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A Dispersive Liquid - Liquid Microextraction based Gas Chromatography-Mass Spectrometry (DLLME-GC-MS) Method for the Simultaneous Determination of Fungicide Residues in Fruit Samples

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A Dispersive Liquid- Liquid Microextraction based Gas Chromatography - Mass Spectrometry (DLLME-GC-MS) Method for the Simultaneous Determination of Fungicide Residues in Fruit Samples

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Keywords : dispersive liquid-liquid microextraction, fungicides, fruits, gas chromatograph, mass spectrometer.

I. Introduction

Nowadays, a large group of fungicides have been introduced in agriculture for the control and the prevention of diseases their by protecting the quantity and quality of agricultural products. Fungicides are sprayed directly on fruits and leaves to prevent the attack of fungi, which reduce the yield of fruit [1]. Some of these fungicides are used for stabilizing fruit during the storage and transport process. Monitoring of the residues in fruits at trace levels is a global regulatory demand as part of environmental safety controls and public health protection.

The crop matrixes are complex and involves extensive procedures to monitor the trace levels of residues. Lower the detection the lower the ruggedness in extraction and measurements.

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There are several methods that describe the multi residue detection of fungicides in fruit samples. Majority of the methods are very specific and suffers from their adoptability to other crops. Sample preparation and extraction plays a major role in the field of pesticide residue analysis. Solid phase extraction (SPE) is the most common method for extraction of fungicides residues [2, 3]. The SPE technique is time consuming, cost effect and labor-expensive. Apart from SPE, the Solid Phase Micro Extraction (SPME) was also applied to the determination of several fungicides [4-6]. The SPME normally provides a higher selectivity than SPE the matrix of samples reduces significantly its extraction efficiency [7, 8]. Some of these drawbacks have been overcome by the DLLME technique was first introduced by Assadi et al, [9]. This method consists of two steps. The first is injection of an appropriate mixture of extraction and dispersion solvent into aqueous sample as very fine droplets and analytes were enriched into it. Because of the infinitely large surface area between extractions solvent and aqueous sample the equilibrium state was achieved quickly and extraction was independent of time. The second step is the centrifugation of cloudy solution. After centrifugation, the determination of analytes in sediment phase can performed by instrumental analysis. Consequently, high enrichment factor simplicity of operation and low cost are some of the advantaged of this method. Some of published reports indicates the extraction of pesticide from fruit samples using DLLME procedure [10-13]. However not much work was reported in determination of azole group of fungicides in fruit samples by DLLME. In this study 12 azole fungicides (Tetraconazole, Penconazole, Tricyclazole, Propiconazole, Tebuconazole, Epoxyconazole, Etoxazole, Fluquinconazole, Difenconazole) were studied in fruit samples. The aim of the present work was to develop and validate the simultaneous determination azoles in three kinds of fruits using DLLME-GC-MS. In addition the effects of different parameters on the efficiency of DLLME method were investigated. The advantage of DLLME method is

its limited use of solvent which is environmental friendly and requires less time and minimal amount of solvent. This environment friendly technique is safe and effective and analytical friendly, once extracted the sample can be directly used without any further clean for the quantification of residues and can also be directly introduced in to the head space injector port.

II. Materials and Methods

a) Chemicals and Reagents

Standards of Tetraconazole (purity-98.9%), Penconazole (purity-98.9%), Tricyclazole (purity-98.9%), Propiconazole (purity-98.9%), Tebuconazole (purity-98.9%), Epoxyconazole (purity-98.9%), Etoxazole (purity-98.9%), Fluquinconazole (purity-98.9%), Difenconazole (purity-98.9%) were purchased from sigma Aldrich, USA.

Extraction solvents, chlorobenzene (C_6H_5Cl), carbon tetrachloride (CCl_4), tetrachloro ethane ($C_2H_2Cl_4$), Chloroform ($CHCl_{3)}$ were purchased from Merck (Merck, Mumbai). Disperser solvents, acetone, acetonitrile, tetrhydrofuron and methanol were obtained from Sigma Aldrich. The water used was ultrapure (Millipore Unit). Sodium chloride (NaCl), was purchased from Merck Chemicals.

b) Instrumentation and GC-MS Conditions

GC-MS analyses was performed using Shimadzu GC MS-QP5000 (Shimadzu, Japan). The HP-1 MS capillary column (30m x 0.25mm i.d with 0.1 μm film thickness) was used for separation. Injection was carried out in the split mode (5:1) at an injector temperature of 290°C. Helium gas was used as a carrier gas with a flow rate of 1.0 mL/min. The column temperature was maintained at 160°C for 13 min and then programmed at 10°C min-1 to 200°C for 5 min followed by a final ramp to 290°C at a rate of 50°C min-1, and held for 6min. The ion source and transfer line temperature was 300°C respectively. All the samples were analysed in Electron Impact Ionization (EI) mode.

c) Method Validation

Specificity, linearity and recovery studies were conducted by injecting the al control samples. Different know concentrations of linearity solutions 1.0, 0.5, 0.1, 0.01, 0.005 and 0.001 μg/mL were prepared by serial dilutions method using acetone and injected in GC-MS. The limit of determination (LOD) was determined as 0.001 μg/mL based on signal noise ratio 3:1. A calibration curve was plotted between the peak area and concentration of the analytes.

