

Development and Validation of an Analytical Method for Tridemorph Determination in Tea Samples by Liquid Chromatograph-Electrospray Ionization-Tandem Mass Spectrometry

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ABSTRACT - Tridemorph is a systemic morpholine fungicide for crops. The objective of this study was to develop reliable and sensitive analytical method for determination of tridemorph residues in tea samples for ensuring the food safety. Tridemorph residues in samples were extracted with acetonitrile after hydration, partitioned with saline water, and then purified using an aminopropyl (NH₂) SPE cartridge. The purified samples were detected and quantified using LC-ESI-MS/MS. The linear detection limits for tridemorph ranged from 0.02 to 1.0 mg L⁻¹ with a correlation coefficient of 0.9999. The method was validated using tea samples spiked with tridemorph at different concentration levels (0.02 and 0.05 µg mL⁻¹). The average recovery ranged between 75.0 and 84.7% with relative standard deviations less than 10%. The LOD and LOQ were 0.01 and 0.02 mg L⁻¹, respectively. The developed method was applied successfully to the identification of tridemorph in real tea samples obtained from different sources, and tridemorph was not detected in any of the samples. The results show that the developed analytical method is accurate and suitable for tridemorph determination in tea samples.

Key words : Tridemorph, LC-ESI-MS/MS, Tea

Tridemorph, 2,6-dimethyl-4-tridecylmorpholine, is a systemic morpholine fungicide that is used to control the fungus *Erysiphe graminis* in cereals, *Mycosphaerella* species in bananas, and *Caticum solmonicolor* in tea by inhibition of ergosterol biosynthesis, steroid reduction, and isomerization¹. Tridemorph is applied onto many crops across the world, but very little information on its usage and production is in the public domain. In the UK, there were 29 approved products, predominately for the control of powdery mildew on cereals and root crops, and in 1997 it was most commonly used on winter barley and wheat². However, in November 1999, the Ministry of Agriculture, Fisheries and Food (MAFF) in the UK banned all uses of tridemorph because of the possible risk of harm to an unborn child if the mother is exposed to products containing the fungicide³. Moreover, in February 2012, Greenpeace conducted pesticide residue monitoring for Lipton-brand teas sold in Beijing, China. Seventeen different pesticides, including tridemorph (0.02 mg kg⁻¹ de-

tected; EU maximum residue limits (MRLs) 0.05 mg kg⁻¹, were found in the samples, while several pesticides in all samples exceeded the EU's MRLs, some of which have been banned by China for use on tea plants and classified as highly toxic by the World Health Organization⁴). The US Environmental Protection Agency, CODEX, and Korea Ministry of Food and Drug Safety did not set MRLs⁵⁻⁷). In Japan, MRLs of 0.05-20 mg kg⁻¹ for tridemorph residues have been established for 160 food items, including rice⁸). In Europe, the MRL set by the EU for tridemorph residues in both orange and banana samples is 0.01 mg kg⁻¹⁹). An accurate and sensitive analytical methodology that can determine the tridemorph concentration in such samples at low ppb levels is thus required. To date, various chromatographic procedures have been recommended for the identification of this fungicide in samples¹⁰⁻¹²).

LC coupled to MS or MS/MS has recently been the preferred approach for pesticide residue analysis. This is because LC is very effective for the separation analytes (selectivity), while MS allows their identification and/or confirmation at low residue levels (sensitivity)¹³⁻¹⁵). By coupling LC with MS/MS, the formed precursor ion undergoes selective fragmentation via collision-induced dissociation. The monitoring of the secondary fragmentation products provides greater discrimination from matrix interferences

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than the use of the primary fragmentation products that are generated in the single-stage MS operation mode. This technique thus enables the analysis of pesticides at trace levels in the presence of many interfering compounds. The controlled MS fragmentation is an essential tool for obtaining confidence in pesticide identification. In addition, this fragmentation generates cleaner signals, which results in an improved signal-to-noise ratio and an expansion of lower LODs.

