Talanta 86 (2011) 221–226

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A sensitive and selective method for the determination of flumorph residues in vegetables and fruits by HPLC–ESI-MS/MS

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ARTICLE INFO

Article history:
Received 8 May 2011
Received in revised form 28 August 2011
Accepted 4 September 2011
Available online 19 September 2011

Keywords:
LC–MS/MS
Conversion
Flumorph residue
Vegetables and fruits

ABSTRACT

A method has been developed and validated for the quantitative determination of flumorph residues in vegetables and fruits using dimoxystrobin as internal standard (I.S.) by high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS). The conversion rule between flumorph (Z) and flumorph (E) has been studied through sunlight photodegradation test of flumorph. Flumorph and I.S. were extracted with ethyl acetate and preconcentrated by Oasis HLB cartridge. Qualitative and quantitative detection for the analytes were carried out under the multiple reaction monitoring (MRM) in positive ionization mode after chromatography separation on a Symmetry C_{18} (150 mm × 2.1 mm × 3.5 μm) column. Studies at fortification level of 0.05–25 μg kg^{-1} gave mean recoveries from 77.6 to 92.9% for flumorph, with relative standard deviation (R.S.D.) ≤8.7%. The limit of quantification (LOQ, S/N = 10) was 0.05 μg kg^{-1}. The proposed method was successfully applied on real samples from different kinds of vegetables and fruits. Flumorph residues were detected in 70% of the samples analyzed and the highest concentration level was 1.83 μg kg^{-1} in tomato sample.

1. Introduction

Flumorph is an oomycete carboxylic acid amide (CAA) fungicide [1–3]. Flumorph [4-[3-(4-fluorophenyl)-3-(3,4-dimethoxyphenyl)-1-oxo-2-propenyl]morpholine] is a structure analogue of dimethomorph, which has two isomers (50% Z-isomer, 50% E-isomer) and has been classified into morpholine group with dimethomorph [4] based on their chemical structures (Fig. 1). In China, it has been widely used as a good fungicide against peronospora and phytophthora diseases in vegetables and fruits. Therefore, it is essential to develop a sensitive and selective method for determination of flumorph residues in vegetables and fruits.

The determination of flumorph using reversed phase high performance liquid chromatography (HPLC) with UV detection has been reported [5]. In which the degradation and residue of flumorph in soil and the degradation kinetics state of flumorph in three kinds of soils under laboratory condition were investigated. The linear range of the proposed method was from 0.1 to 5.0 mg L^{-1}. Hu et al. [3] proposed a method for the determination of flumorph residues in vegetables, soil and natural water by solid phase extraction cleanup and high performance liquid chromatography with UV detection, in which two isomers of flumorph were extracted with 70 mL acetonitrile and purified by a salting out process, the limit of detection (LOD) was 10 μg kg^{-1}. However, LC–UV method had high probabilities of false positives especially at low residual levels. Nowadays, liquid chromatography coupled with mass spectrometry (LC–MS/MS) is becoming one of most powerful techniques for the residue analysis of polar, ionic or low volatility fungicides in fruits and vegetables, which is capable of discriminating more efficiently than LC-UV between analyte and matrix signal [6–13], and improve selectivity and sensitivity making this technique appropriate for analysis at low residue levels [14–23]. Recently, Tian et al. [24] developed a method for determination of chloramphenicol, enrofloxacin and 29 pesticides residues including fluomorph in bovine milk by liquid chromatography–tandem mass spectrometry, analytes were extracted with 30 mL acetonitrile and dehydrated with 3 g of anhydrous sodium sulfate. The linear range of flumorph is from 3.9 to 30 μg kg^{-1}, the LOD was 1.0 μg kg^{-1}.

