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Determination of tridemorph and other fungicide residues in fruit samples by liquid chromatography–electrospray tandem mass spectrometry☆

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Abstract

A rapid and sensitive liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS–MS) method for the determination of tridemorph and other pre- and post-harvest fungicides (carbendazim, thiabendazole, imazalil, propiconazole and bitertanol) in banana and orange samples has been developed and validated. The sample preparation was a simple extraction step with acetone using a high-speed blender prior to the injection of the five-fold diluted extract into the LC system with no other previous sample pre-treatment. Quantification was carried out using a matrix matched calibration curve which was linear in the range of 1–100 ng ml^{-1} for all the compounds. The limit of quantification was 0.05 mg kg^{-1} for all studied compounds, whereas limits of detection ranged between 0.005 and 0.025 mg kg^{-1} (0.01 mg kg^{-1} for tridemorph). Recoveries for tridemorph from spiked banana and orange samples at 0.05 and 1 mg kg^{-1} were satisfactory, with values between 83 and 99% and relative standard deviations (R.S.D.s) lower than 13% ($n = 5$). For the other fungicides, recoveries between 75 and 95% with R.S.D.s lower than 12% were obtained. The developed method has been applied to the determination of selected fungicides in real samples of bananas and oranges from different origin. Thiabendazole and imazalil have been detected in almost all orange samples analyzed, and in around of 30% of banana samples. Bitertanol residues exceeded the maximum residue level (0.05 mg kg^{-1}) in three banana samples while tridemorph was only detected in one sample.

Keywords: Fruits; Food analysis; Tridemorph; Carbendazim; Thiabendazole; Bitertanol; Propiconazole; Imazalil; Pesticides

1. Introduction

Tridemorph is the common name of the fungicide 2,6-dimethyl-4-tridecylmorpholine. Although it was originally thought that tridemorph consisted of only of tridecyl (C_{13}) isomers, it is now known that the active ingredient in the commercial product is a reaction mixture that comprises C_{11} to C_{14} homologues containing 60–70% of 4-tridecyl isomers, 0.2% C_9 and C_{15} homologues and 5% of 2,5-dimethyl isomers [1].

The US Environmental Protection Agency (EPA) has established a tolerance for residues of 2,6-dimethyl-4-tridecylmorpholine in banana samples of 0.1 mg kg^{-1}. In Europe, the maximum residue level (MRL) fixed by the European Union for tridemorph residues in both banana and orange samples is 0.05 mg kg^{-1}. Accurate and sensitive analytical methodology able to determine tridemorph residues in such samples at low ppb level are required. However, very few analytical methods are referenced in the scientific literature. As far as we know, only in the Analytical Methods for Pesticide Residues in Foodstuffs proposed by the Ministry of Public Health, Welfare and Sport of The Netherlands, is a reference found on a method that has been applied to the determination of tridemorph residues in grain [2]. This method is based on that proposed by the manufacturer (BASF) and it requires exhaustive sample handling, including four extraction steps previously to the spectrophotometric determination at 510 nm. Recoveries between 77 and 103% and a limit of determination of 0.05 mg kg^{-1} are reported. The manufacturer also proposed a GC–mass

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spectrometry (GC–MS) method for cereal straw and soil, but detailed information is not available. Thus, the need for analytical methods to determine tridemorph residues in vegetable samples is evident.

Coupling LC to MS (LC–MS) has became a powerful tool in recent years for pesticide residue analysis of foods and vegetables due to its inherent selectivity and sensitivity [3–5]. The combination of LC with tandem mass spectrometry (LC–MS–MS), where the formed precursor ion undergoes selective fragmentation achieved by collision-induced dissociation, leads to improve selectivity and sensitivity making this technique appropriate for analysis at low residue levels. In this way, LC–MS–MS methods have been successfully applied for the determination of pesticides in vegetables samples [6–8]. These methods usually involve a previous clean up step such as gel permeation chromatography [6], size-exclusion step [7] or a solvent switch [8]. However, the high specificity of the LC–MS–MS methods could allow to reduce this sample pretreatment allowing to achieve satisfactory results even without making use of cleanup treatments [9–12].

