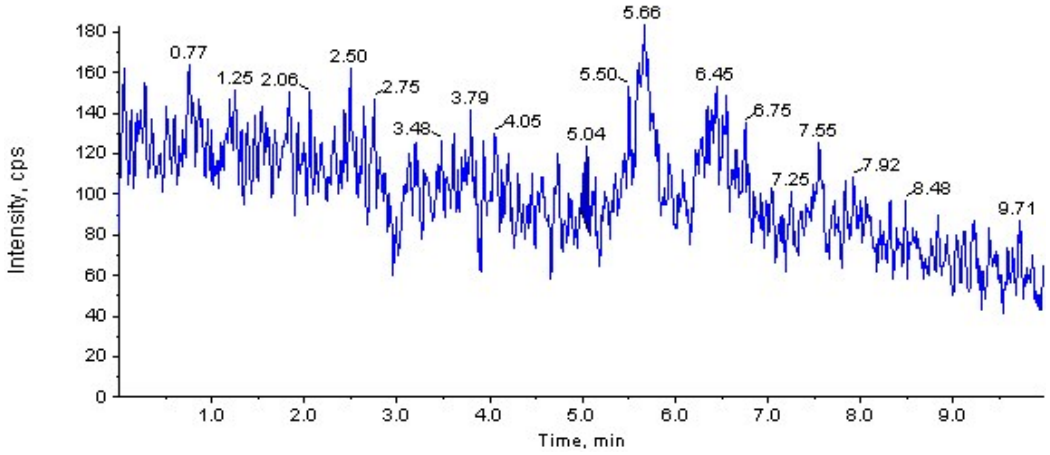
B



C



D



E

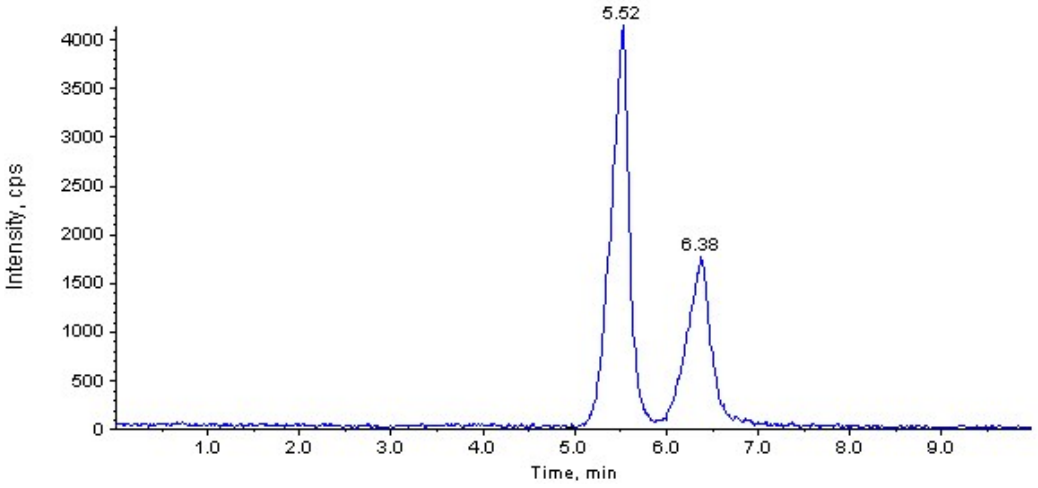
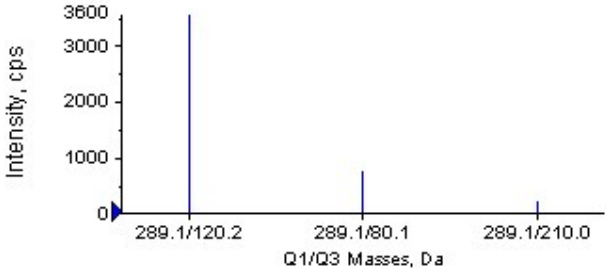
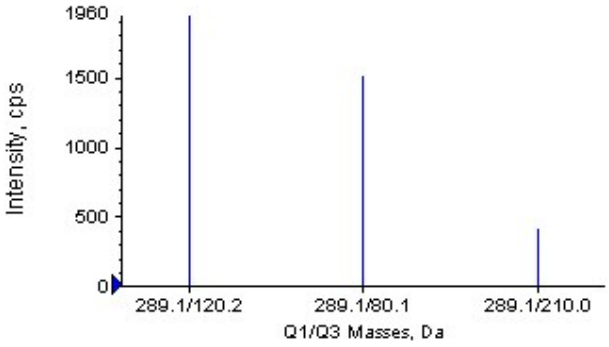


Fig.2 HPLC-MS/MS ion chromatograms of (A) mixed standard of 3R, 5R-pyrisoxazole and 3R, 5S-pyrisoxazole (B) blank soil sample, (C) soil spiked sample at 10 μg kg^{-1}, (D) blank tomato fruit sample, (E) tomato fruit spiked sample at 10 μg kg^{-1}.

.



3R, 5S-pyrisoxazole



3R, 5R-pyrisoxazole

Fig. 3 Product ion scan of the two isomers of pyrisoxazole (m/z 289.1) measured on an API 3200 triple quadrupole MS equipped with an ESI source under the positive ion mode.