Recovery studies in fruit samples were conducted by fortifying different concentrations of standard solutions (0.005 μg/g and 0.05 μg/g) of analytes.

For the repeatability analysis, five replicated determinations were made at each concentration level. After fortification of standards, the samples were homogenized as per extraction procedure and analysed GC-MS. The method has a limit of quantification (LOQ) 0.005 μg/mL. The RSD% for each concentration was calculated.

d) Sample Pretreatment Procedure

The representative sample (200 g of fruit) was homogenized by miller. A 20 g of homogenized sample was weighed and transferred into a centrifuge tubes. The sample was centrifuged for 5 minutes at 15,000 RPM using REMI cooling centrifuge. The supernant was filtered through 0.45μm PTFE Nylon filter into 10-mL volumetric flask with doubly distilled water to the volume for the DLLME procedure.

e) Extraction Procedure - Dllme

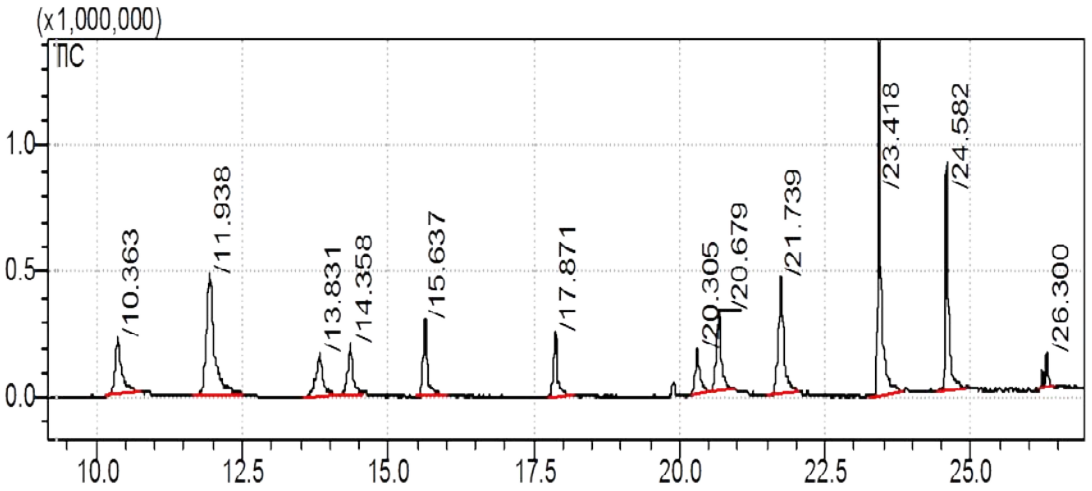
A 5.0 mL of fruit sample solution previously obtained was placed into a 10.0 mL glass vial with conical bottom and 1% (w/v) of sodium chloride (NaCl) was added to the glass vial. The organic solution containing 0.7 mL acetonitrile as dispersive solvent and 15.0 μL $C_2H_2Cl_4$ as extraction solvent was rapidly injected into the sample solution. Then the sample solution was gently shaken for 30 sec, and a cloudy solution was formed in the glass vial. In this step the analytes in sample solution were extracted into the fine droplets of $C_2H_2Cl_4$ rapidly.

In order to separate the organic phase from the aqueous phase the sample was centrifuged for 5 min at 3,500 RPM. After this process, the dispersed fine droplets of $C_2H_2Cl_4$ were sedimented at the bottom of the glass vial. The sedimented phase (8 ± 0.5 μL) was transferred in to sample vial and 1 μL of sedimented phase was injected to GC-MS for analysis.

III. Result and Discussion

a) Analytical Data- Linearity, Recovery and Repetability

The method was found to linear with a correlation coefficient of 0.9962 – 0.9997 when the tested in the range 0.001 to 1.0 μg/mL. The limit of determination (LOD) was determined as 0.001 μg/mL based on signal noise ratio 3:1. The method has a limit of quantification (LOQ) 0.005 μg/mL. The recovery details are presented in Table 2.



