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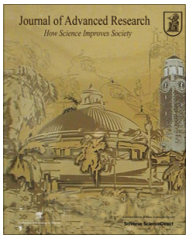

ORIGINAL ARTICLE

Determination of kresoxim-methyl and its thermolabile metabolites in pear utilizing pepper leaf matrix as a protectant using gas chromatography

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ABSTRACT

Kresoxim-methyl and its two thermolabile metabolites, BF 490-2 and BF 490-9, were analyzed in pear using a pepper leaf matrix protection to maintain the metabolites inside the gas chromatography system. Samples were extracted with a mixture of ethyl acetate and *n*-hexane (1:1, v/v) and purified and/or separated using a solid phase extraction procedure. The pepper leaf matrix was added and optimized with cleaned pear extract to enhance metabolite sensitivity. Matrix matched calibration was used for kresoxim-methyl in the pear matrix and for metabolites in the pear mixed with pepper leaf matrix. Good linearity was obtained for all analytes with a coefficient of determination, $r^2 \geqslant 0.992$. Limits of detection (LOD) and quantification (LOQ) were 0.006 and 0.02 mg kg^{-1} and 0.02 and 0.065 mg kg^{-1} for kresoxim-methyl and the metabolites, respectively. Recoveries were carried out at two concentration levels and were 85.6–97.9% with a relative standard deviation $<2.5\%$. The method was successfully applied to field incurred pear samples, and only kresoxim-methyl was detected at a concentration of 0.03 mg kg^{-1}.

Introduction

Kresoxim-methyl(methyl(E)-2-(methoxyimino)-2-[2-(*o*-tolyloxymethyl)phenyl]acetate), a strobilurin fungicide, is used to control powdery mildew and scab in apples, pears, grapes, cucumbers, strawberries, and vegetables [1]. The mode of action of strobilurins is to inhibit mitochondrial respiration by binding to the ubihydroquinone oxidation center of the mitochondrial bc1 complex and thereby blocking electron transfer [2,3]. The major reasons for the success of strobilurins

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vary between individual active ingredients, but consist of one or more of the following: broad spectrum activity, control of fungal isolates resistant to other fungicide modes of action, low use rate, and excellent yield and quality benefits [4]. However, residues may remain in the crops and environment and might constitute a public health hazard to consumers. Thus, residues are regulated in different countries in terms of maximum residue limits (MRLs) to maintain food quality and prevent consumer health problems.

Kresoxim-methyl is registered in the Republic of Korea for application on pear with maximum residue limits of 1.0 mg kg^{-1} [5]. The European Commission has revised the residue definition of kresoxim-methyl and proposed the sum of total kresoxim-methyl and its metabolites, α-[(*o*-hydroxymethyl)phenoxy]-*o*-tolyl(methoxyimino) acetic acid (BF 490-2) and α-(*p*-hydroxy-*o*-tolyloxy)-*o*-tolyl(methoxyimino) acetic acid (BF 490-9) for risk assessment [6]. The chemical structures of kresoxim-methyl and its two metabolites are shown in Fig. 1.

Kresoxim-methyl has been analyzed by gas chromatography/nitrogen phosphorus detector (NPD)/mass spectrometry (MS) or a liquid chromatography/electrospray tandem mass spectrometry in different matrices [7–9]. No analytical method has been reported to analyze the metabolites by gas chromatography until a method was developed for total residue analysis in Korean plum in our laboratory [10]. An unpublished complex analytical method was found to analyze – kresoxim-methyl and its metabolites using liquid chromatography [11]. However, we showed in our previous study that the two metabolites of kresoxim-methyl, BF 490-2 and BF 490-9, had poor responses or peak broadening when injected into GC-μECD in pure solvent. It was really tough to integrate and analyze these types of peak due to higher detection limit and also for overestimation when compared with solvent calibration. Erney and his co-workers explained this overestimation and named it the "matrix-induced response enhancement effect." Finally, they tried to remove this effect using single additive (as a protectant) aiming to protect the analyte in solvent and subsequently equalize the response between solvent and in matrix. However, their efforts were not successful, and they suggested to use matrix matched calibration [12,13]. Anastassiades et al. in 2003 [14] re-introduce the concept of additives as analyte protectant and evaluated 93 compounds with strong hydrogen bonding capability, whereas Mastovska et al. in 2005 [15] determined that a combination of three compounds (ethylglycerol, gulonolactone, and sorbitol) among the 93 compounds provided perfect protection for the thermally affected compounds in gas chromatography-mass spectrometry. However, their application range was limited due to the solubility of the protectant was polar-dependent, here up to 20% water was needed to be mixed with acetonitrile to dissolve them. Furthermore, the applicability of the combined analyte protectant was not examined for other detectors, including ECD (electron-capture detector), FPD (flame photometric detector), or NPD (nitrogen–phosphorus detector). On the other hand, in our early studies, pepper leaf matrix was a promising analyte protectant for thermolabile metabolites such as terbufos metabolites (terbufos sulfoxide and terbufoxon sulfoxide) and kresoxim-methyl metabolites (BF 490-2 and BF-490-9) using a FPD and a ECD, respectively. A pepper leaf matrix was incorporated with the pepper and plum matrix and provided complete protection for the metabolites inside the GC system [16,17].