In this paper we developed a sensitive and selective method based on LC–MS/MS for the determination of flumorph residues in vegetables and fruits, studied the conversion rule between flumorph (Z) and flumorph (E), and confirmed their chromatographic behavior. To our knowledge, there is no report on trace analysis of flumorph residues using mass spectrometry with dimoxystrobin

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doi:10.1016/j.talanta.2011.09.004

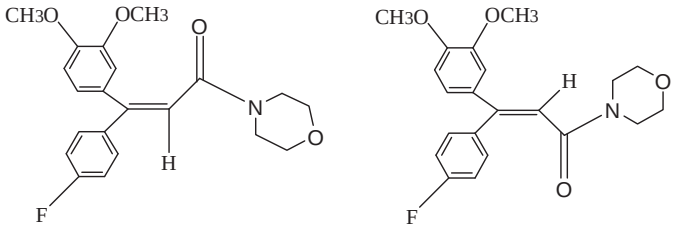

Fig. 1. Chemical structures of flumorph (Z) and flumorph (E).

as I.S. and study of conversion rule between flumorph (Z) and flumorph (E). This proposed method is a very appropriate technique for the determination of flumorph residues in vegetables and fruits, in which the simpler extraction process was employed with a small amount of organic solvent than reference [3], the LOD was improved 50 times compared with reference [24], gave a LOQ of 0.05 $\mu g\,kg^{-1}$ and LOD of 0.02 $\mu g\,kg^{-1}$. The proposed method was successfully applied for the determination of analyte residues in real samples of tomato, cucumber, grape and strawberry from the local markets.

2. Experimental

2.1. Chemicals and materials

Flumorph standard (purity ≥99.8%) was obtained from China's Shenyang Research Institute of Chemical Industry (Shenyang, China). Dimoxystrobin standard (purity ≥99.8%) was obtained from Sigma Aldrich (Steinheim, Germany). HPLC grade methanol, acetonitrile and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals and solvents were of analytical grade. HPLC grade water was prepared from deionized water. The samples of vegetables (tomatoes and cucumbers) and fruits (grapes and strawberries) were obtained from local markets.

2.2. Standard preparation

A stock standard solution of flumorph (1000 $\mu g\,mL^{-1}$) was prepared in methanol. The solutions required for preparing five standard solutions (2, 10, 200, 500, and 1000 $ng\,mL^{-1}$) were prepared from the stock solution by serial dilutions with methanol. A stock solution of I.S. (dimoxystrobin, 1000 $\mu g\,mL^{-1}$) in methanol was diluted with methanol to prepare an I.S. standard solution (20 $ng\,mL^{-1}$). All solutions were stored in brown flask at −18 °C to avoid degradation.

2.3. Sample extraction and clean-up

A 4.00 g aliquot of chopped and homogenized samples was weighed into a 50 mL disposable screw-capped polypropylene tube, then 100 μL I.S. solution (20 $ng\,mL^{-1}$) and 100 μL sodium carbonate solution (1 $mol\,L^{-1}$) were added. The mixture was vigorously shaking for 10 min with 10 mL ethyl acetate followed by centrifugation for 5 min at 5000 × *g*. The upper organic layer was decanted into a 100 ml round bottom flask. This extraction step was repeated twice and the extracts were evaporated under vacuum to dryness at a bath temperature of 45 °C.

Residues were dissolved in 5 mL water (5% methanol) and further processed through Oasis HLB SPE cartridge that has been activated with 5 mL methanol followed by 5 mL water. The cartridge with the adsorbed extract was washed with 5 mL water–methanol (95 + 5, v/v). The analyte and I.S. were eluted with 5 mL methanol. After drying under nitrogen gas, the samples were dissolved in 1 mL methanol and filtered through a 0.22 μm nylon filter before analysing with LC–MS/MS.

2.4. Chromatographic condition

A Shimadzu Prominence LC-20A series system consisting of two LC-20ADsp isocratic pumps, a CTO-20AC column oven, a SIL-20AC autosampler, a DGU-20A3 degasser, and a CBM-20A controller was used for chromatographic studies (Shimadzu, Kyoto, Japan). Separation was carried out on a Waters symmetry C_{18} analytical column (150 mm × 2.1 mm × 3.5 μm) (Milford, MA, USA).