The main problem in order to use the direct injection in the analysis of complex matrices is the presence of interferences that could inhibit or enhance the analyte ionization, hampering correct quantification [10,13–17]. This matrix effect depends on the analyte–sample combination and obviously could be likely present when performing direct injection of raw extracts. Different approaches have been used to minimize the matrix effect, as increase the sample pretreatment [7,8], or to correct it, as matrix matched calibration [12,18].

Confirmation of the identity of residues in unknown samples is of utmost importance in order to avoid the presence of false positives. Following a recent European Union working group recommendation, the European Union has adopted the concept of identification points (IPs) as quality criteria for the determination of contaminants residues [19]. For compounds with an established MRL, a minimum of three IPs is required for satisfactory confirmation of the compound's identity. When LC–MS–MS technique is used with one precursor ion and two products ions (as in this paper), four IPs are earned, fulfilling the requirements of these criteria.

Due to the absence of analytical methodology available in the literature for tridemorph residue analysis, the first purpose of this paper was to develop a new, rapid, sensitive and selective method for tridemorph residue analysis in banana and orange samples based on direct injection of raw extracts in the LC–electrospray ionization (ESI)-MS–MS system, avoiding any sample treatment apart from the extraction. The applicability of the method developed was assayed for other pre- and post-harvest fungicides (carbendazim, thiabendazole, imazalil, propiconazole and bitertanol) widely used in banana and orange crops, and several real world samples were analyzed in order to evaluate the presence of fungicide residues.

2. Experimental

2.1. Reagents and chemicals

The 97.0–99.8% fungicide standards (tridemorph, carbendazim, thiabendazole, imazalil, propiconazole and bitertanol) were purchased from Dr. Ehrenstorfer (Promochem, Wesel, Germany). Individual standard stock solutions (around 500 $\mu g\,ml^{-1}$) were prepared by dissolving standards in methanol and stored in a freezer at −20 °C. Stock solution mixtures were prepared in methanol and stored at 4 °C. Working solutions of fungicides used for sample fortification and for injection in the LC system were prepared by diluting stock solutions mixtures in methanol.

LC grade methanol, acetonitrile, and acetone (pesticide residue analysis-grade) were purchased from Scharlab (Barcelona, Spain). LC grade water was obtained by purifying distilled water in a Nanopure II system (Barnstead, Newton, MA, USA). Analytical-grade formic acid (98%) and ammonium acetate were supplied by Scharlab.

2.2. Instrumentation

A high-performance liquid chromatography (HPLC) system Waters Alliance 2690 (Waters, Milford, MA, USA) was interfaced to a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK). The LC separation was performed using a Nucleosil C_{18} (125 mm × 2.1 mm i.d., particle size of 5 μm) (Scharlab), at a flow-rate of 300 $\mu l\,min^{-1}$. The mobile phases was a 2.5 mM ammonium acetate in water–0.01% formic acid in methanol gradient in which the percentage of organic modifier was changed linearly as follows: 0 min, 5%; 2 min, 5%; 14 min, 90%; 16 min, 90%; 17 min, 5%; and 25 min, 5%.

A Quattro LC (quadrupole–hexapole–quadrupole) mass spectrometer with an orthogonal Z-spray–electrospray interface (Micromass) was used. Drying gas, as well as nebulizing gas, was nitrogen generated from pressurized air in a NG-7 nitrogen generator (Alquilo, Etten-Leur, The Netherlands). The nebulizer gas flow was set to approximately 80 $l\,h^{-1}$ and the desolvation gas flow to 800–900 $l\,h^{-1}$. Infusion experiments were performed using a Model 11 single syringe pump (Harvard, Holliston, USA), directly connected to the interface. MS optimization was performed by infusion of standard solutions of each individual compound, at a concentration of 5 $\mu g\,ml^{-1}$, in methanol–water (50:50) at a flow rate of 10 $\mu l\,min^{-1}$.