S.No.	Name of the Compound	Retention Time (min)	Molecular Mass (m/Z)
1	Tetraconazole	10.3	372.2
2	Penconazole	11.9	284.2
3	Tricyclazole	13.8	189.2
4	Paclobutrazole	14.3	293.8
5	Hexaconazole	15.6	314.2
6	Diniconazole	17.8	326.2
7	Propiconazole	20.3	342.2
8	Tebuconazole	20.6	307.8
9	Epoxyconazole	21.7	329.8
10	Etoxazole	23.4	359.4
11	Fluquinconazole	24.6	376.2
12	Difenconazole	26.3	406.3

Figure 1 & Table 1 : Representative Chromatogram of twelve azole fungicides, retention times and molecular Mass

b) Optimization of Dispersive Liquid – Liquid Micro extraction

The effect of various experimental parameters were studied and optimized, including Selection of extraction solvent and dispersion solvent, extraction time and ionic strength. To evaluate the extraction efficiency under different conditions, extraction recovery and enrichment factor were used. The following equations 1and 2 were used for calculation of enrichment factor (EF) and extraction recovery (R)

$$EF = \frac{Csed/C_0}{C_0} \quad \text{---------- 1}$$

$$R = \frac{Csed \times Vsed}{Co \times Vo} \quad \text{------- 2}$$

Where,

Csed = concentration of analyte in sedimented phase

Co = initial concentration of analyte in aqueous sample

Vsed = colume of sedimented phase

Vo = volume of aqueous sample

c) Selection of Extraction Solvent

The extraction solvents were selected on the basis of higher density than water. The extraction capability of interested compounds immiscibility with water but miscibility in the dispersive solvent and good gas chromatography behavior. Based on this consideration C_6H_5Cl, CCl_4, $C_2H_2Cl_4$ and $CHCl_3$ were selected as potential extraction solvents. A series of sample solutions was test by using 0.7 mL of acetonitrile containing different volume of extraction solvent to achieve 10.0 μL volume of sediment phase. There by 16.0, 20.0 25.0 and 30.0 μL of C_6H_5Cl, CCl_4, $C_2H_2Cl_4$ and $CHCl_3$ were used respectively. $C_2H_2Cl_4$ had the highest extraction efficient in comparison to the other tested solvents. Consequently $C_2H_2Cl_4$ was selected as the optimal extraction solvent.

d) Selection of dispersive solvent

Miscibility of dispersive solvents in organic phase and aqueous phase is the critical for selection of dispersive solvent. Accordingly acetonitrile, acetone methanol and tetrahydrofuran were evaluated for this purpose. The enrichment factors using acetonitrile, acetone and tetrahydrofuran as dispersive solvents. According to the results acetonitrile was chosen as dispersive solvent.

e) Effect of extraction solvent volume

To examine the effect of extraction solvent volume, 0.7 mL of acetonitrile containing different volume of $C_2H_2Cl_4$ (15.0, 20.0 24.0 and 28.0 μL) was subjected to the same DLLME procedure. The extraction recoveries and enrichment factors verses volume of extraction solvent.

It was obvious that extraction recoveries for most of the analysts varied slightly, but enrichment factors decrease by increasing the volume of $C_2H_2Cl_4$. As a consequent 15.0 μL $C_2H_2Cl_4$ was selected to obtain high enrichment factor, good recovery and low detection limit.

f) Effect of dispersive Solvent volume

To obtain optimized volume of acetonitrile 0.4, 0.6, 0.9, 1.2 and 1.5 mL of acetonitrile containing the corresponding volume of $C_2H_2Cl_4$ were studied to attain the constant volume of the sedimemted phase (6.0 $\pm$ 0.5 μL). The enrichment factors increased with the increase of volume of acetonitrile when it was less than 1.0 mL but decreased after the volume of acetonitrile exceeded 1.0 mL. Therefore, 1.2 mL was chosen as the optimum volume of the dispersive solvent.

g) Effect of Extraction Time

The effect of extraction time was examined in the range of 3-30 min with constant experimental conditions. It is revealed that after formation of a cloudy solution the surface area between extraction solvent and fruit sample phase was infinitely large, there by transition of analytes from fruit sample phase to extraction solvent was fast. Subsequently equilibrium state was established rapidly so that the extraction time very short. In this 5 min as suitable for the procedure.

h) Effect of Salt Concentration (Ionic strength)

The salting-out effect is an important parameter in DLLME. Generally addition of salt decrease the solubility of target compounds in the aqueous sample and enhances their partitioning into the organic phase. For investigating the influence of ionic strength on extraction efficiency of DLLME, various experiments were performed by adding different amount of NaCl (0-10%w/v). The increase in ionic strength led to decrease in $C_2H_2Cl_4$ solubility in aqueous phase which increased the volume of sediment phase but decreased the enrichment factors. The enrichment factor decreases when the salt addition exceeded 1%, but extraction recoveries were almost constant. As a result a salt concentration of 1% was utilized.

i) Real Fruit Sample Analysis

Three Batches of Grape, Apple and Strawberry were collected from local super markets. The samples were pretreated as described in sample preparation extracted using DLLME procedure and analyzed by GC-MS. The results are summarized in Table 3.