The mobile phase consisted of (A) water and (B) methanol. The gradient elution was as following: started with 12% B for 2 min, followed by a linear gradient up to 52% B in 22 min, up to 90% B in 30 min, down to 12% B in 33 min, finishing with 12% B for 2 min, After this 35 min run time, 2 min of post-time followed using the initial 12% B. The flow rate was 0.25 $mL\,min^{-1}$ and the injection volume was 25 μL.

2.5. Mass spectrometric conditions

An Applied Biosystems/MDS SCIEX API 4000 equipped with ESI source was used for all MS/MS studies (Concord, Ontario, Canada). The triple quadrupole was operated in the MRM mode. ESI source parameters were optimized in positive ionization mode for flumorph and I.S. by injecting individual standard solution directly into the MS system through a syringe pump, at the same time, HPLC pumps were set up with methanol–water (50:50, v/v) and flow rate of 0.25 $mL\,min^{-1}$ through column. The ion spray voltage was operated at 5500 V. Nitrogen was used as ion source gas1 (65 psi; 1 psi = 6894.76 Pa), gas2 (60 psi) curtain gas (15 psi) and collision gas (6 psi). The TurboIonSpray probe temperature was maintained at 550 °C.

2.6. Validation study

The accuracy of the proposed method was studied by means of recovery assays. Recovery experiments were carried out in six replicates at 3 fortification levels (0.25, 5, and 12.5 $\mu g\,kg^{-1}$) by adding known volumes (100 μL) of flumorph standard solutions in methanol to different blank matrixs (tomato, cucumber, grape, and strawberry). These were extracted and cleaned-up as described in Section 2.3 after being mixed for 20 min. The recoveries were calculated using matrix-matched single-level calibration standards, at concentration levels corresponding with the recovery of 100% of the fungicide.

Precision (intra-day) was evaluated at each recovery level and was calculated in terms of R.S.D. for six replicates. The sensitivity of the method was expressed in terms of practical LOQ, which was determined from injections of matrix-matched standards at concentration levels corresponding to a signal-to-noise ratio of 10. Linearity was evaluated in individual matrix, using matrix-matched calibration curves prepared in concentration range of 0.05–25 $\mu g\,kg^{-1}$. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in matrix.

3. Results and discussion

3.1. Conversion between flumorph (Z) and flumorph (E)

Preliminary experiments were conducted with the purposes of finding the conversion rule and identifying the chromatogram retention times of flumorph (Z) and flumorph (E), and confirmed

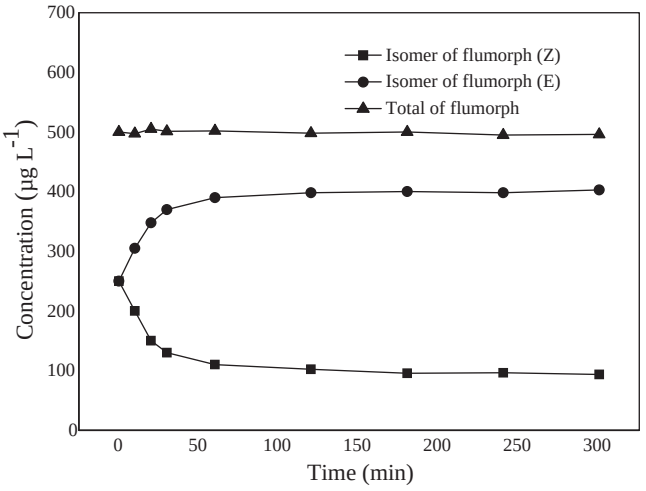

Fig. 2. Conversion curves of two isomers concentration with an increase of photodegradation time.

their chromatographic behavior by monitoring concentrations of two isomers at room temperature under sunlight. Instantaneous concentration was determined by LC–MS/MS in a certain interval of time. The trends of conversion are shown in Fig. 2. The concentration of one isomer of flumorph increased along the growth of sunlight irradiation time, which is flumorph (E) accordance with the inferences, whose retention time is 27.03 min (Fig. 3). On the contrary, the concentration of the other isomer decreased, which is flumorph (Z), whose retention time is 26.40 min (Fig. 3). However, the total concentration of the two isomers of flumorph remained constant. Therefore, flumorph residues were calculated by the total of flumorph in this paper.