For operation in the MS–MS mode, the collision gas was argon 99.995% (Carburos Metálicos, Valencia, Spain) with a pressure of 1×10^{-3} mbar in the collision cell. Capillary and extractor voltages of 3.5 kV and 3 V, respectively, were used in the positive ionization mode. The RF lens were set to 0.2 and the interface and source temperatures were set to 350 and 120 °C, respectively. Dwell times of 0.2 s per scan were chosen. Resolution of (low mass resolution/high mass resolution) 13/13 were set for both quadrupoles and

ion energies of 1.5 and 1 were set for the first and the second quadrupole, respectively. Finally, the electron multiplier was set to 650 V and entrance and exit voltages to 3 V to perform MS–MS methods.

Masslynx NT v 3.5 (Micromass) software was used to process the quantitative data obtained from calibration standards and from samples.

In all experiments, the first part of the chromatogram was sent to waste using the built-in divert valve in the mass spectrometer controlled by the Masslynx software. This solvent delay gave an additional cleanup step, and avoided the overload of the interface with early-eluting interferences that could decrease the analyte ionization.

2.3. Sample preparation

Whole orange and banana samples, including the skins, were cut into small pieces without any pretreatment and triturated. Homogenized sample (25 g) was accurately weighed (precision: 0.1 mg) and mixed with 70 ml of acetone. After extraction for 2 min with a high-speed blender Ultraturrax T25 (Janke & Kunkel, Staufen, Germany) at 8000 rpm, the entire extract was filtered through a filter paper and washed with 25 ml of acetone, and the final volume was adjusted to 100 ml with acetone. Then, the raw extract was diluted five-fold with methanol (i.e., a 2 ml aliquot was diluted to 10 ml with LC-grade methanol) and a 20 μl aliquot was injected into the LC–ESI-MS–MS system under the experimental conditions described above.

Quantification of samples was carried out by matrix-matched standards calibration prepared from a sample blank.

2.4. Validation study

The matrix-matched calibration curve was obtained by analyzing in duplicate seven standard solutions at concentrations between 1 and 100 ng ml^{-1} for all the compounds. Blank banana and orange samples were extracted accordingly with the sample preparation procedure. A 2 ml aliquot of the acetone sample extract was transferred to a 10 ml volumetric flask, and 1 ml of methanolic mixture standard solutions (fungicide concentration: between 10 and 1000 ng ml^{-1}) was added. Finally, the volume was adjusted to 10 ml with methanol.

The specificity of the method was evaluated by injecting a procedure blank, orange and banana blank samples, and blank samples spiked at the lowest fortification level assayed (limit of quantification (LOQ)), i.e. 0.05 mg kg^{-1}. Under these conditions, the response obtained for the blank samples should not exceed 30% of the response corresponding to LOQ.

The accuracy of the method was studied by means of recovery assays, which were performed at two levels of concentration (0.05 and 1 mg kg^{-1}) for the selected fungicides. Blank orange and banana triturated samples were spiked by delivering appropriate volumes of mixture standard solutions in methanol (between 0.5 and 1 ml). The spiked samples were equilibrated for 1 h prior to extraction as described above. Recovery experiments were performed in quintuplicate at every concentration level.

Precision was evaluated within the same day at each recovery level and was calculated in terms of relative standard deviation (R.S.D.) for five replicates.

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was obtained when the signal was three times the average of background noise in the chromatogram at the lowest analyte concentration assayed. The LOQ was established as the lowest concentration validated that gave acceptable recoveries (between 70 and 110%) and precision (<15%) [20].

2.5. Data evaluation

To ensure the quality of the analysis when processing real-world samples, these were injected in duplicate, and blank samples fortified at two levels (0.05 and 1 mg kg^{-1}) were used as a quality control (QC) alternately inserted every six injections. The quantification of the sample list was considered satisfactory if the QC recoveries were in the range of 70–110%. The results in unknown samples were confirmed by means of the two transitions selected for each compound, accepting a value of 0.8–1.2 as ion ratio between the two transitions (see Section 3.1).

