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Simultaneous Determination of Dimethomorph and Chlorothalonil in Pesticide Formulation: HPLC Method Development and Validation

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Abstract: The identification and accurate quantification of pesticides is important to verify the recommended concentration of active content of each pesticide in formulated products to avoid adverse effects on human life due to over dosage. In this study, method of quantitative determination of Dimethomorph and Chlorothalonil in pesticide formulation was developed and validated by using ICH guidelines. Chromatographic separations with good resolution were performed on Beckman C-18 column (5 µm x 150 mm x 4.6 mm), using 80:20, v/v – (CH_3CN:H_2O) as mobile phase in isocratic mode at 230 nm. The retention time for Dimethomorph and Chlorothalonil at flow rate 1.2 mL/min was 6.21 and 9.63 minutes, respectively. Calibration curves of both studied fungicides (Chlorothalonil and Dimethomorph) were linear showing coefficient of determination greater than 0.996. %RSD value of inter-day precision was found to be less than 3 for both pesticides and for intra-day precision these values were less than 2. Inter-laboratory comparison (ILC) method was applied to evaluate the accuracy of the proposed method and Z-score values were found to be less than 2. The proposed method is therefore efficient, accurate, and cost-effective and can suitably be used for simultaneous quantitative determination of Dimethomorph and Chlorothalonil in pesticide formulated products.

Keywords: Liquid chromatography, fungicides, quantitative determination, isocratic mobile phase, optimization.

Introduction

Insecticides, fungicides and herbicides are one of the most commonly applied chemical compounds (Yusa et al., 2009). These pesticides are utilized through different routes for nourishment production, regulating weeds and prevention of crops from insects, rodents and molds (Ozkan, 2015). They are practiced on vegetable and fruit crops in their formulated forms. Therefore, several formulated products (containing various pesticides) are available in market with different brand names (Hafeez et al., 2015). At present, almost 860 active contents have been formulated and are commercially sold.

Among these pesticides, Dimethomorph and Chlorothalonil are commonly used fungicides. Dimethomorph, (E, Z)-4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl) acryloyl] morpholine, is a systemic fungicide which is applied against fungal-originated diseases such as late blights, downy mildews and root rots. It is effective on tomatoes, cucumbers, and other vegetable crops (Walorczyk, 2013). Therefore, ensuring claimed quantity of active content in formulation for controlled use of pesticides on agricultural crops is essential. In this regard, numerous protocols have been documented for quantitative determination of active content of Dimethomorph in its formulations (Yong, 2000; Xinjin, 2006; Yu, 2007; Yang et al., 2008; Yan-hua et al., 2009; Wei, 2010; Yan et al., 2010; Yi-fei et al., 2010; Cuicui, 2013; Miao-jin, 2013; Jian-ting et al., 2014). Similarly, Chlorothalonil, 2,4,5,6-tetrachloroisophthalonitrile, is a fungicide with broad-spectrum, structurally based on substituted benzene, that has been widely used to control a large number of contagious pathogens, which harm cabbages throughout their growth process (Hou et al., 2015). A number of methods have also been reported for quantitative determination of chlorothalonil in its respective formulations (Vargyas et al., 2000; Xiaolan, 2005; Aijun et al., 2007; Qiong et al., 2007; Kin and Huat, 2011; Xingfa et al., 2012; Catalá et al., 2016). In addition, these pesticides have not only protected agricultural products from different diseases, pests, and weeds, but they have also helped to prevent human life from several disease-carrying insects (Hafeez et al., 2016). Due to this fact, nowadays a large amount of these pesticides are consumed by the world (Palenikova et al., 2015). But excessive use or over dosage of these formulated products may cause adverse effects on human life. This issue addresses the need of checking the recommended concentration of active content of each pesticide in formulated products (Hafeez et al., 2016). For this purpose, chromatography is the most important tool for analysis of pesticide in environmental matrices and as active content in formulations due to its good resolution ability (Khan et al., 2016). As mentioned

above, numbers of methods for quantitative determination of Dimethomorph and Chlorothalonil in available formulations have already been reported in literature, but no method has been found in literature for simultaneous determination of Dimethomorph and Chlorothalonil in pesticide formulation. Therefore, it was necessary or there was a need to establish a reliable, efficient, cheap and sensitive method that executes the requirement of identification and accurate quantification of Dimethomorph and Chlorothalonil in pesticide formulation.

