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Monitoring of multi-class pesticide residues in fresh grape samples using liquid chromatography with electrospray tandem mass spectrometry

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Abstract

A sensitive and selective liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS–MS) method was developed for the routine analysis of 10 multi-class pesticides residues (imidacloprid, thiamethoxam, chlorpyrifos, dimethoate, monocrotophos, metalaxyl, methomyl, hexaconazole, myclobutanil, carbendazim) in fresh grape samples. A miniaturized extraction-partition procedure that requires small amounts of non-chlorinated solvents was used. The extracts were analyzed by LC–ESI–MS–MS without any further cleanup step. The pesticides are separated on a reversed phase non-polar column using a gradient elution. Mean recoveries obtained at fortification levels of 0.010–0.100 mg/kg were 78–104% for all compounds, with relative standard deviations (RSDs) of $\leqslant$15%. The LC–MS–MS method allowed sensitive and selective quantification and identification at low levels in different matrices. The method was applied for analysis of fresh grape samples collected from an agricultural area in Hyderabad, South India. The results revealed that the concentrations of studied pesticide residues in grape samples were in the permissible limits except monocrotophos.

Keywords: Pesticide residues; Grapes; Non-chlorinated solvents; LC–ESI–MS–MS

1. Introduction

Application of agrochemicals like pesticides at various stages of cultivation and during post-harvest storage has become a usual practice in modern agriculture. These chemicals help to control a wide range of pests and plant diseases, and consequently increase in the productivity thereby playing an important role in food production and quality preservation. The toxic chemicals taken up by plants during cultivation or contaminated during preservation are passed on in the food chain causing serious health effects in human beings. The toxicity of these compounds necessitated the monitoring of pesticide residues in food products in order to assess the human exposure to pesticides through foods. European Union Commission (EU) has set the maximum residue limit (MRLs) in foodstuffs to guarantee consumer safety and to regulate international trade (Commission Regulation (EC), 1990). Thus the analytical methodologies employed must be capable of residue measurement at very low levels and must also provide clear-cut evidence to confirm both identity and quantity of any residues detected.

The most frequently used methods rely on gas chromatographic (GC) separation and detection with selective and sensitive detectors such as electron-capture detection (ECD), nitrogen–phosphorus detection (NPD) and mass spectrometry (MS), because most of the pesticides are volatile and thermally stable (Fillion, Sauve, & Selwyn, 2000; Sannino, Bandini, & Bolzni, 1999; Sannino, Bandini,

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& Bolzni, 2003). In the last few years a tendency towards the use of more polar pesticides than non-polar compounds is observed due to their less persistence and higher toxicity. Analysis of polar compounds using gas chromatography is less suitable hence use of alternative technique gained importance (Frenich, Vidal, Lopez, Aguado, & Salvador, 2004).

Liquid chromatography (LC) coupled to mass spectrometry (MS) is the most powerful technique and preferred approach for analysis of compounds that are of low volatility, high polarity and thermal liability in nature. LC is very effective in separating analytes, while MS allows their identification and/or confirmation at trace-levels. In recent years, application of LC–MS has been widely used for the analysis of pesticide residues in fruits, vegetable and other food samples like honey (Barnes et al., 1997; Fernandez-alba, Tejedor, & Aguera, 2000; Fernandez, Rodriguez, Pico, & Manes, 2001; Obana, Okihashi, Akutsu, Kitagawa, & Hori, 2003; Pico, Font, Motto, & Manes, 2000; Pous, Ruiz, Pico, & Font, 2001; Rissato, Galhiane, Almeida, Gerenutti, & Apon, 2007).

LC–MS analysis employing atmospheric pressure ionization (API) provides excellent sensitivity and selectivity for target analytes at trace levels. More recently, coupling of LC with tandem mass spectrometry detection (MS–MS) has gradually become significant for pesticide residue analysis (Bester et al., 2001; Hogenboom, Niessen, & Brinkman, 2001, 2002; Mol, Van Dam, & Steijiger, 2003; Soler, Manes, & Pico, 2005; Taylor, Hunter, Hunter, Lindsay, & Le Bouhellec, 2002). This technique is capable of discriminating analyte and matrix signal more efficiently than LC–MS (Hernandez et al., 2006). Taylor et al. (2002) reported LC–MS–MS method for the analysis of pesticide residues in crude extracts from fruits and vegetables. Electrospray ionization tandem MS (ESI–MS–MS) for the simultaneous determination of about 100 pesticides in crops at concentrations below 0.010 mg/kg was reported by Klein and Alder (2003). Thus the LC–MS–MS with electrospray ionization (ESI) has become a suitable technique in the pesticide residue analysis.