3. Results and discussion

3.1. MS optimization

All compounds presented positive ionization and MS parameters were optimized in order to have at least two transitions with acceptable sensitivity. The most sensitive was selected as quantitative transition and the other one was used as a confirmative transition.

As an example, Fig. 1 shows the full scan and the MS–MS spectra for tridemorph. The full scan spectrum (optimized at a cone voltage of 50 V) showed an abundant ion at *m*/*z* 298 corresponding to the $[M + H]^+$ ion obtained from the most abundant C_{13} isomer, which was selected as precursor ion. Additionally, it can be also seen in Fig. 1a the $[M + H]^+$ ions corresponding to the less abundant C_{11}, C_{12} and C_{14} homologues.

The MS–MS spectrum presented a major peak at a *m*/*z* 130 optimized at a collision energy of 25 eV, corresponding this peak to the fragmentation of the tridemorph into the 2,4,6-trimethylmorpholine. By increasing the collision energy to 30 eV, several peaks related with the alkyl group fragmentation (*m*/*z*: 98, 85, 71, 57 and 43) and with fragmentation into the dimethylmorpholine (*m*/*z*: 116) appeared in the spectra favoring the inclusion of additional transitions

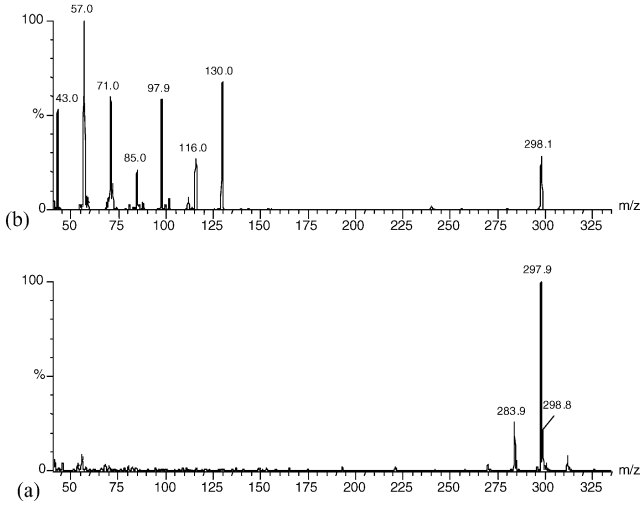

Fig. 1. Positive ion electrospray full scan mass spectrum at 50 V (a), and product ion spectra with a collision energy of 30 eV (b), of tridemorph acquired by infusion of 5 μg ml^{-1} standard solution.

(Fig. 2). Finally, 298 → 130 and 298 → 98 were selected as quantitative and confirmative transitions, respectively. In the same way, MS–MS parameters were optimized for all selected fungicides and the results obtained are shown in Table 1.

3.2. LC optimization

Initially, the mobile phase assayed for the determination of tridemorph consisted of methanol: water mixtures. We