The study undertaken here developed a single HPLC-UV method to simultaneously determine Chlorothalonil and Dimethomorph in formulation and then validated the method by following the ICH guideline (ICH, 1996).

Materials and Methods

Chemicals and Reagents

The reagents and chemicals which were utilized during the experimental work were of HPLC grade and procured from Merck, Darmstadt (Germany). Deionized water was used for all the solution preparations. Filtration of solutions was performed through 0.45 µm filter membrane and sonicator was used for degassing. The standards of Dimethomorph (purity 99.5%, Fig. 1a) and Chlorothalonil (purity 96.2%, Fig. 1b) were obtained from Pakistan Agricultural Research Council, Karachi and the pesticide formulation was purchased from local market.

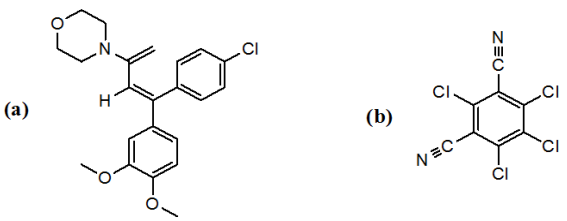

Fig. 1 Chemical structures: (a) Dimethomorph, (b) Chlorothalonil.

Instrumentation

Shimadzu HPLC attached with UV-visible detector was used for experimental work. The specification of instrument includes bi-gradient delivering pumps with 20 µL loop connected with a rheodyne. As a stationary phase, C-18 (5 µm x 150 mm x 4.6 mm) column was used in this experiment. Shimadzu SPD-10AV UV-detector was used for the integration and analysis of chromatographic data using communication bus module. The experimental data were subjected to evaluate on chromatography software (version 1.7 DLL, S# 11-8199, Build 160502).

Chromatographic Conditions and Optimization

To select mobile phase, solubility of analytes was checked in methanol and acetonitrile. The analytes showed acceptable solubility in both green solvents (acetonitrile and methanol), so different ratios of methanol and acetonitrile separately with deionized water were checked so that maximum resolution of analytes with good separation can be obtained. Different flow rates of mobile phase were checked so that shortest retention time for the quantitative determination of analytes can be achieved without affecting the resolution and shape of peak of analytes. For analytes separation the C-18 (5µm x 150 mm x 4.6mm) column was utilized. The chromatographic work was done by applying isocratic mode for the flow of mobile phase. A range of UV wavelength (between 200 to 290 nm) was used to fix detection wavelength, and optimum experimental conditions were set where the interference of inert material (observed in some formulations of pesticides) was minimum.

Preparation of Solutions

Standard Solutions: 0.01g of Dimethomorph and 0.05g of Chlorothalonil were weighed and transferred into 10 mL and 25 mL volumetric flasks (VF) respectively and final volumes of solutions were marked up by diluent (mixture of acetonitrile and water – 80:20). Both solutions were degassed by using sonicator and all the solutions were filtered using 0.45 micro meter (µm) filter membrane prior to injecting into HPLC. Standard mixture was made by transferring 4.0 mL (1000 µg/mL – stock solution) Dimethomorph and 10.0 mL (2000 µg/mL – stock solution) Chlorothalonil in 50 mL volumetric flask. To prepare working standard solutions, same stock solutions of Dimethomorph and Chlorothalonil were used.

Calibration Standards Preparation: Six (06) working standard solutions of Dimethomorph and Chlorothalonil were prepared in the working range of 12.5–400 µg/mL and 2.5–80 µg/mL respectively, to plot the calibration curve. Throughout the experimental work the analysis was done in triplicate.