It is revealed that the recommended method could be applied for the trace analysis of selected fungicides in real fruit samples.

IV. Conclusion

This work highlights an easy and quick simultaneous analytical method DLLME-GC-MS to quantify 12 azole fungicides in fruit samples (Tetraconazole, Penconazole, Tricyclazole, Propiconazole, Tebuconazole, Epoxyconazole, Etoxazole, Fluquinconazole, Difenconazole).

The results of this study demonstrate that the prosposed method provides the high enrichment factor and acceptable extraction recovery and repeatability. The proposed method was fits the requirement for the determination of selected fungicides in real fruit samples.

Table 2 : The recovery and relative standard deviations (n=5) for spiked fruit samples at two different concentration levels of fungicides from fruit samples

Fungicides	Spiked	Grape		Apple		Strawberry	
	(μg/g)	RR (%)	(n=5) (%)	RR (%)	(n=5) (%)	RR (%)	(n=5) (%)
Tetraconazole	0.005	92.3	3.3	88.0	3.4	89.7	3.4
	0.05	88.3	4.0	89.3	3.4	91.3	3.3
Penconazole	0.005	88.3	4.6	88.3	4.0	85.3	4.1
	0.05	86.0	3.1	88.3	4.6	90.3	4.6
Tricyclazole	0.005	88.3	3.5	87.3	3.3	88.0	3.4
	0.05	89.0	3.4	88.0	3.4	88.0	4.5
Paclobutrazole	0.005	86.0	3.1	88.0	3.4	85.7	4.1
	0.05	91.7	3.3	92.7	3.5	88.7	4.0

Hexaconazole	0.005	88.0	3.0	92.7	3.5	89.3	2.8
	0.05	92.3	3.8	94.3	3.7	90.3	3.4
Diniconazole	0.005	85.3	3.6	84.7	3.0	85.3	3.6
	0.05	88.3	4.0	90.0	4.0	88.0	4.1
Propiconazole	0.005	88.3	4.0	91.7	3.3	91.0	4.0
	0.05	91.3	2.8	92.0	3.3	93.0	3.9
Tebuconazole	0.005	87.3	2.9	91.7	2.7	92.3	2.7
	0.05	91.3	3.8	92.7	3.5	94.3	2.7
Epoxyconazole	0.005	86.0	2.3	86.0	3.1	86.0	3.1
	0.05	89.0	3.0	91.7	3.8	93.3	4.5
Etoxazole	0.005	94.0	3.8	91.0	2.9	94.3	3.2
	0.05	95.0	3.2	94.3	2.7	95.0	3.2
Fluquinconazole	0.005	86.3	2.9	86.3	2.9	89.3	2.8
	0.05	88.7	4.0	93.7	3.7	92.3	2.7
Difenconazole	0.005	87.3	3.5	92.3	2.7	90.0	4.0
	0.05	95.0	2.8	92.7	3.5	90.7	4.2

RR = Relative Recovery

Table 3 : Concentrations of twelve fungicides in three batches of fruit samples

Fungicides Founded (μg/g)	Grape (Batch)-(μg/g)			Apple(Batch)-(μg/g)			Strawberry(Batch)-(μg/g)		
	1	2	3	1	2	3	1	2	3
Tetraconazole	ND	ND	ND	ND	ND	ND	0.05	ND	ND
Penconazole	ND	0.05	ND	N D	ND	ND	ND	ND	ND
Tricyclazole	ND	ND	ND	ND	ND	ND	ND	ND	ND
Paclobutrazole	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hexaconazole	ND	0.02	ND	ND	ND	ND	ND	0.01	ND
Diniconazole	ND	ND	ND	ND	0.005	ND	ND	ND	ND
Propiconazole	0.01	ND	ND	ND	ND	ND	ND	ND	ND
Tebuconazole	ND	ND	0.02	ND	ND	ND	ND	ND	ND
Epoxy conazole	ND	ND	ND	ND	ND	ND	ND	ND	ND
Etoxazole	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluquinconazole	ND	ND	ND	ND	ND	ND	ND	ND	ND
Difenconazole	ND	ND	0.01	ND	ND	ND	ND	0.005	ND

ND- Not Detected

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