3.2. Optimization of LC–MS/MS parameters

In order to optimize chromatographic separation results, the mixtures of water-acetonitrile and water-methanol were investigated as mobile phases. The experimental results indicated that the use of acetonitrile as organic modifier did not improve the sensitivity and separation efficiency of the two isomers. However, the mixture of water-methanol successfully achieved the separation purpose. Therefore, methanol was selected as organic solvent in the mobile phase.

Usually, formic acid was added into mobile phases to support positive ionization. In this experiment three different concentrations of formic acid (0.1%, 0.3%, and 0.5%) were added respectively into the mobile phase to obtain a good peak shape and to improve the sensitivity. However, displayed asymmetric peaks (tail peaks) were observed with the increase of formic acid concentration. A well peak shape and high sensitivity was obtained for flumorph and I.S. without formic acid. Therefore, water–methanol without formic acid was used as the mobile phase in this experiment. The representative MRM chromatograms of different samples are shown in Fig. 3.

The mass optimization of flumorph and I.S. were made by flow injection analysis (FIA) of the individual standard solutions at a concentration of 100 ng mL^{-1} in methanol. In this process the parention ions and the product ions were chosen, along with the optimum declustering potentials for the parention ion and the collision energies for the product ions. The most abundant product ions were selected as quantitative transition and the others in abundance, for identification. The full scan spectrum of flumorph presented positive ionization and showed an abundant ion at m/z 371.9 corresponding to the $[M+H]^+$ ion, which was selected as parention ion. The MS/MS spectrum presented a major peak at m/z 284.9 optimized at a collision energy of 26 eV. By increasing the collision energy to 44 eV, a peak at m/z 164.8 appeared in the spectra