Preparation of Sample Solutions: Accurately weighted 0.0516 g of formulated product of Dimethomorph and Chlorothalonil was transferred into a volumetric flask (50 mL) and then the volume of the flask was marked up by diluent/mobile phase (80:20 acetonitrile: water). For the complete solubilization of analyte in diluent, the flask content was then sonicated. This solution was then filtered using millipore filter membrane and then injected into HPLC.

Optimization of Experimental Conditions for Method Development

An HPLC based method to simultaneously determine Dimethomorph and Chlorothalonil in pesticide formulation has been developed using UV detector and optimized by following discussed parameters.

Stationary Phase Selection: Two different columns

with different specifications were checked for simultaneous determination of Dimethomorph and Chlorothalonil. The column with the shortest retention time and good separation was selected for the study.

Mobile Phase Selection: For the best separation and resolution of pesticides at room temperature, different ratios of acetonitrile and methanol in different compositions with deionized water were checked. The composition of mobile phase which gave best resolution and separation was selected as mobile phase.

Flow Rate: A suitable flow rate of mobile phase was selected by considering changes in chromatographic response of both pesticides on analyzing the standard solution with flow rate in the range of 1.0 to 1.4 mL/min.

Wavelength Selection: Separate UV scan of Dimethomorph and Chlorothalonil was obtained on Shimadzu HPLC with UV detector. The point of wavelength where the response of both pesticides was relatively stable, selected as suitable wavelength for the simultaneous determination of Dimethomorph and Chlorothalonil.

Method Validation

For this newly developed method of simultaneous determination of Dimethomorph and Chlorothalonil in pesticide formulation, following parameters were validated by using ICH guidelines (Khan et al., 2016).

System Suitability Test: A system suitability criteria tells about the performance and resolution of peaks. It was determined by ten consecutive injections of a same standard. The results are evaluated based on tailing factor 2 or under 2.0.

Linearity: By constructing the calibration curve between area of peaks and concentration of standards, the linearity of the method was checked. Different parameters from these calibration curves including slope, coefficient of determination and intercept were also calculated.

Limit of Detection and Quantification: To estimate the limit of detection (LoD) and limit of quantification (LoQ) of the newly developed method, the following formulae were used at signal to noise ratios three times and ten times of the base line respectively.

$LoD = 3.3 \times \sigma / S$

$LoQ = 10 \times \sigma / S$

Where,

S = slope obtained from calibration curve
σ = standard deviation

Precision: The precision of the newly developed method was checked by repeating the experiment within a day (intra-day) and by repeating the same experiment for three days (inter-day). The analyses were done in triplicate, and %RSD values were calculated.

Accuracy: The accuracy of the developed method was checked by analyzing the formulation of pesticide having Dimethomorph and Chlorothalonil by Inter-Laboratory Comparison (ILC) among three different laboratories. All the results were then compared to calculate Z-score.

Robustness: Deliberate changes in some parameters of newly developed method were made to check the flexibility of the method. Wavelength was changed to ±2.0 from 230 nm. Mobile phase composition was changed to ±2.0 from 80:20 ratio and flow rate varied from 1.1–1.3 mL/min.

Results and Discussion

HPLC Method Optimization

Different ratios of mobile phase composition (acetonitrile and water) were checked for good separation and resolution of peaks. On increasing the concentration of acetonitrile, a continuous decrease in the retention time of both pesticides were observed, while on increasing the concentration of acetonitrile more than 80%, tailing factors start increasing. therefore, the mobile phase composition which yielded good separation and acceptable tailing factor with less retention time was 80:20 (acetonitrile: water) (Table 1).

Table 1. Selection of mobile phase composition.