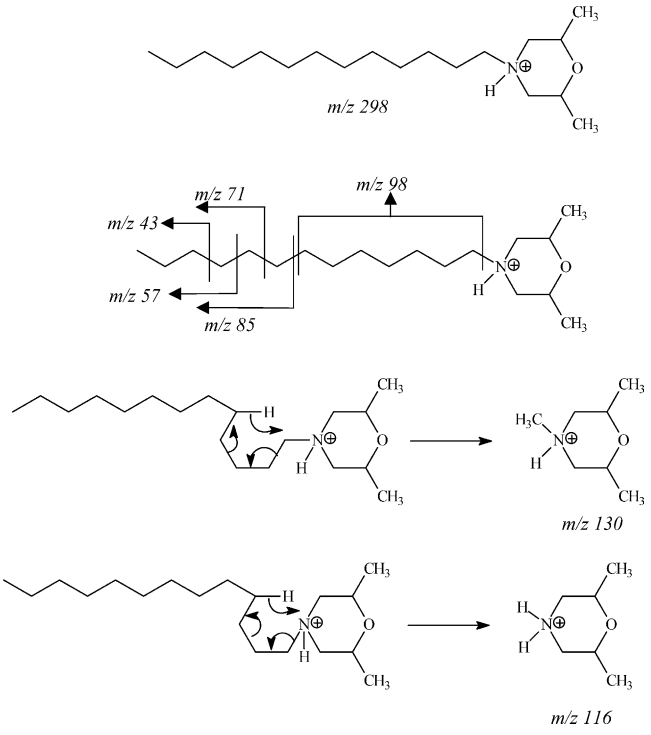

Fig. 2. Fragmentation pathways of tridemorph.

Table 1
Optimized MS–MS parameters for the determination of tridemorph and others fungicides in banana and orange samples

Fungicides	Cone voltage (V)	Precursor ion (*m/z*)[a]		Product ion (*m/z*)	Collision energy (eV)
Carbendazim	30	192	*Q*	160	15
			q	132	30
Thiabendazole	40	202	*Q*	175	25
			q	131	35
Imazalil	35	297	*Q*	41	25
			q	159	25
Tridemorph	50	298	*Q*	130	25
			q	98	30
Bitertanol	20	338	*Q*	99	15
			q	70	10
Propiconazole	40	342	*Q*	69	20
			q	159	25

[a] *Q*, quantitative transition; *q*, confirmative transition.

proved that the addition of 0.01% formic acid to the mobile phase was necessary to obtain a good peak shape for this compound. However, when we included other fungicides, some of them (carbendazim and thiabendazole) displayed asymmetric peaks (tail peaks). A correct peak shape was obtained for these compounds when ammonium acetate was added to the mobile phase instead of formic acid, but the results were not satisfactory for tridemorph under these conditions.

Moreover, it was observed that compounds which required the presence of ammonium to obtain good chromatographic behaviour eluted earlier than tridemorph. Then, we decided to use as the mobile phase, 2.5 mM ammonium acetate in water–0.01% formic acid in methanol gradient in which the percentage of methanol was increased linearly and, consequently, also the content of formic acid, which was necessary for a good tridemorph peak shape. The use of this gradient allowed fast elution and correct peak shape for all the compounds.

3.3. Method optimization

Firstly, matrix effect was evaluated by comparing the response of fungicide standards prepared in orange and banana blank methanolic extracts with standards in methanol.

Response in extracts was substantially lower as a result of ionization suppression from the coextracted components of the matrix. Dilution of the sample extracts with methanol was assayed in order to reduce these interferences. Results obtained for five-fold diluted extracts were better, but the average signal was still approximately half of the methanolic standards. In order to compensate the matrix effect, the use of matrix-matched calibration using blank extracts diluted five-fold with methanol was required.

Recoveries were found to the acceptable (70–110%) for almost all compounds, but the method was not enough

reproducible. Thus, other solvents, as acetronitrile and acetone, were checked for extraction. The best results were obtained for acetone, and the response of matrix-matched standards was found similar to that of methanolic extracts. In this way, the five-fold dilution approach was also applied after extraction with acetone, leading to satisfactory results.

3.4. Method validation

The optimized method described above was validated for the determination of tridemorph and the other selected fungicides in both banana and orange samples.

Linear calibration curves were obtained for matrix-matched standards of tridemorph in the range of 1–100 ng ml^{-1}, with a correlation coefficient >0.999 and residuals lower than 30%. Studying the specificity, no responses were detected for either the procedure blank nor the fruit sample blanks, showing the high specificity of MS–MS detection. The method was found to be precise (R.S.D. <13%) and accurate, with satisfactory recoveries (83–99%) for both types of fruit samples at both fortification levels. The LOQ corresponded to the lowest fortification level assayed, 0.05 mg kg^{-1}, and a LOD as low as 0.01 mg kg^{-1} was estimated for both orange and banana samples from the chromatograms at the lowest concentration level assayed. Table 2 summarizes data obtained for the validation of the method.