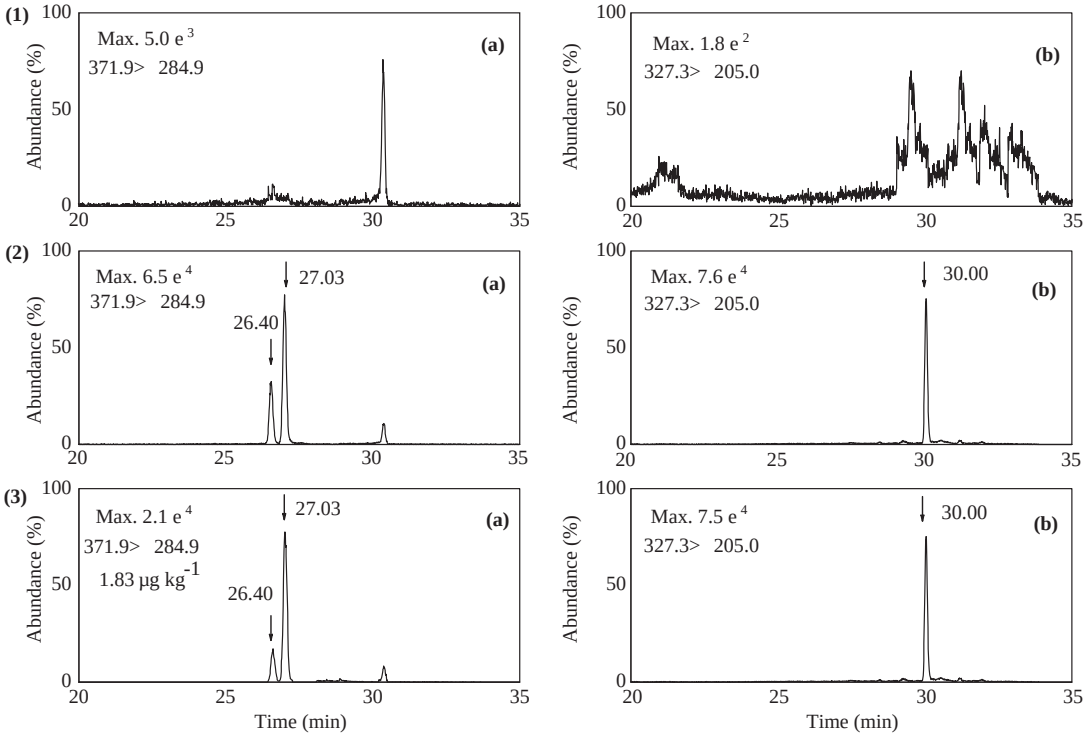

Fig. 3. Representative MRM chromatograms of (1) blank tomato; (2) blank tomato spiked with 5.0 μg kg^{-1} flumorph and 0.5 μg kg^{-1} I.S.; (3) a tomato sample spiked with 0.5 μg kg^{-1} I.S. (peaks (a) flumorph; (b) dimoxystrobin).

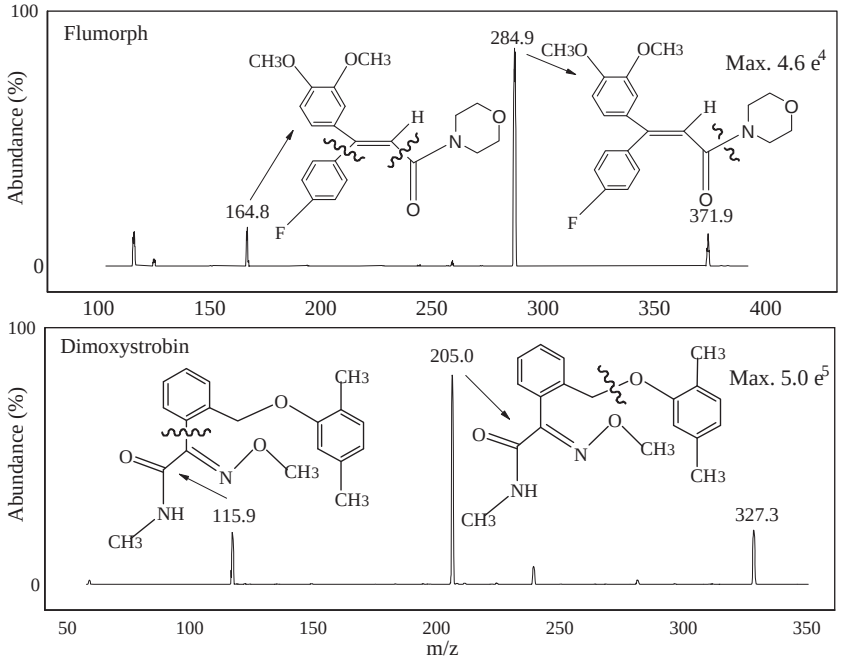

Fig. 4. Full-scan product ion spectra of $[M+H]^+$ ions of flumorph and I.S., whose structures and speculated fragments showed by black arrows.

was used as additional transition (Fig. 4). Finally, 371.9 → 284.9 was selected as quantitative transition and 371.9 → 164.8 was confirmative transition. In the same way, MS/MS parameters were optimized for I.S. The optimization results are shown in Table 1 and speculated fragmentation scheme are shown in Fig. 4.

3.3. Optimization of the extraction procedure

Extraction procedure is often the most critical part of a new method due to the direct impact on the results of accuracy and precision. First the extraction solvent was considered. For polar to very polar fungicides, three extraction solvents are used frequently, ethyl acetate, dichloromethane and diethyl ether. In this paper these extraction solvents were compared, the extraction recoveries of ethyl acetate were more stable and higher than the other two extraction solvents. Therefore, ethyl acetate was selected as the extraction solvent.