Therefore, the aim of this study was to adapt and optimize our previous method for analyzing kresoxim-methyl and its metabolites to determine the total field incurred residues in pear.

Material and methods

Chemicals and reagents

Analytical standard kresoxim-methyl (purity 99.9%) and two metabolites, BF 490-2 (purity 94.6%) and BF 490-9 (purity 99.7%), were purchased from Badische Anilin-und Soda-Fabrik (BASF, Seoul, Republic of Korea). High performance liquid chromatography grade ethyl acetate (EtOAc), acetone, and *n*-hexane were supplied by Burdick and Jackson (SK Chemical, Ulsan, Republic of Korea). Anhydrous magnesium sulfate ($MgSO_4$) was of analytical grade and obtained from Junsei Chemicals Co., Ltd. (Tokyo, Japan). A C_{18}-E solid phase extraction (SPE) cartridge (500 mg, 6 mL) was provided by Phenomenex (Torrance, CA, USA).

A standard stock solution of kresoxim-methyl and two metabolites (BF 490-2 and BF 490-9) were prepared individually in EtOAc at a concentration of 100 mg L^{-1} and stored at −24 °C. An intermediate solution was prepared by diluting kresoxim-methyl to 10 mg L^{-1} and mixing metabolites together to attain 10 mg L^{-1} using the same solvent. Finally, intermediate solutions were diluted separately to 0.05 mg L^{-1} using EtOAc to make a working solution. Both intermediate and working solutions were kept in a refrigerator at 4 °C pending analysis.

Field experimental design

As Naju (Southern part of Gwangju, Republic of Korea) is famous for pear cultivation, a field study was conducted at the Naju Agricultural Farm affiliated with Chonnam National University, Gwangju, Republic of Korea. Thirteen mature trees (14 years old) in the same row were selected for applying a commercial pesticide after dividing the rows in different segments for various application times. The experimental

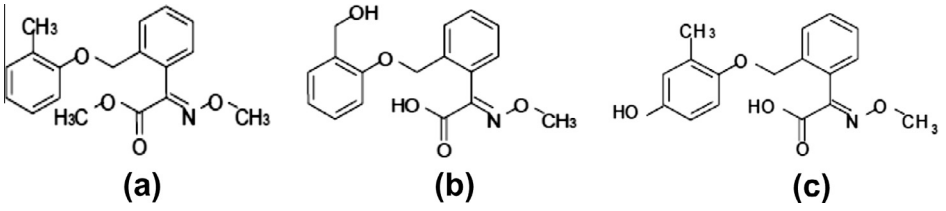

Fig. 1 Chemical structure of kresoxim-methyl (a) and its metabolites BF 490-2 (b) and BF 490-9 (c).

row was divided into five segmented plots where two plots were used for triple doses of applications and two plots were used for quadruple doses of applications. The remaining plot was considered a control and did not receive any pesticide treatment. Commercial pesticide (Allready®, suspension concentrate containing 20% kresoxim-methyl, provided by kyung Nong Co., Seoul, Republic of Korea) was diluted 2000 times with water and sprayed at a.i. 0.05 kg 10 a^{-1} during fruit maturation according to the manufacturer recommendations. Samples were collected from the first two plots after 14, 21, and 30 days and 21, 30, and 40 days post-application. Similarly, samples were collected after 21, 30, 40, and 50 days and 14, 21, 30, and 40 days post-application from the second two plots. The collected pear samples (12 pear samples from each plot) were transferred to the laboratory, chopped, and blended. The homogenized samples were then stored at −24 °C until analysis.