Satisfactory results were also obtained in the validation for the other fungicides. The response was linear in all cases in the range of 1–100 ng ml^{-1} (r > 0.99) and recoveries varied between 75 and 95%, with R.S.D. always lower than 12%. The LOQ for all compounds was set up at 0.05 mg kg^{-1}, and the estimated LOD ranged between 0.005 and 0.025 mg kg^{-1} depending on compound and type of sample (Table 2).

As an example of the excellent sensitivity and selectivity of the method, Fig. 3 shows typical LC–MS–MS chromatograms for a matrix-matched standards solution (2.5 ng ml^{-1}) and orange sample extracts (blank and fortified at 0.05 mg kg^{-1}). From this figure, it can be concluded that a lower LOQ could have been established in case that concentrations level below 0.05 mg kg^{-1} would have been tested.

Table 2
Detection limit (mg kg^{-1}), mean recoveries (%) and relative standard deviations (%) (n = 5) for the analytical procedure

	Banana samples			Orange samples		
	0.05	1	LOD	0.05	1	LOD
Carbendazim	83 (6)	84 (7)	0.005	96 (12)	106 (6)	0.005
Thiabendazole	85 (3)	88 (5)	0.005	83 (8)	86 (5)	0.010
Imazalil	81 (5)	81 (5)	0.005	96 (8)	99 (4)	0.010
Tridemorph	83 (13)	93 (9)	0.010	99 (12)	83 (6)	0.010
Bitertanol	75 (9)	96 (12)	0.025	91 (12)	80 (9)	0.025
Propiconazole	76 (5)	93 (2)	0.005	93 (4)	91 (9)	0.005

Table 3
Fungicides detected in banana and orange samples

No. of sample	Thiabendazole	Carbendazim	Bitertanol	Imazalil	Tridemorph
Banana samples					
1	nd		nd	nd	nd
2	<0.05		nd	0.13	nd
3	nd		nd	nd	nd
4	nd		0.50	nd	nd
5	0.09		nd	nd	nd
6	0.37		nd	0.25	nd
7	0.37		nd	0.39	<0.05
8	nd		1.58	nd	nd
9	nd		0.67	nd	nd
Orange samples					
1	6.58	nd		7.83	
2	1.69	nd		1.58	
3	3.20	nd		2.50	
4	3.89	nd		4.39	
5	2.80	nd		2.93	
6	0.17	nd		1.24	
7	3.10	nd		2.06	
8	0.40	<0.05		2.56	
9	<0.05	nd		0.66	
10	1.45	nd		0.90	
11	0.52	nd		2.74	
12	nd	nd		0.42	
13	nd	0.05		1.14	

Concentration: mg kg^{-1}; nd: not detected.

3.5. Analysis of real-world samples

The validated method was applied to the analysis of nine banana samples and 13 orange samples taken from different local markets. Two of the samples were obtained from a banana plantation in Ecuador. Every three samples (six injections) two QC (one at the LOQ level and the other at the 20 × LOQ level) were inserted. Satisfactory QC recoveries were obtained for all the compounds (between 70 and 110%) demonstrating the robustness of the method.

The results for positive samples are summarized in Table 3. Compounds not shown in this table presented concentration levels lower than LOD.

For the banana samples, positive detection occurred for thiabendazole, bitertanol and imazalil. These three fungicides were found and quantified in three different samples each one. The concentration levels were lower than the MRL for thiabendazole and imazalil, but bitertanol exceeded the MRL (0.05 mg kg^{-1}) in the three positive samples, reaching values between 0.50 and 1.58 mg kg^{-1}. Tridemorph was only detected in one commercial sample, but at a level of concentration lower than LOQ. The high sensitivity of the method and the satisfactory data obtained for tridemorph in the validation allowed us to estimate a residue level close to 0.02 mg kg^{-1} of tridemorph in banana sample no. 7.