Grape cultivation is one of the intensively cultivated commercial crops in India and multi-class pesticides (imidacloprid, thiamethoxam, chlorpyrifos, dimethoate, monocrotophos, metalaxyl, methomyl, hexaconazole, myclobutanil, carbendazim) are extensively applied for the control of pests at various stages of grape cultivation.

Due to this, it is necessary to monitor the grapes quality, in order to avoid risks to consumers, as well as to promote international trade. The aim of the present study was to (a) develop a sensitive LC–MS–MS approach for the determination of selected multi-class pesticide residues, that requires small amounts of non-chlorinated solvents (b) evaluate the method developed for analyzing extracts from fresh grapes using triple quadrupole instrument with an ESI interface without any sample pre-treatment except extraction-partition and (c) apply the method for monitoring the pesticide residues in fresh grapes collected from an agricultural area in Hyderabad, South India.

2. Experimental

The selected compounds in this study belong to different chemical classes that are commonly used pesticides at different stages in cultivation of grapes. The common name, main activity and chemical class are listed in Table 1.

2.1. Chemicals and materials

Certified pesticide standards (purity $\geqslant$97.5%) were obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany. HPLC-grade solvents (ethyl acetate and methanol) were obtained from Merck Limited, Mumbai, India. HPLC-grade water was obtained from Merck Limited, Mumbai, India. The anhydrous sodium sulfate AR grade (Merck) was heated at 550 °C at least for 4 h, cooled in a desiccator and stored in sealed bottle. A total of 10 fresh grape samples were obtained from an agriculture area in Hyderabad.

2.2. Standard preparation

Individual stock solutions (1000 μg/ml) were prepared by dissolving standard pesticides in methanol. Working standards were prepared by diluting appropriate aliquots of stock solutions with methanol and stored in a refrigerator at 2–8 °C.

2.3. Sample preparation

Fresh grapes (10 samples) were obtained from an agricultural area in Hyderabad, South India. Grapes from different bunches were collected in a glass tray and a composite sample was prepared. One kilograms of composite sample was transferred into the jar of the blender and homogenized for 3 min. Accurately weighed 50 g of homogenized sample was taken into a clean beaker, 75 g anhydrous sodium sulfate and 100 ml ethyl acetate were added and blended in a jar for about 3 min. The homogenized mixture was collected in a conical flask, the ethyl acetate layer was transferred to ria vials and centrifuged at 3000 rpm for 10 min. Two milliliter of the supernatant layer were transferred into a ria vial, evaporated to dryness

Table 1
Common name, activity and chemical class of the pesticides studied

Common name	Activity	Chemical class
Imidacloprid	Insecticide	Nicotinoids
Thiamethoxam	Insecticide	
Chlorpyrifos	Insecticide	Organophosphorus
Dimethoate	Insecticide	
Monocrotophos	Insecticide	
Metalaxyl	Fungicide	Acylaminoacid
Methomyl	Insecticide	Carbamate
Hexaconazole	Fungicide	Conazoles
Myclobutanil	Fungicide	
Carbendazim	Fungicide	Benzimidazole