MP Ratio	Dimethomorph			Chlorothalonil		
	RT	Area	TF	RT	Area	TF
50:50	7.55	83993.0	1.179	12.06	1279449.5	1.208
60:40	7.21	94301.5	1.187	11.64	1214711.0	1.167
70:30	6.36	85505.0	1.039	11.22	1281012.0	1.226
80:20	6.43	83120.5	1.119	10.65	1288024.0	1.239
90:10	5.87	77766.0	1.110	8.34	1251258.5	1.246
100:00	5.72	71833.5	1.186	7.10	1209880.0	1.302

MP = Mobile phase (acetonitrile:water), RT = retention time (min), TF = tailing factor

For the optimization of the stationary phase of the developed method, two different reversed phase columns from different companies with different specifications, that is C-18 Beckman column (150 mm x 4.6 mm x 5 µm) and a C-18 discovery column (250 mm x 4.6 mm x 5 µm) were checked. Among them, C-18 from the company Beckman responded the best result for the separation of both fungicides without disturbing the peak shape and was selected and proposed for the method.

Different flow rates of mobile phase were studied in the range of 1.0–1.4 mL/min. Flow rate was suitably adjusted to 1.2 mL/min with good separation (Table 2).

Table 2. Selection of flow rate.

Flow Rate (mL/min)	Dimethomorph		Chlorothalonil	
	R.T* (min)	Area	R.T* (min)	Area
1.0	6.55	81029.0	10.05	1214711
1.1	6.23	88332.5	9.73	1323410
1.2	6.21	94301.5	9.63	1611814
1.3	6.23	92191.0	9.45	1391980
1.4	6.17	94606.0	9.41	1279517

* Retention time

A UV scan between 200 and 300 nm by HPLC program was employed to determine detection wavelength. Optimized chromatographic responses of two studied pesticides depicted 230 nm as detection wavelength (Fig. 2).

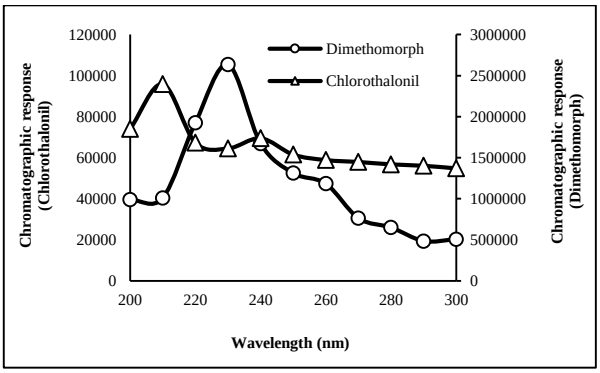

Fig. 2 Selection of detection wavelength for two studied pesticides.

Validation of HPLC Method

A representative chromatogram showing good separation and resolution of both analytes is depicted in Fig. 3. The method developed for the simultaneous determination of Dimethomorph and Chlorothalonil has been validated considering the international ICH guidelines (ICH, 1996), by means of different parameters including robustness, system suitability, linearity, precision and accuracy.

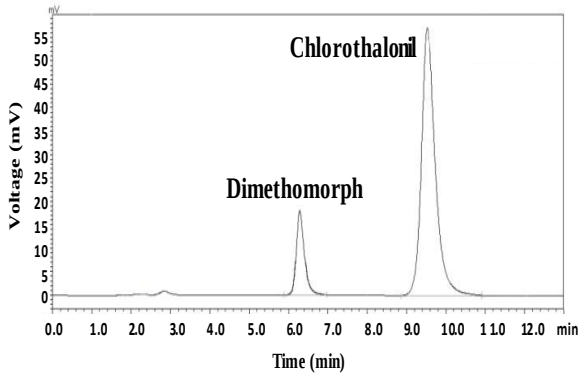

Fig. 3 HPLC chromatogram of studied pesticides: Dimethomorph and Chlorothalonil.

System Suitability Test

This test is associated with the performance and resolution of chromatographic separation. The precision among the replicate results confirms the chromatographic system suitability for proposed method. Tailing factor and number of theoretical plates were also measured for this test (Table 3).