Tridemorph was not detected in any of the orange samples analyzed. However, typical post-harvest fungicides usually used in citrus crops, as thiabendazole and imazalil,

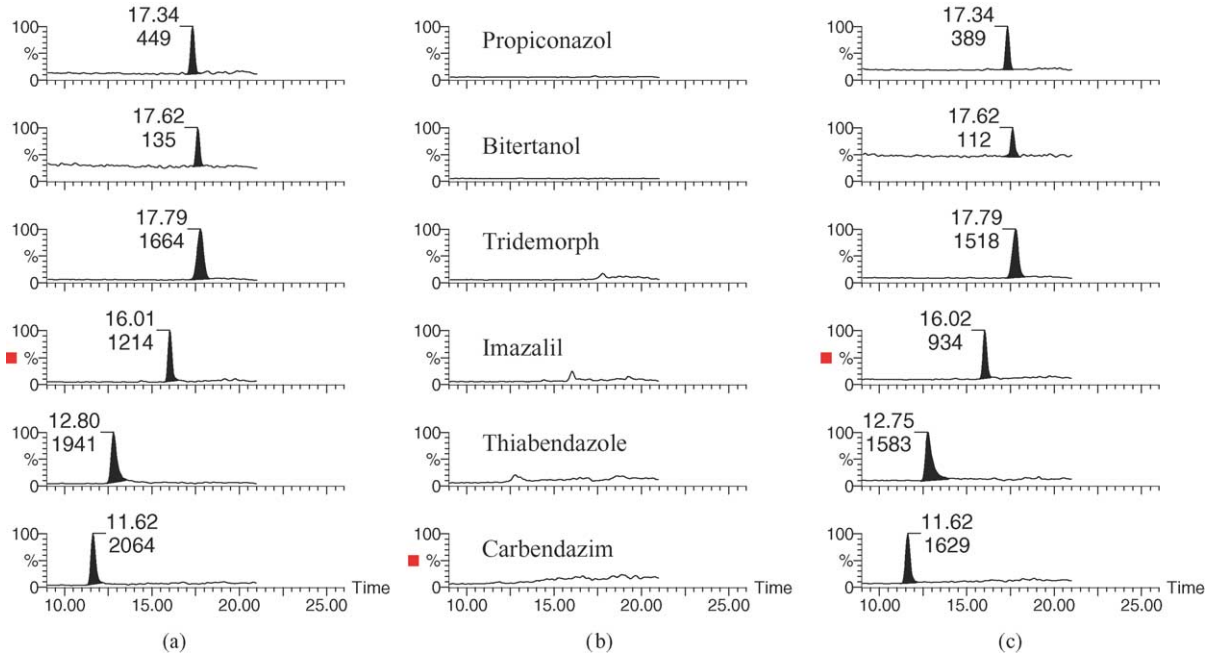

Fig. 3. LC–ESI-MS–MS chromatogram corresponding to: (a) standard solution at 2.5 ng ml^{-1}; (b) blank orange sample; and (c) an orange sample spiked at 0.05 mg kg^{-1} with carbendazim, thiabendazole, imazalil, tridemorph, bitertanol and propiconazole. Injection with optimized conditions.

were detected in almost all the samples (100% of samples for imazalil). Concentration levels were lower than MRL (5 mg kg^{-1}), except for sample no. 1, where residue levels of 6.6 and 7.8 mg kg^{-1} were found for both compounds, respectively. Carbendazim was detected in only two samples, and the concentration levels were lower than MRL (5 mg kg^{-1}). The results are in agreement with other reported in the literature [3,21], where the majority of the