Table 2
Analyte MS–MS transitions, retention time and instrument conditions

Peak number	Pesticide	Retention time (Minimum)	M[a]	MS/MS m/z (amu)	DP (V)[b]	CE (V)[c]	CXE (V)[d]
1	Monocrotophos	4.95	223.17	224.2/127.2	50.87	24.51	8.66
2	Thiamethaoxam	4.96	291.7	292.1/211.2	49.31	19.48	18.24
3	Methyomyl	5.03	162.21	163.2/88.2	37.53	15.00	14.93
4	Imidacloprid	5.07	255.66	256.3/175.4	69.95	22.01	11.42
5	Dimethoate	5.28	229.26	230.2/198.9	55.30	14.87	9.00
6	Carbendazim	5.80	191.19	192.1/160.1	44.56	26.85	9.00
7	Metalaxyl	6.33	279.34	280.3/220.2	66.68	21.04	19.54
8	Myclobutanil	6.73	288.78	289.1/70.1	61.13	38.15	10.0
9	Hexaconazole	8.79	314.21	314.1/70.3	62.93	43.23	5.00
10	Chlorpyrifos	13.07	350.59	350.2/197.8	43.05	28.84	15.34

[a] M is monoisotopic molecular mass.
[b] Declustering potential (similar to the cone voltage of other manufacturers).
[c] Collision energy.
[d] Cell exit potential.

in Turbo evaporator under stream of nitrogen at 45 ± 2 °C. The residue was reconstituted with 0.45 ml methanol. Vortexed to dissolve the residue, and 5 mM ammonium formate buffer (0.05 ml) was added. Mixed well with the help of cyclomixer and loaded into LC–MS–MS. A reagent blank was run by taking 50 ml of HPLC grade ethyl acetate instead of grape sample by following the procedure described above.

2.4. High performance liquid chromatography

The high performance liquid chromatography was performed using SCL10AVP series liquid chromatograph system equipped with DGU 14AM degaser, LC10A Dvp pump, SILHTC auto sampler (Shimadzu Scientific Instruments Inc., Japan). Inertsil ODS-3V column (150 mm × 4.6 mm × 5μm) was operated at a flow rate of 0.350 ml/min. The following elution program was used: at the start 90% solvent A (methanol) and 10% solvent B (water containing 5 mM ammonium formate); initial flow rate at 0.35 ml/min for 10 min and then at 0.45 ml/min. Prior to use, the solvents were filtered through 0.22 μm filter with applied vacuum. 25 μl of either fortified or real grape sample extract was injected.

2.5. Mass spectrometry operating conditions

API-MS detection was achieved using AB Sciex API 3000 triple quadrapole mass spectrometer (AB Sciex Instruments, New Jersey, USA) equipped with TurboIonSpray interface (ESI). The instrument was operated in positive ion electrospray mode with 55 MS–MS transitions monitored during LC separation in the multiple reaction monitoring (MRM) modes.

Selection and tuning of transitions as well as analyte-dependent parameters, DP (declustering potential), CE (collision energy) and CXP (cell exit potential) were performed by direct infusion of individual pesticide solution in methanol at a concentration of 1 mg/l. Analyte MS–MS transitions, retention times and instrument conditions are presented in Table 2. A dwell time of 200 ms per transition was used.

ESI source parameters were optimized for all compounds by flow injection experiments. For this purpose, HPLC pumps were set-up with MeOH:H_2O (90:10, v/v) containing 5 mM ammonium formate, then the autosampler and HPLC system were connected to the MS with no column on-line (Flow rate 0.350 ml/min, 25 μl injection volume, analyte concentration 0.1 mg/l).

The capillary voltage was 5500 V. Nitrogen was used as nebulizer gas (7 psi; 1 psi = 6894.76 Pa), curtain gas (10 psi), heater gas (50 psi) and collision gas (4 psi). The TurboIonSpray probe temperature was maintained at 450 °C.

2.6. Recovery study

The recovery rate of each pesticide at two different fortification levels was evaluated in order to assess the extraction efficiency of the proposed method. For this, 50 g of blank sample (grapes grown without application of any pesticide) were spiked with 0.010 mg/kg and 0.100 mg/kg of pesticides. Resulting samples were mixed and allowed to stand for 15 min before extractions. Six replicates at each fortification level were prepared. Concentrations of pesticides were calculated by measuring peak areas from extracted-ion current profile and by comparing them with those obtained from matrix-matched standards of a concentration similar to that of sample. Sample data were processed by external standard technique and five-point calibration.