Flumorph and I.S. are alkaline compounds, so it is helpful for improving extraction recovery to add a proper amount of alkaline substances in extraction procedure. In this paper sodium hydroxide or sodium carbonate was tested to improve the extraction recovery. The experimental results showed that sodium carbonate was more suitable for obtaining satisfactory extraction recoveries than sodium hydroxide.

The extraction efficiency for three solid phase extraction (SPE) columns, HLB, MAX and MCX, were evaluated in the experiment. The experimental results showed that three SPE columns achieved the same purpose of removing interference respectively. However, HLB gave better results than MAX and MCX, obtained higher recoveries than MAX and MCX, for the column based on reverse phase chromatographic retention mechanisms with higher adsorption capacity and wider pH applicable scope for its packing materials. Therefore, HLB was chosen as it provided relatively clean extracts, improved peak shape, improved S/N compared to crude extracts.

3.4. Method validation

The proposed method was validated for the determination of flumorph residues in fruit and vegetable samples. Validation was performed in accordance with EU Guidelines [25]. Performance characteristics studied were accuracy (expressed as recovery), precision, selectivity, linearity, matrix effects, LOQ and LOD.

Under the optimal conditions, the linear calibration curves were constructed from calibration solutions in extracts of four representative, blank matrices (tomato, cucumber, grape, strawberry), at five different concentrations (0.05, 0.25, 5, 12.5, and 25 $\mu g\,kg^{-1}$) in the range of 0.05–25 $\mu g\,kg^{-1}$ for flumorph, with individual correlation coefficient >0.997. The limit of quantification (LOQ, S/N = 10) corresponded to the lowest fortification level assayed was 0.05 $\mu g\,kg^{-1}$, and the limit of detection (LOD, S/N = 3) was 0.02 $\mu g\,kg^{-1}$. Linear regression equations and LOQ of the different matrices are given in Table 2.

The accuracy of the method was verified by measuring recoveries from spiked blank samples of the different matrices investigated at three concentration levels, 0.25, 5, and 12.5 $\mu g\,kg^{-1}$, six replicates at each fortification level. These fortification levels were selected according to the expected concentration in real samples.

Table 1
MS/MS parameters for the determination of flumorph and dimoxystrobin. (I.S.)

Compound	Parention (*m*/*z*)	Daughterion (*m*/*z*)	Collision energy (eV)	Declustering potential (V)
Flumorph	371.9	284.9[a]	26	81
		164.8	44	81
Dimoxystrobin (I.S.)	327.3	205.0[a]	14	50
		115.9	26	50

[a] Quatitative ions.

Table 2
Linear regression equations, correlation coefficients and limits of quantification for the analytical procedure.

Matrix name	Linear range ($\mu g\,kg^{-1}$)	Correlation coefficient (r)	Linear regression equations	LOQ ($\mu g\,kg^{-1}$)
Tomato	0.05–25	0.9990	$y = 0.26x + 0.0086$	0.05
Cucumber	0.05–25	0.9984	$y = 0.35x + 0.0038$	0.05
Grape	0.05–25	0.9978	$y = 0.24x + 0.0019$	0.05
Strawberry	0.05–25	0.9989	$y = 0.31x + 0.0051$	0.05

Table 3
Mean recoveries and precision ($n = 6$) for the analytical procedure.