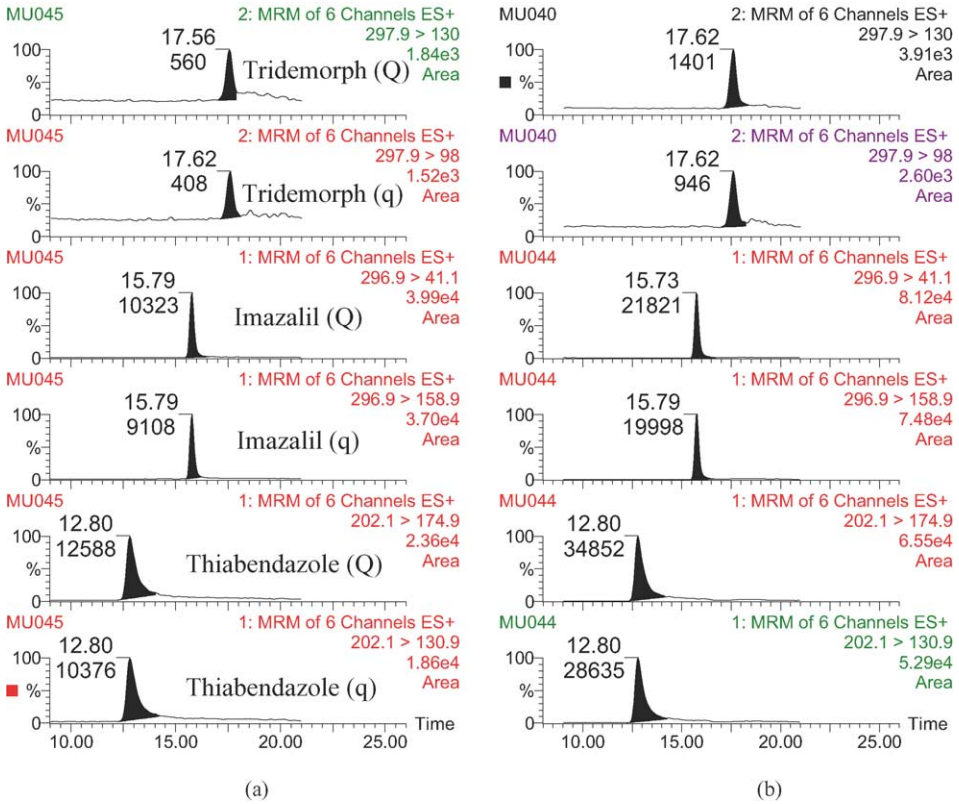

Fig. 4. LC–ESI-MS–MS chromatogram corresponding to: (a) a real banana sample with detection of thiabendazole (0.37 mg kg^{-1}); imazalil (0.39 mg kg^{-1}) and tridemorph (<LOQ); and (b) standard solution of 1 ng ml^{-1} for tridemorph and 50 ng ml^{-1} for imazalil and thiabendazole.

orange samples analyzed contained imazalil and thiabendazole residues, and occassionally carbendazim [3]. Except for sample no. 1, which was the most contaminated, the rest of data are similar to the other data reported for orange samples.

Fig. 4 shows the chromatograms corresponding to the banana sample no. 7, that contained thiabendazole and imazalil at concentration level of 0.37 and 0.39 mg kg^{-1}, respectively. In this sample, tridemorph was also detected at a concentration level close to the LOD (0.01 mg kg^{-1}).

All the detections were confirmed by the qualification transition (*q*) selected, obtaining an ion ratio within the accepted tolerance in all cases, even at the low levels found for tridemorph.

4. Conclusions

This work has shown that LC–ESI-MS–MS is a rapid, sensitive and selective technique for the determination of several fungicides in fruit samples. A method has been developed for the residue determination of tridemorph, a fungicide for which analytical methodology is not available in the scientific literature. The method allows the determination of this compound in banana and orange samples without cleanup steps, injecting directly the five-fold diluted raw extracts in the LC system. This method also gives satisfactory results for other fungicides normally used in banana and citrus crops.

The use of the two MS–MS transitions, one for quantification and the other for confirmation, together with the high sensitivity reached make of this analytical methodology applicable to monitoring the compliance of MRL in Europe and the USA, and also minimises the possibility of reporting false positives.

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