3. Results and discussion

3.1. Optimization of the detection system

The extraction of different pesticide residues in grape samples was performed with ethyl acetate, using small volumes of solvent per sample, shorter analysis time and with out using any chlorinated solvents. The extracts were

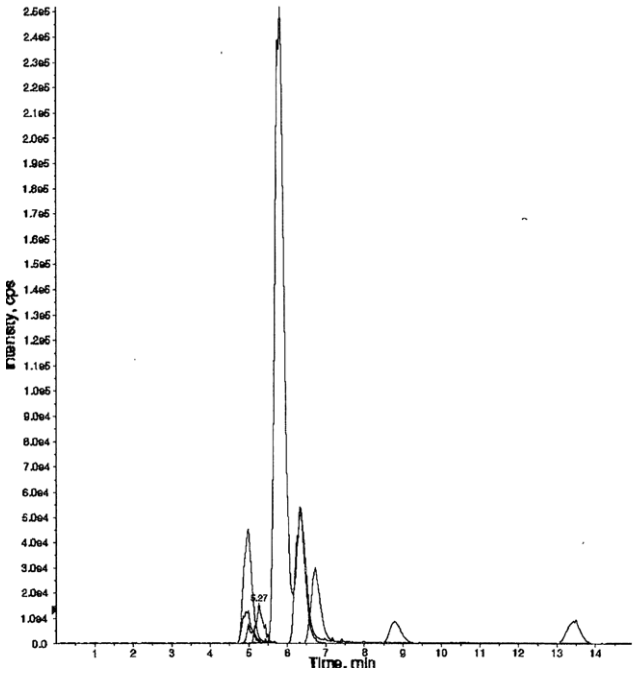

Fig. 1. Total ion chromatogram of the monitored pesticides using LC–MS–MS.

analyzed by LC–MS–MS without any further cleanup step. Suitable transitions from precursor to product ion (MRM transitions) were identified for each compound as described in experimental section. Thus, in a single injection all the ten pesticides of the present study were screened using only one retention window.

Total ion chromatogram (TIC) of all ten ions monitored from a 10 μg/kg spiked grape sample was satisfactory, except for Imidacloprid and chlorpyrifos, which were poorly resolved (Fig. 1). However they could be easily identified and quantified on individual ion chromatograms (in the SIM Mode) due to different pseudo-molecular and fragment ions. The background obtained for chromatograms of real samples was very low and thus the extracts did not require further cleanup. Analysis of blank samples revealed no traces of the pesticides studied.

The typical chromatograms of individual MRM transitions for ten pesticides in grape extracts at concentration 10 μg/kg are shown in Fig. 2. The pesticides were eluted within 14 min. under the LC gradient conditions described. The total run time was 20 min. including column conditioning. These chromatograms demonstrated how the selectivity was enhanced by MS–MS detection and allowed discrimination between target pesticides that were marginally separated by liquid chromatography.

3.2. Matrix effect

Although interferences are not visible in the LC–MS–MS chromatograms, co-eluting matrix component could inhibit or enhance the analyte signal. Therefore the influence from the matrix can be very variable. The effect of one specific combination of pesticide and matrix can vary