Tridemorph is widely used over numerous crops in many countries around the world. Hence, it is necessary to determine the levels of tridemorph residues in domestic and imported agricultural commodities with a very reliable and sensitive analytical method. A number of methods have been reported in the literature for individual or simultaneous determination of tridemorph in agricultural commodities. LC-MS/MS methods have been reported including a method for tridemorph quantification in fruit and vegetable samples¹⁶⁻²⁰. One key challenge of all of these methods is that they are limited to the analysis of agricultural commodities such as banana, lettuce, apple, and orange. Therefore, this study was conducted to develop an accurate and sensitive method for tridemorph residue analysis in tea samples using an LC-ESI-MS/MS system. The applicability of the developed method was assayed in tea samples sold in Korea to evaluate the presence of the fungicide residue.

Experimental

Reagents, Chemicals, and Samples

A certificated tridemorph analytical standard (98.5%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Pesticide grade acetonitrile, methanol, and dichloromethane were purchased from Merck (Darmstadt, Germany). LC grade water was obtained by purifying distilled water in a high-performance reverse osmosis system (Sartorius Stedim Biotech, Göttingen, Germany). Other reagents, such as sodium chloride and sodium sulfate anhydrous were purchased from Wako (Osaka, Japan). An aminopropyl SPE cartridge (NH₂, 6 CC, 1 g) and a 0.45 µm GHP syringe filter were purchased from Waters (Milford, MA, USA) and General Electric Company (Fairfield, CT, USA), respectively.

Twelve dried tea samples collected from different local markets in Korea, consisting of powdered teas and bagged teas, were analyzed for tridemorph residues.

Stock and Working Standard Solutions

A standard stock solution of tridemorph was prepared in acetonitrile at 500 µg mL⁻¹ and stored in a freezer at -18°C. Suitable concentrations of working standards were prepared from the stock solution by serial dilution using acetonitrile,

immediately prior to sample preparation.

Extraction Procedure

A homogenized tea samples 5 g (± 0.1 g) was placed in a 400-mL capped beaker. The sample was hydrated with distilled water (20 mL) for 15 min and then extracted with acetonitrile (100 mL) for 10 min. Next, the sample was subjected to vacuum filtration, and then transferred to a 500 mL separatory funnel. Sodium chloride (10 g) added, the mixture was vigorously shaken for 3 min, and then eluent (the acetonitrile layer) was passed through anhydrous sodium sulfate for dehydration. Next, an aliquot of the acetonitrile layer (20 mL) was transferred to a round bottom flask and evaporated to near dryness using a rotary evaporator at 40°C. Finally, the concentrated sample was reconstituted with a 1% methanol in dichloromethane solution (4 mL).

Purification Procedure

To condition the NH₂ SPE cartridge, dichloromethane (5 mL) was introduced and discarded. An extracted tea sample (4 mL in a 1% methanol in dichloromethane solution) was introduced into the NH₂ cartridge and the eluate was collected in a 50 mL round bottom flask. The analytes were then eluted with 1% methanol in dichloromethane (7 mL). The eluate was collected and evaporated to dryness using a rotary evaporator, and the concentrated residue was dissolved in acetonitrile (2 mL) and filtered through a 0.45 µm GHP syringe filter.

Chromatographic Separation Parameters

A Waters Acquity (Waters, Milford, MA, USA) LC system was interfaced with a Quattro premier XE LC mass spectrometer (Waters, Milford, MA, USA). The LC separation was performed using a Cpacell Pak UG 120 C₁₈ column (150 mm × 2 mm i.d., 5 µm particle size, Shiseido, Tokyo, Japan) at a flow rate of 200 µL min⁻¹. The mobile phase was a 0.1% formic acid in water-0.1% formic acid in acetonitrile gradient in which the percentage of organic modifier was changed linearly as follows: 0min, 30%; 3min, 30%; 4min, 70%; 10min, 70%; and 15min, 30%.

A Quattro LC (quadrupole-hexapole-quadrupole) MS with an orthogonal Z-spray-electrospray interface (Waters, Milford, MA, USA) was used. Nitrogen generated from pressurized air in a nitrogen generator (Peak Scientific, Billerica, MA, USA) was used for the drying, and nebulizing gases. The nebulizer gas flow was set to approximately 20 L h⁻¹ and the desolvation gas flow to 600 L h⁻¹. Infusion experiments were performed using a single syringe pump, directly connected to the interfaces. MS optimization achieved by infusion of standard solutions of each individual compound, at a concentration of 1 µg mL⁻¹, in acetonitrile at a flow rate of 20 µL

min⁻¹.