Matrix name	Spiked level ($\mu g\,kg^{-1}$)	Recovery (%)	R.S.D. (%)
Tomato	0.25	84.36	4.53
	5	92.83	8.40
	12.5	79.85	6.79
Cucumber	0.25	86.43	5.88
	5	90.24	8.64
	12.5	84.80	6.57
Grape	0.25	77.64	4.62
	5	79.32	6.67
	12.5	80.62	5.39
Strawberry	0.25	90.20	4.31
	5	84.31	5.77
	12.5	78.92	7.98

Acceding the EU guideline (recovery in 70–110%, R.S.D. ≤20%), the proposed method was found to be accurate, with satisfactory recoveries for both types of fruit samples and vegetable at three fortification levels. The precisions were evaluated by six replicates per concentration for four different matrix samples. Mean recovery data and R.S.D. are shown in Table 3.

Matrix effect was tested in 4 representative matrices: tomato, cucumber, grape and strawberry. Matrix effect ((1 − (slope matrix/slope solvent)) × 100) was expressed by employing matrix-matched standards and was also evaluated depending on the percentage of signal suppression or enhancement for different matrix. Four blank matrices were extracted and blank extracts were fortified at 0.1, 0.25, 0.5, 5 and 12.5 $\mu g\,kg^{-1}$ at the end of the sample preparation. In this study four matrixes presented very mild signal suppression, for each percentage of them was between −20% and 0%. It was considered to be a mild signal suppression or enhancement effect between −20% and 0% and between 0% and +20%; it was considered to be of medium effect when the slope values were between −50% and −20% or +20% and +50%; and it was considered to be a strong effect of signal suppression or enhancement below −50% or above +50% [26]. Cucumber and strawberry presented milder signal suppression than tomato and grape, the value of percentages are shown in Table 4. In general, matrix effect was compensated by preparing matrix-matched calibration, or by the use of isotope internal standards. However, for the most fungicides these compounds are not available. Therefore, dimoxystrobin was selected as I.S. and matrix-matched calibration to eliminate the matrix effects and obtained a more reliable determination results in this study.

Selectivity was evaluated by extracting and analyzing blank samples of the different matrices. The absence of any signal at the same elution time as the flumorph and I.S. indicated that no matrix or chemical compounds were extracted and gave a false positive signal.

Table 4
Percentage of signal suppression or enhancement for different matrix.

Matrix name	Matrix effect (%) (1 − (slope matrix/slope solvent)) × 100
Tomato	−18.6
Cucumber	−13.3
Grape	−17.4
Strawberry	−13.8

Table 5
Determination results for real samples.

Sample	Flumorph residues ($\mu g\,kg^{-1}$)				
	1#	2#	3#	4#	5#
Tomato	0.12	0.20	1.83	nd	0.19
Cucumber	0.11	0.28	nd	0.20	nd
Grape	0.30	0.34	0.36	nd	0.34
Strawberry	nd	0.21	0.23	0.13	nd

nd: not detected.

3.5. Real samples analysis

The developed method was applied to the determination of selected fungicides in 20 real samples of vegetables and fruits (tomato, cucumber, grape and strawberry) obtained from local markets. Table 5 summarizes the levels of flumorph residues in the real samples analyzed. Flumorph residues have been detected in around of 70% of samples analyzed. The highest concentration level was 1.83 $\mu g\,kg^{-1}$ in a tomato sample and the MRM chromatograms are shown in Fig. 3.

4. Conclusion

A new method has been developed for the quantitative determination of flumorph residues in vegetables and fruits using dimoxystrobin as I.S. by LC–MS/MS. To our knowledge, this is first report on trace analysis of flumorph residues in vegetables and fruits using I.S. and study of conversion rule between flumorph (Z) and flumorph (E). The analytical sensitivity was improved 50 times, compared with reference [24]. The Limit of quantification for the target compound in the proposed method was 0.05 $\mu g\,kg^{-1}$, which was 400 times lower than that in reference [3]. So far as we know, this is most sensitive method for the detection of flumorph. The method has been validated for various vegetable and fruit matrices with good sensitivity and selectivity and meets the current requirements for trace analysis of fungicide residues by monitoring two transitions and their relative ion intensity within maximum permitted tolerances. In ongoing studies, the new method will be evaluated for the determination of other fungicides on vegetable and fruit.

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