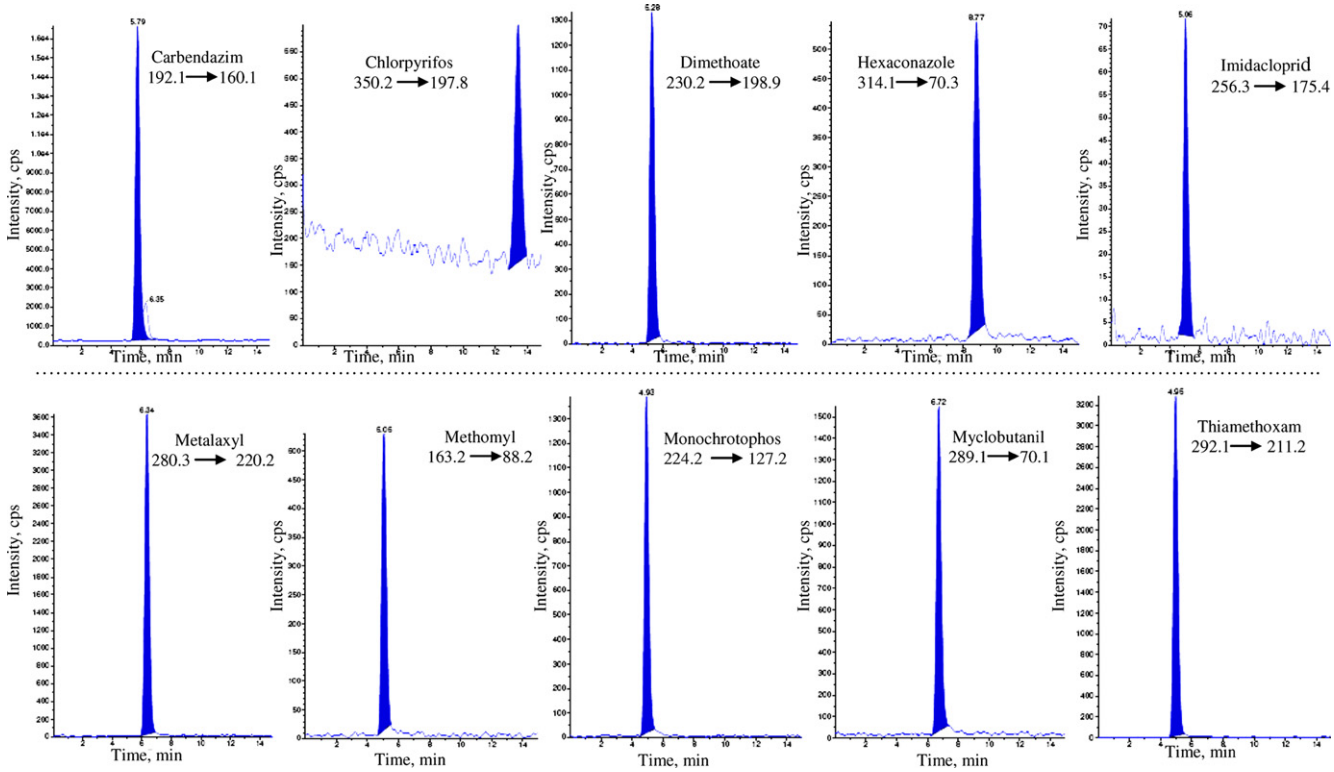

Fig. 2. Typical MRM profile of a fortified grape sample at 10 μg/kg, injection volume 25 μL.

Table 3
Recoveries of pesticides from fortified grape samples

Pesticide	Added (mg/kg)	Recovery (%)[a]	RSD (%)[a]	LOQ (mg/kg)
Imidacloprid	0.010	78	12	0.005
	0.100	85	10	
Thiamethoxam	0.010	92	12	0.005
	0.100	98	9	
Chlorpyrifos	0.010	89	15	0.01
	0.100	85	10	
Dimethoate	0.010	89	14	0.005
	0.100	98	10	
Monocrotophos	0.010	94	8	0.01
	0.100	98	10	
Metalaxyl	0.010	92	15	0.01
	0.100	98	10	
Methomyl	0.010	92	12	0.01
	0.100	96	9	
Hexaconazole	0.010	89	10	0.01
	0.100	92	8	
Myclobutanil	0.010	94	12	0.01
	0.100	98	9	
Carbendazim	0.010	82	8	0.01
	0.100	102	6	

RSD (%): relative standard deviation; LOQ: limit of quantification.
[a] Each value in the mean of six determinations.

from one time to another. This means that testing matrix effect only once and using the same results for future calculations is not possible. For example, a pesticide that is affected by 30% suppression on one occasion can be affected by 30% enhancement on the next occasion. The matrix effect is compound-dependent which is often due to interaction of co-eluting matrix components with target pesticide in the ionization step (Jansson, Pihlsstrom, Osterdahl, & Markides, 2004). Matrix effects can be tested as a ratio of analyte response in matrix-matched standard to its response in solvent (Zrostlikova, Hajslova, Kovalczuk, Stepan, & Poustka, 2003).

The matrix effect on method validation was measured at the highest levels of fortification of grape samples. The results revealed that no matrix effect on signal reduction was detected for all compounds. LC–MS–MS response suppression caused by sample matrix component has been widely discussed (Klein & Alder, 2003; Zrostlikova et al., 2003).