For operation in the MS/MS mode, argon (99.999%) was used as the collision gas with a pressure of 1.2×10^{-3} mbar in the collision cell. Capillary and extractor voltages of 1 kV and 5 V, respectively, were used in the positive ionization mode. The RF lens was set to 0.2, and the interface and source temperatures were set to 300 and 120°C, respectively. Dwell times of 0.2s per scan were chosen. A resolution (low-mass resolution/high-mass resolution) of 15/15 was set for both quadrupoles, and ion energies of 0.5 and 1 were set for the first and the third quadrupoles, respectively. Finally, the electron multiplier was set to 655 V, and the entrance and exit voltages were each 2 V for performing the MS/MS methods analysis. Masslynx NT v 4.1 software was used to process the quantitative data obtained from for the calibration standards and the samples. The conditions for the LC-ESI-MS/MS analysis are summarized in Table 1.

Method Validation

Method validation ensures analysis credibility. In this study, the linearity, specificity, accuracy, precision, LOD and LOQ were considered.

The linearity was determined using different known concentrations (0.02, 0.05, 0.1, 0.5, and 1.0 $\mu\text{g mL}^{-1}$), which were prepared by diluting the stock solution.

The specificity of the method was evaluated by injecting a procedure blank, tea blank samples, and blank samples spiked at the lowest fortification level assayed (LOQ), i.e., 0.02 mg kg^{-1} .

The accuracy of the method was determined by means of

a recovery test using samples spiked at two concentration levels of (0.02 and 0.05 mg kg^{-1}). The recovery experiments were performed in six replicates at each concentration level.

Precision was evaluated within the same day at each recovery level and was calculated in terms of RSD for six replicates.

The LOD ($\mu\text{g mL}^{-1}$) was determined as the lowest concentration giving a response of three times the baseline noise defined from the analysis of a control (untreated) sample. The LOQ ($\mu\text{g mL}^{-1}$) was determined as the lowest concentration validated that gave acceptable recoveries (70-120%) and precision (< 10%) and a response of 10 times the baseline noise.

Data Evaluation

To ensure the quality of analysis when processing the samples, each sample was injected in duplicate. Blank samples fortified at two levels (0.02 and 0.05 mg kg^{-1}) were alternately used as a quality control and inserted every six injections. The quantification of the sample list was considered satisfactory if the quality control recoveries were in the range 70-120%.

Results and Discussion

Extraction and Purification Optimization

Acetone^{21,22}, acetonitrile^{23,24}, methanol^{25,26}, and ethyl acetate^{27,28} have been commonly used as extraction solvents for optimizing and improving pesticide residue analysis. In this study, acetonitrile was used as the extraction solvent because fewer co-extracted compounds, such as lipids, pigments, and starches were obtained. In addition, acetonitrile is completely miscible with water, yet it can be readily separated from water, not only by liquid-liquid separation with nonpolar solvents but also by the addition of salts such as sodium chloride²⁹. The use of acetonitrile followed by the addition of salts proved to be better suited for the extraction of both nonpolar and polar pesticides from vegetable samples³⁰. This extraction technique is simple and fast, but the extracts still required further purification steps. Therefore, in this study, the acetonitrile layer was cleaned up using an aminopropyl (NH₂) SPE cartridge. The aminopropyl cartridge is suitable for the purification of slightly polar tridemorph ($\text{LogP}_{\text{ow}} = 4.2$ at pH 7, 20°C) because it has (CH₂)₃NH₂ groups at the chain ends; these groups have polar properties³¹.