Table 3. System suitability test for Dimethomorph and Chlorothalonil.

	Tailing Factor	Efficiency (th.pl)	HETP*	Capacity factor	Resolution
Dimethomorph	1.152	4226.341	35.492	1.940	1.271
	1.157	4236.713	35.405	1.947	1.267
	1.156	4490.249	33.406	1.736	1.239
	1.104	4456.349	33.66	1.760	1.231
Chlorothalonil	1.269	4529.347	33.117	4.354	5.911
	1.276	4530.820	33.107	4.359	5.915
	1.252	5187.271	28.917	3.724	5.827
	1.251	5176.152	28.979	3.762	5.866

* Height equivalent to the theoretical plate

Linearity

Linearity for Dimethomorph and Chlorothalonil was checked by constructing a calibration curve between peak area and concentration (ranging at 12.5–400 µg/mL for Dimethomorph and 2.5–80 µg/mL for Chlorothalonil). The values of regression coefficient of all calibration curves were found to be greater than 0.996 indicating good linearity (Table 4).

Table 4. Regression characteristics and sensitivity of the method.

	Linearity*	Intercept	Slope	R^2
Dimethomorph	12.5-400	22114	372.36	0.9991
Chlorothalonil	2.5-80	8078.5	409.01	0.9965
	SE	**SEE**	**LoD***	**LoQ***
Dimethomorph	22549.17	1829.006	9.22	27.93
Chlorothalonil	4960.294	803.2442	3.69	11.17

* = µgmL^{-1}, SE = standard error, SEE = standard error estimate

Limit of Detection and Quantification

To determine the limits (LoD and LoQ) for the developed method, ICH guidelines were followed based on standard deviation (σ) and their respective slopes (S). These standard deviations were calculated from calibration curve data and formulae for LoD and LoQ are provided in experimental part. LoD of Dimethomorph and Chlorothalonil were estimated as 9.22 and 3.69 µg/mL, whereas LoQ were 27.93 and 11.17 µg/mL, respectively.

Precision

The precision of the developed method was observed as percent RSD value among results obtained in inter-day and intra-day analysis. The analytes repeatability (Intra-day precision) was observed using triplicate analysis within a day. Reproducibility (Inter-day precision) was checked by repeating the analysis for a

period of three days. The %RSD for both types of precision was < 3, further confirming that the precision of developed method is acceptable for simultaneous analysis (Table 5).

Table 5. Inter-day and intra-day precision.

	Intra-day Analysis			Inter-day Results		
	Results* *(percentage)*	**%RSD**	**% Recovery**	**Results*** *(percentage)*	**%RSD**	**% Recovery**
Dimethomorph	8.11	1.85	100.41	8.03	2.60	101.43
Chlorothalonil	38.50	1.53	99.73	38.89	2.17	98.72

*Average of three replicate results

Accuracy

Inter-laboratory comparison (ILC) was used to check the accuracy of newly developed method. Pesticide formulation containing Dimethomorph and Chlorothalonil was also analyzed in two other laboratories. Results for the quantitative analysis of Chlorothalonil and Dimethomorph from other laboratories and results obtained from the newly developed method were compared with each other. Table 6 indicates that the developed method is suitable for determination (simultaneous) of Dimethomorph and Chlorothalonil in formulations of pesticides.

Table 6. Inter-laboratory comparison test for formulation of Dimethomorph and Chlorothalonil.

	Lab 1[a]		Lab 2[b]		Lab 3[c]	
	Result	%RSD	Result	%RSD	Result	%RSD
Dimethomorph - 8%	7.93	0.3895	7.95	1.385	8.17	1.1
Chlorothalonil - 39%	39.27	1.2498	38.13	0.835	39.03	1.483

[a] Food Quality and Safety Research Institute, Pakistan Agricultural Research Council.
[b] Syngenta Pakistan Limited, Pakistan
[c] Pakistan Council of Scientific & Industrial Research (PCSIR)

By using the given formula, Z-score values of each analysis of all participating laboratories were calculated (Fig. 4).