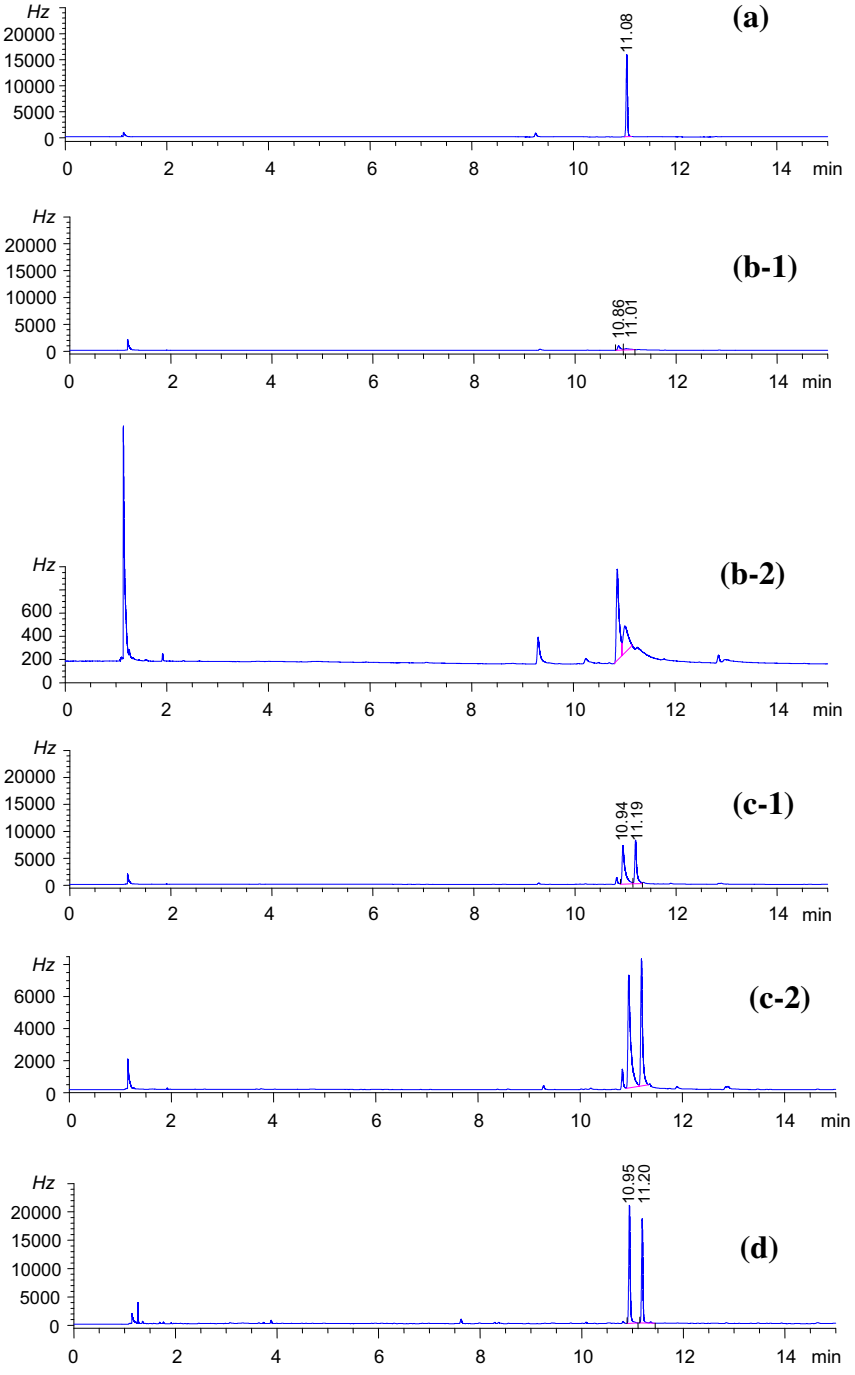

Fig. 2 Gas chromatography-μECD chromatograms of (a) 5 ppm standard kresoxim-methyl insolvent; standard mixture of 5 ppm BF 490-2 and BF 490-9 in solvent (b-1) at large window, (b-2) at short window; in pear matrix (c-1) at large window, (c-2) at short window, and in pear and pepper leaf matrix (d).