Instrument Optimization

Tridemorph undergoes positive ionization, and the MS parameters were optimized to have at least two transitions with acceptable sensitivity. The most sensitive was selected as the quantitative transition, while the second most was

Table 1. LC-MS/MS conditions for the determination of the tridemorph residue

Instrument	LC-MS/MS (Quattro Premier XE, Waters, USA)		
Column	HPLC packed column C ₁₈ (2 mm I.D. × 150 mm, 3 μm)		
	Time (min)	0.1% formic acid in water (%)	0.1% formic acid in acetonitrile (%)
	0	70	30
Mobile phase	3	70	30
	4	30	70
	10	30	70
	15	70	30
Flow	0.2 mL min ⁻¹		
Column temperature	40°C		
Ionization mode	ESI positive-ion mode		
Cone voltage	50 V		
Collision energy	32 eV		
Injection volume	5 μL		

used as a confirmative transition.

Fig. 1 shows the full-scan MS/MS spectrum 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.

The MS/MS spectrum also presented major peaks at m/z 98 and 130 when optimized at a collision energy of 32 eV, which correspond to the fragmentation of tridemorph into 2,4,6-trimethylmorpholine. Therefore, $298 \rightarrow 98$ and $298 \rightarrow 130$ were selected as the quantitative and confirmative transitions, respectively (Fig. 2 and 3).

Initially, the mobile phase consisted of acetonitrile and water mixtures. However, such mixtures displayed broad and asymmetric peaks. The addition of 0.1% formic acid to the mobile phase, however, resulted in sharp peaks with good peak shapes. The use of a gradient enabled fast elution and a correct peak shape for tridemorph.

Linearity

In recent years, external matrix-matched standard calibration has been widely applied in pesticide residue analysis in plant matrices by LC-electrospray mass spectrometry. However, in this study, external solvent standard calibration was used because it was found that the response of the analyte in a pure solvent standard ($y = 8742 \times -5.2442$) did not differ from that obtained using external matrix-matched standard calibration ($y = 8666 \times -3.3492$). This similarity was obtained due to the small sample size (5 g) and the aliquot step (5

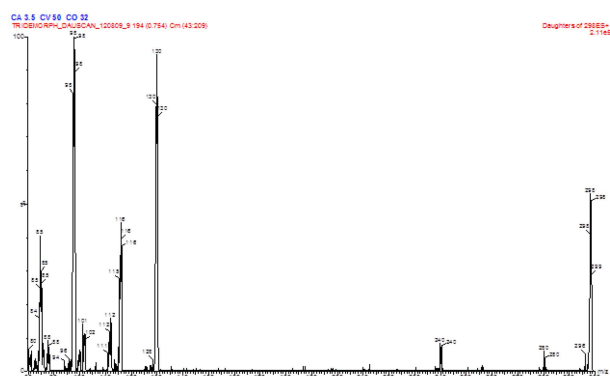


Fig. 1. Positive ion electrospray full-scan mass spectrum of tridemorph at 50 V.

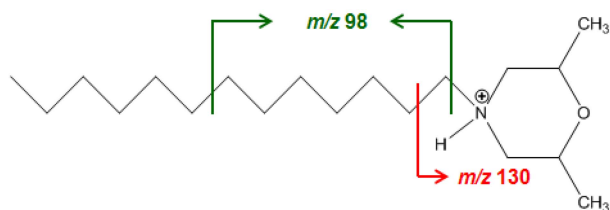


Fig. 2. Fragmentation of tridemorph.

times dilution), which resulted in elimination or compensation of the matrix effect.

Different known concentrations of tridemorph (0.02, 0.05, 0.1, 0.5, and $1.0 \mu\text{g mL}^{-1}$) were prepared in acetonitrile by diluting the stock solution. Each solution was prepared in triplicate. Standard solutions were injected into the LC column and the peak area was measured to calculate the tridemorph residue content. A calibration curve was plotted for the infected standard concentrations versus the peak area observed, and the linearity of the method was evaluated by analyzing five working solutions. The peak area obtained for each concentration of tridemorph was then used to calculate the residues ($r = 0.9999$).

Specificity

The specificity of the analytical method was judged from the absence of interfering peaks at the retention time of the analyte. The retention time of tridemorph was constant at 5.4 ± 0.1 min, and there were no interfering peaks at this retention time (Fig. 3).