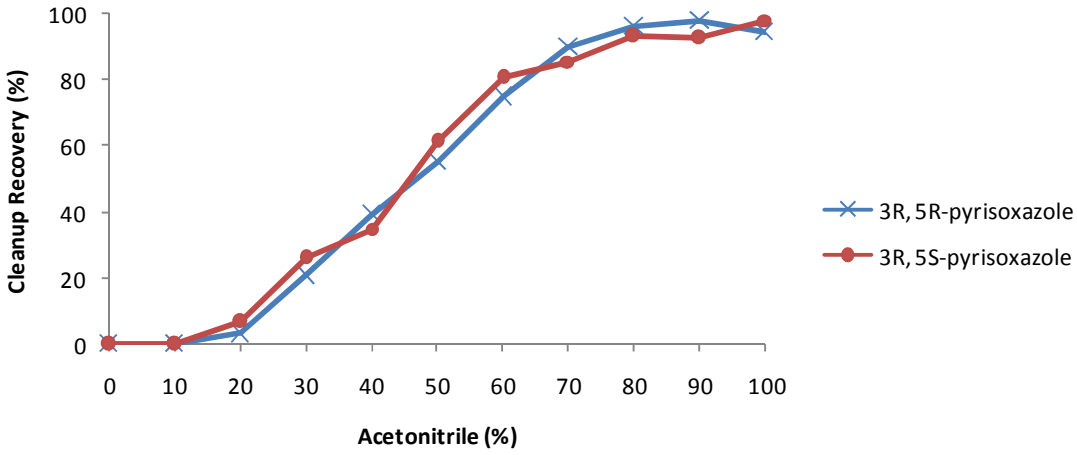


Fig. 4 SPE elution curve for the two isomers of pyrisoxazole on a C18 SPE tube

Table 1 Recoveries (n =5, percent) for the two isomers of pyrisoxazole from tomato fruit and soil at 100 μg kg^{-1} using three different extraction solvents

		Acetonitrile		Methanol		Acetone	
Compound	Matrix	Average recoveries (%)	RSD_r (%)	Average recoveries (%)	RSD_r (%)	Average recoveries (%)	RSD_r (%)
3R, 5S-	Soil	88.1	4.0	82.1	4.2	73.8	1.7
pyrisoxazole	Tomato fruit	85.7	6.2	79.7	8.1	75.3	4.9
3R, 5R-	Soil	84.7	4.9	80.4	6.5	78.5	6.8
pyrisoxazole	Tomato fruit	88.1	5.4	81.1	3.7	74.7	7.3

Table2 Linear regression equations, LOD, LOQ and matrix effect of the two isomers of pyrisoxazole

Analyte	Matrix	Regression equation	R	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	Matrix effect (%)
3R, 5S-pyrisoxazole	Acetonitrile	y=7370x-2870	0.9991	0.3	0.9	-
3R, 5S-pyrisoxazole	Soil	y=6100x+2910	0.9989	0.3	1.0	-17.2
3R, 5S-pyrisoxazole	Tomato fruit	y=6050x+3530	0.9987	0.4	1.4	-17.9
3R, 5R-pyrisoxazole	Acetonitrile	y=4190x-2960	0.9993	0.6	2.1	-
3R, 5R-pyrisoxazole	Soil	y=3470x+1240	0.9995	0.5	1.8	-17.2
3R, 5R-pyrisoxazole	Tomato fruit	y=3360x-943	0.9991	0.8	2.8	-19.8

Table 3 Recoveries (n =15, percent) and RSD_r and RSD_R for the two isomers of pyrisoxazole from tomato fruit and soil at three concentration levels[a]

			Intra-day (n =15)						Inter-day (n =15)	
			Day 1		Day 2		Day 3			
Compound	Matrix	Spiked level ($\mu g\ kg^{-1}$)	Average recoveries (%)	RSD_r (%)	Average recoveries (%)	RSD_r (%)	Average recoveries (%)	RSD_r (%)	Average recoveries (%)	RSD_R (%)
3R, 5S-	Soil	10	80.7	6.8	81.8	4.3	84.7	6.8	82.4	6.0
pyrisoxazole		100	88.1	4.0	87.0	4.4	83.4	5.9	86.2	5.0
		1000	91.5	4.1	90.9	3.1	87.7	4.2	90.1	4.0
	Tomato fruit	10	84.9	5.4	83.4	5.4	80.0	4.3	82.8	5.4
		100	85.7	6.2	86.7	4.5	85.0	6.3	85.8	5.4
		1000	83.6	3.1	85.5	6.5	85.4	6.1	84.8	5.2
3R, 5R-	Soil	10	81.8	1.8	81.6	7.7	82.1	6.7	81.8	5.5
pyrisoxazole		100	84.7	4.9	85.9	3.9	84.0	8.5	84.9	5.7

	1000	88.5	2.8	85.6	1.8	86.0	3.6	86.7	3.1
Tomato fruit	10	83.8	4.0	86.0	2.1	86.4	5.9	85.4	4.2
	100	88.1	5.4	86.2	3.0	86.7	6.1	87.0	4.8
	1000	88.7	6.2	89.6	2.5	85.5	2.4	87.9	4.4

[a] RSDr stands for the intra-day precision, the relative standard deviations for repeatability (n =5); RSD_R stands for the inter-day precision, the relative standard deviations for reproducibility (n =15)