Sample extraction and cleanup

Approximately 10 g of homogenized sample was weighed into a 50-mL Teflon centrifuge tube, and 20 mL of EtOAc-*n*-hexane (1:1, *v*/*v*) was added to the tube and vigorously shaken by hand for 1 min. Six grams of anhydrous $MgSO_4$ was then added and shaken again for 30 s. The extract was centrifuged for 5 min at 3000 rpm. Ten milliliter of the upper layer was transferred to a 20-mL vial and evaporated to dryness under vacuum at a temperature < 40 °C.

A C_{18}-E SPE cartridge was conditioned with 6 mL of acetone. The dried extract was dissolved in 6 mL *n*-hexane and loaded onto the cartridge. First, kresoxim-methyl was eluted with 8 mL 1% acetone in *n*-hexane. The remaining analytes were then washed with 6 mL 5% acetone in *n*-hexane. Finally, BF 490-2 and BF 490-9 were eluted with 10 mL 20% acetone in *n*-hexane. The first and second eluates were separately evaporated under a vacuum; the first eluate (kresoxim-methyl) was dissolved in 2 mL EtOAc and the second (BF 490-2 and BF 490-9) was dissolved in pepper leaf extract (0.25 g mL^{-1}) [10].

Instrument

An Agilent gas chromatography model 7890A (Palo Alto, CA, USA) equipped with an Agilent 7683 B autosampler and a microelectron-capture detector (μECD, 63Ni) were used for analysis. A standard split/splitless injector was used in the split injection mode at a ratio of 10:1 at 270 °C with an injection volume of 1 μL. A HP-5 capillary column (30 m × 0.25 mm id, 0.25 μm film thickness with nitrogen gas flowing at 2 mL/min) was employed for separation. The detector was maintained at 300 °C with makeup gas (N_2) flowing at 60 mL/min. The oven temperature was set to 100 °C for 1 min, ramped to 280 °C at 15 °C /min, and held for 2 min. Under these conditions, BF 490-2 and BF 490-9 appeared at average retention times of 10.95–11.20 min. An Agilent Chemstation was used for data acquisition.

Method validation

The method was validated by a recovery experiment in triplicate at two fortification concentrations equivalent to 0.2 mg kg^{-1} and 1.0 mg kg^{-1} and compared with the matrix matched external standard calibration, which was previously assessed by linearity and accuracy was expressed as a percentage of recovery. The sensitivity of the method was determined from limit of detection ($S/N \geqslant 3$) and quantification ($S/N \geqslant 10$). The precision (repeatability) of the method was evaluated via the relative standard deviation (RSD)% obtained from the replicated analysis during recovery experiment.

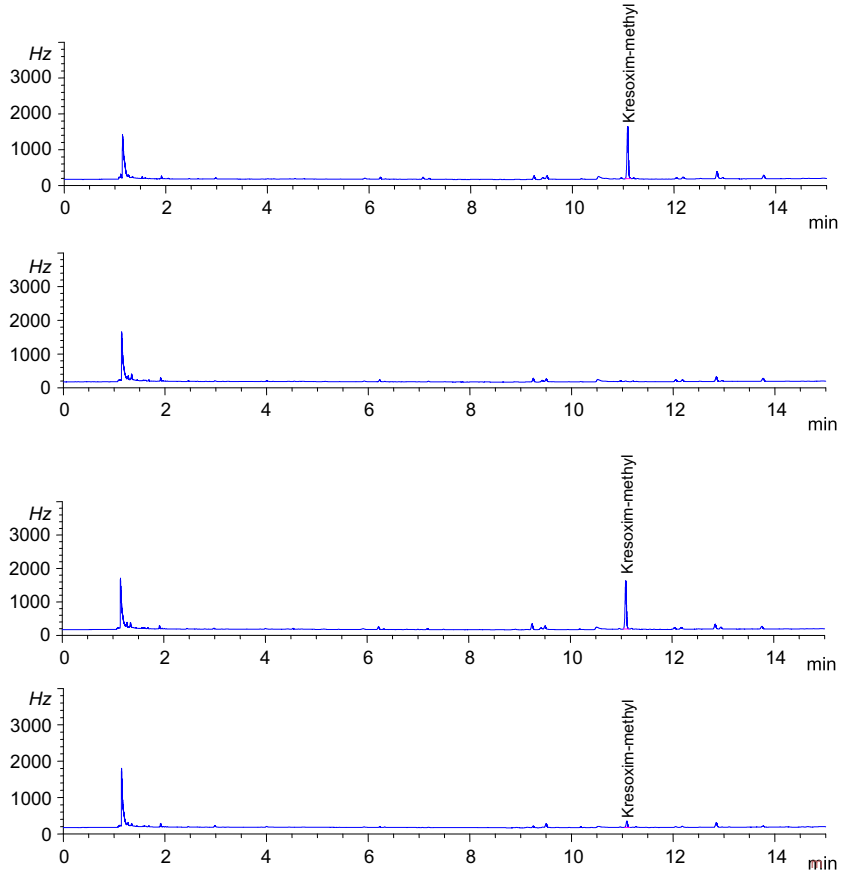

Fig. 3 Chromatogram of kresoxim-methyl (a) standard 0.5 mg kg^{-1} in matrix, (b) blank sample, (c) recovery equivalent to 0.5 mg kg^{-1}, and (d) field incurred pear sample.