Accuracy and Precision

Recovery studies were conducted at 0.02 and 0.05 mg kg^{-1}

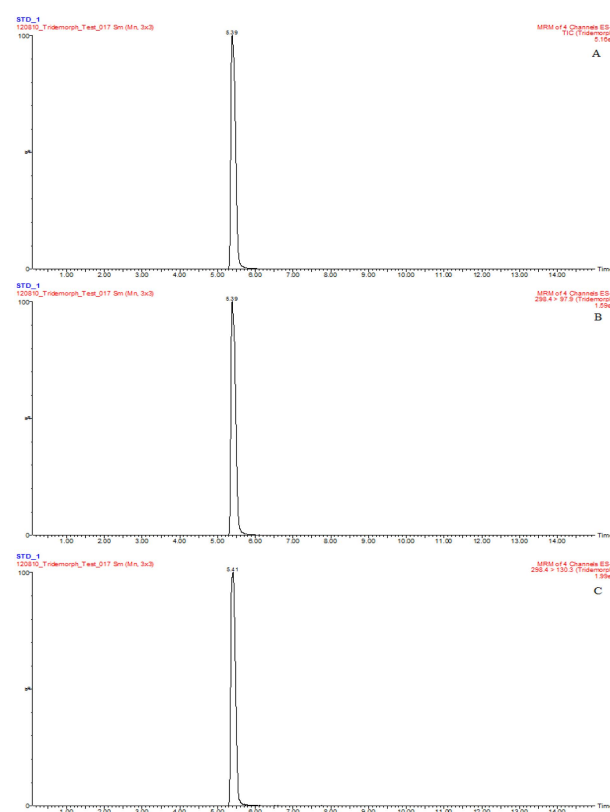


Fig. 3. LC-ESI-MS/MS chromatograms of standard solutions at $1 \mu\text{g mL}^{-1}$ corresponding to: A, the total ion chromatograph (TIC); B, m/z 298 \rightarrow 98; and C, m/z 298 \rightarrow 130.

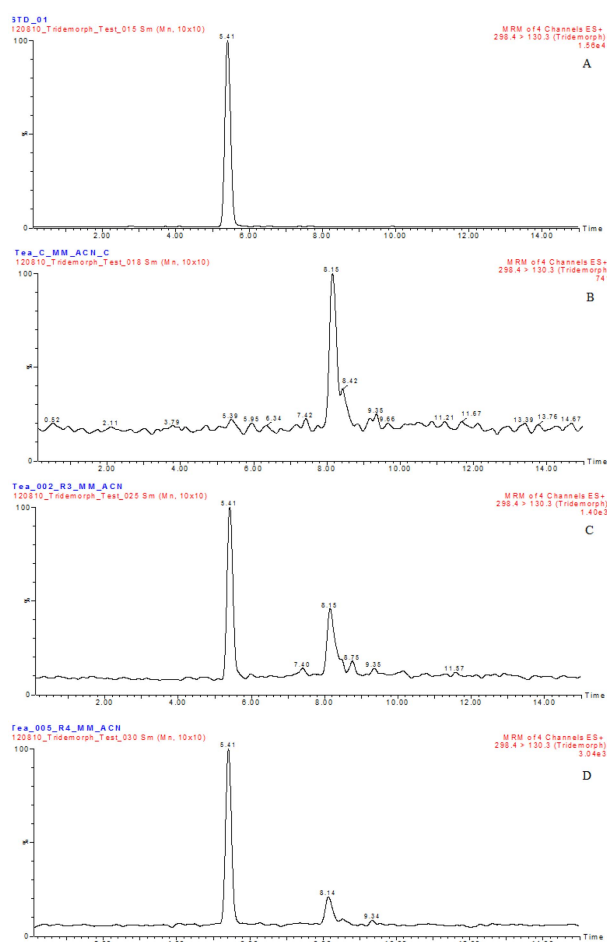


Fig. 4. LC-ESI-MS/MS chromatograms corresponding to: A, a tridemorph standard solution at 0.1 mg L^{-1} ; B, a blank tea sample; C, a tea sample spiked at 0.02 mg kg^{-1} ; and D, a tea sample spiked at 0.05 mg kg^{-1} .

fortification levels for tridemorph in control tea samples (Fig. 4). The recovery data and relative standard deviations obtained using this method is summarized in Table 2. These values were calculated from six replicate analyses of the given samples. The repeatability of the method was satisfactory ($\text{RSD} < 10\%$).