3.3. Method validation

The method validation was carried out by assessing selectivity, accuracy and precision.

The selectivity of the method was tested by the analysis of real samples (without spiking). The absence of any chromatographic signal at the same retention time of the target pesticides demonstrated that there were no false signals due to matrix-matched compounds.

The accuracy of the method was calculated in terms of recoveries. The recovery rate of each pesticide at two different fortification levels was evaluated in order to assess the extraction efficiency of the proposed method. For this, 50 g

Table 4
Concentration of pesticide residues in grapes (mg/kg) collected from an agricultural area, Hyderabad, South India

Pesticide	GS-1	GS-2	GS-3	GS-4	GS-5	GS-6	GS-7	GS-8	GS-9	GS-10	Maximum residue limits (MRLs)	
											European union	Netherlands
Imidacloprid	ND	0.012 ± 0.001	ND	ND	ND	0.018 ± 0.002	0.012 ± 0.001	ND	ND	ND	#	1.00
Thiamethoxam	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	#	0.05
Chlorpyrifos	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.50	0.50
Dimethoate	0.013 ± 0.001	ND	0.018 ± 0.002	ND	0.013 ± 0.002	0.021 ± 0.003	ND	0.010 ± 0.001	ND	ND	0.02	0.02
Monocrotophos	0.185 ± 0.001	0.102 ± 0.009	0.088 ± 0.007	0.120 ± 0.009	0.086 ± 0.008	0.147 ± 0.001	0.137 ± 0.001	0.107 ± 0.001	ND	ND	#	0.05
Metalaxyl	0.037 ± 0.005	ND	0.035 ± 0.004	ND	0.015 ± 0.002	ND	ND	ND	ND	ND	2.00	2.00
Methomyl	ND	ND	0.016 ± 0.002	ND	ND	ND	ND	ND	ND	ND	0.05	0.05
Hexaconazole	ND	0.032 ± 0.003	ND	ND	ND	ND	ND	0.064 ± 0.005	ND	ND	0.10	0.10
Myclobutanil	0.051 ± 0.004	0.110 ± 0.009	ND	ND	ND	0.010 ± 0.001	0.034 ± 0.003	0.123 ± 0.011	0.043 ± 0.005	ND	1.00	1.00
Carbendazim	0.101 ± 0.008	ND	ND	ND	ND	ND	0.024 ± 0.001	ND	0.043 ± 0.002	ND	2.00	2.00

ND: not detected.
(Mean ± SD) ($n = 3$).

of blank grape samples were spiked with the pesticides at each fortification level. Satisfactory results were found with relative recoveries between 78 and 102% (Table 3). According to the EU guidelines LOQs were defined as lowest concentration that provided acceptable recoveries between 70 and 110% and RSDs (<19%) (Zrostlikova et al., 2003).

The precision was assessed in terms of repeatability and LOQs to validate the procedure. LOQs corresponding to the lower calibration level are reported in Table 3. The values were empirically verified by analyzing samples spiked with the pesticides at two concentration levels. A good repeatability ($n = 6$) with RSDs ranging from 6 to 15% at LOQ level was observed.

3.4. Application of the method to real samples

The proposed method has been applied for the routine analysis of approximately 10 real samples collected from different agricultural fields near Hyderabad, South India. The results (Table 4) showed that the concentration of pesticide residues in all the samples analyzed was below the EU Maximum Residue Limits (MRLs) except monocrotophos for which there is no MRL, because it was completely banned in EU. Therefore, monocrotophos results were compared with those of the Netherlands' MRL norms.

4. Conclusions

The present multi-residue method is simple and LC–MS–MS analytical technique allows the simultaneous determination of pesticides that are commonly found in grape sample. The method involves miniaturized extraction-partition procedure that requires small amounts of non-chlorinated solvents and no cleanup procedure. It combines the advantage of MS–MS detection and allowed discrimination between target pesticides that were marginally separated by liquid chromatography in real samples. Moreover, it is rapid and allows the routine analysis of large number of samples. The optimized method was used for monitoring of pesticide residues in fresh grape samples collected from an agricultural area. All the samples contained residues lower than their respective MRLs.

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