$Z_i = (X_i - \bar{X})/ S$
Z_i = Z-score value
X_i = reported result of laboratory
$\bar{X}$ = mean of all laboratory results
S = standard deviation among results

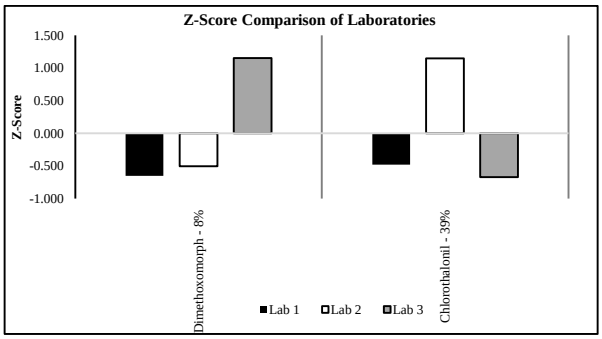

Fig. 4 A comparison of Z-score values among laboratories: Lab 1, Food Quality and Safety Research Institute, Pakistan Agricultural Research Council; Lab 2, Syngenta Pakistan Limited, Pakistan; and Lab 3, Pakistan Council of Scientific & Industrial Research (PCSIR).

The Z-score values (Fig. 4) ensure the reproducibility of the analytical results and also the reliability of proposed method. The results interpreted in terms of Z-score are taken as gratifying questionable and de-gratifying when $|Z| \leq 2$, $2 < |Z| \leq 3$ and $|Z| \geq 3$ respectively (Hafeez et al., 2016). The Z-score values of our laboratory (for both pesticides in formulations) were found to be < 2. Therefore, the results produced by proposed method are reliable and gratifying.

Robustness

Some deliberate changes were made in the optimized conditions of proposed method to test out the authenticity and flexibility of the method. Minor changes in the wavelength, and flow rate and composition of mobile phase imposed negligible effect on the results (Table 7). In general, the newly developed method to quantify Dimethomorph and Chlorothalonil in pesticides formulation is robust.

Table 7. Robustness of HPLC method for Dimethomorph and Chlorothalonil.

Parameters	**Variations**	**Dimethomorph**		**Chlorothalonil**	
		N	**T**	**N**	**T**
Mobile Phase	78:22	711.302	1.237	2956.034	1.163
	80:20	702.233	1.022	2333.386	1.135
	82:18	703.458	1.267	2939.37	1.184
Wavelength (nm)	228	783.364	1.038	2517.725	1.235
	230	598.225	0.984	2803.367	1.241
	232	664.911	1.146	2776.043	1.034
Flow rate (mL/min)	1.1	739.374	0.977	2320.662	1.056
	1.2	800.155	0.942	2964.576	1.235
	1.3	723.356	1.145	2696.494	1.173

N = Theoretical plate, T = tailing factor

Conclusion

In the current study, a new chromatography-based method was developed, validated and proposed for quantitative determination of Dimethomorph and Chlorothalonil in pesticide formulation. This proposed method allows good chromatographic separation and quantification of studied fungicides along with good precision (%RSD < 3) and accuracy. Coefficient of determination (> 0.996) derived from calibration curve depicts good linearity of the method. The LoD values were observed as 9.22 and 3.69 µg/mL, whereas LoQ values were determined as 27.93 and 11.17 µg/mL for Dimethomorph and Chlorothalonil respectively. Inter-laboratory comparison was also employed among three laboratories. The z-score values for both pesticides in formulation were found to be < 2. For validation, all the important parameters, such as robustness, system suitability, precision, linearity and accuracy were considered for the reliability of the method. Based on the results, it is inferred that the proposed analytical method is efficient, quick, precise, cost effective and free of determinate error and therefore can be appropriately applied for analytical purpose in quality control laboratories.

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