Detection and Quantification Limits

The LOQ was determined to be $0.02 \text{ } \mu\text{g mL}^{-1}$. The quantification limit was defined as the lowest fortification level evaluated at which acceptable average recoveries ($75.0\text{--}84.7\%$, $\text{RSD} < 10\%$) were achieved. This quantification limit also reflects the fortification level at which the analyte peak was consistently generated at approximately 10 times the baseline noise in the chromatogram. In addition, at a level of approximately three times the background of a control injection near the retention time of the peak of interest, the LOD was determined to be $0.01 \text{ } \mu\text{g mL}^{-1}$.

Table 2. Quantification limit (mg kg^{-1}), mean recoveries (%), and relative standard deviations (%) ($n = 6$) for the analytical procedure

Sample	Spiked (mg kg^{-1})	Recovery \pm RSD (%)	LOQ (mg kg^{-1})
Tea	0.02	84.7 ± 8.9	0.02
	0.05	75.0 ± 4.4	

Table 3. Tridemorph detected in real tea samples

Sample	Tridemorph
	(mg kg^{-1})
Herb tea - Lime	ND*
Herb tea - Lemon	ND
Herb tea - Jasmine	ND
Herb tea - Rosemary	ND
Herb tea - Chamomile	ND
Herb tea - Peppermint	ND
Herb tea - Rooibos	ND
Yellow label tea	ND
Ice tea - Apple	ND
Ice tea - Raspberry	ND
Ice tea - Lemon	ND
Ice tea - Peach	ND

*ND, not detected

Application of the Method to Real Tea Samples

The developed and validated method was applied to the routine analysis of 12 real tea samples taken from different local markets. The results for these samples are summarized in Table 3. Tridemorph was detected in one commercial sample, but at a concentration lower than the LOQ. The detection was confirmed by the selected qualification transition, however, and the obtained ion ratio was within the accepted tolerance in this case.

Conclusion

This study has demonstrated that LC-ESI-MS/MS is an accurate and sensitive technique for the determination of tridemorph residues in tea samples. The tridemorph extraction procedure for the described method is based on the Korean Food Standards Codex as a multi-class pesticide multi-residue method using an acetonitrile. An NH_2 SPE cartridge was used for sample preparation. The process was simple and easy, providing adequate clean-up of the matrix. Most of the sample extracts were very clean, with no interfering peaks at the retention time of the target compound, indicating good sensitivity for the proposed method. Satisfactory validation parameters, such as linearity, recovery, precision and very low detection limits, were obtained according to the Korean Food Standards Codex³². For

tridemorph the sensitivity of the method was suitable enough to ensure reliable determination of levels lower than the respective MRLs. In addition, the evaluation time is sufficiently short to enable the routine analysis of many samples. Therefore, the proposed analytical method should be useful for the regular monitoring of tridemorph residues in tea samples.

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요 약

차 중에 있는 살균제 tridemorph의 잔류량을 검사하기 위해 LC-ESI-MS/MS를 이용한 정확하고 감도가 좋은 분석방법을 개발하였다. Tridemorph 잔류물은 샘플을 수화한 후 acetonitrile로 추출하고, NaCl을 이용한 액-액 분배, NH₂ 카트리지 정제를 거쳐 기기분석을 수행하였다. 직선성은 0.02~1.0 µg mL⁻¹ 범위에서 상관계수(r^2) 0.9999로 높은 직선성을 보였다. 0.02와 0.05 mg kg⁻¹ 처리수준으로 회수율을 실험한 결과는 75.0~84.7%이었으며, 상대표준편차는 10% 미만이었다. 분석방법의 검출한계와 정량한계는 각각 0.01와 0.02 mg L⁻¹이었다. 이러한 결과들을 통해 확립된 분석법은 차 중 tridemorph의 잔류량 분석에 적합함을 확인할 수 있었다.

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