Results and discussion

Matrix protection for sensitive GC analysis

Due to the high temperatures inside the injection port, column oven, and detector in a gas chromatograph, the analyte may undergo decomposition. Thus, poor peak/peak broadening/or altered peaks are quite impossible to be integrated and analyzed. As the GC system (injection port, column, and detector) is not completely inert, the principal cause of analyte degradation/decomposition is reaction with active sites (silanols, metal ions, and other active sites on the surfaces) during their journey from the injector to detector. The matrix can deactivate these active sites and increase the transfer of analyte to the detector; consequently, a good response with sharp peak will be attained. However, deactivation capacity varies from matrix to matrix, as it depends on the components in each matrix. In our previous studies, we showed that a pepper leaf matrix had more deactivating capability than other matrices and protects thermolabile compounds inside the GC system [10,12]. Therefore, the pepper leaf matrix was optimized and added with the pear matrix in the present study to protect thermolabile BF 490-2 and BF 490-9 because the solvent or pear matrix alone could not protect them against decomposition (Fig. 2).

Validation of analytical method

Selectivity and specificity of the method was achieved from standards, blanks, and recovery, and identical retention times were found for all except the blank. Moreover, no significant noise was detected in a blank chromatogram within the retention times of the standards. The chromatograms of standard, blank, recovery, and field incurred sample for both kresoxim-methyl and its metabolites are shown in Figs. 3 and 4.

Linearity of the calibration curve was established for all analytes. The squared correlation coefficient (r^2) both in pure solvent-based and matrix matched was $\geqslant 0.992$ for all compounds except the metabolites in pure solvent. The limit of quantification (LOQ) for all of the analytes was $\leqslant 0.065\ \mathrm{mg\ kg^{-1}}$, which was 10 times lower than the MRL established by the Korea Food and Drug Administration (KFDA) [5]. The matrix-induced enhancement in target signals was prominent for metabolites; however, clean pear matrix alone failed to provide sufficient sensitivity after being enhanced. Therefore, an optimized amount of pepper leaf matrix ($0.25\ \mathrm{g\ mL^{-1}}$) was added as a protectant for the metabolites.

Table 1 shows the recovery data and repeatability (RSD) for kresoxim-methyl and the two metabolites analyzed at two different spiking levels. The recoveries and RSD were

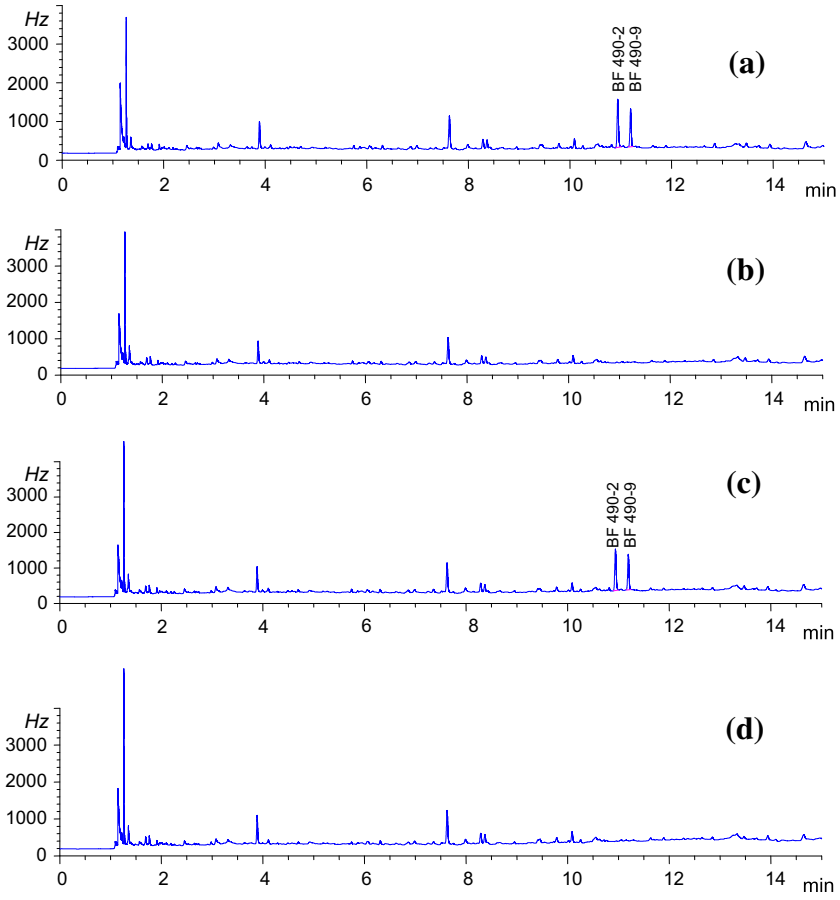

Fig. 4 Chromatogram of BF 490-2 and BF 490-9 (a) standard $0.5\ \mathrm{mg\ kg^{-1}}$ in matrix, (b) blank sample, (c) recovery equivalent to $0.5\ \mathrm{mg\ kg^{-1}}$, and (d) field incurred pear sample.

Table 1 Correlation coefficient (r^2), limit of detection (LOD), limit of quantification (LOQ), and recovery of kresoxim-methyl, BF 490-2, and BF 490-9 in pear.

Compound	r^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Recovery (mean, RSD%)	
				0.2 mg kg^{-1}	1 mg kg^{-1}
Kresoxim-methyl	0.999	0.006	0.02	92.5 (1.9)	92.4 (2.3)
BF 490-2	0.995	0.02	0.065	93.3 (2.0)	88.7 (1.1)
BF 490-9	0.992	0.02	0.065	97.9 (0.6)	85.6 (0.9)

RSD, relative standard deviation.

Table 2 The residues of kresoxim-methyl, BF 490-2, and BF 490-9 in pear (mg kg^{-1}).

Spray frequency	Spraying day before harvest	Kresoxim-methyl	BF 490-2	BF 490-9	Total
3	21–30–40	BQL	ND	ND	–
	14–21–30	0.03	ND	ND	0.03
4	21–30–40–50	BQL	ND	ND	–
	14–21–30–40	0.03	ND	ND	0.03

ND, not detected.
BQL, below the quantification limit.

85.6–97.9% and 0.6–2.3%, respectively, which were considered satisfactory according to the SANCO guideline [18].

Optimization of extraction and cleanup

In our previous study, only 4 mL of the upper aliquot equivalent to 2 g was evaporated for purification following the extraction of 10 g Korean plum sample with 20 mL of solvent [10]. However, in the case of pear, 10 mL of the upper layer, equivalent to 5 g, was evaporated. This is because the pear extract was comparatively cleaner than the plum extract. The cartridge method was optimized and redeveloped for separation and elution of the analytes as shown in experimental extraction and cleanup section.

Method application

Treated pear samples were analyzed according to the developed methodology. Kresoxim-methyl was detected at a residue of 0.03 mg kg^{-1} after 30 days of triple application and 40 days of quadruple application. No metabolites were found in field treated samples. In another study, kresoxim-methyl was found to be the dominant parent compound residues in apple and wheat [19]. The residual amount of kresoxim-methyl in various samples has also been previously assessed by researchers. Cabras et al. [7] found very low residue (0.15 mg kg^{-1}) in grapes after low doses treatment of kresoxim-methyl, which were completely disappeared after a couple of weeks. Jian-Zhong et al. [20] revealed that the residues in cucumber were below the MRL (0.05 mg kg^{-1} fixed by EU after 7 days of application. Liu et al. [21] investigated the residues of kresoxim-methyl in melon and found the residues were below the MRL value (0.2 mg/kg in melon fixed by EU) following 7 days of last application. However, in the present study, kresoxim-methyl was considered as safe in terms of application rate and pre-harvest interval because the residue was 30 times lower than the MRL [1 mg kg^{-1}, KFDA [5]] (Table 2).

Conclusions

In conclusion, the pepper leaf matrix mixed with the pear matrix protected the compounds in the sample to produce a sharp and sensitive outcome for kresoxim-methyl thermolabile metabolites during gas chromatography analysis.

Conflict of interest

The authors have declared no conflict of interest.

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