

# Analysis of Multiclass Pesticide Residues in Vegetables using Microwave Assisted Extraction Followed by High Performance Liquid Chromatography with ultraviolet Detection

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**Abstract** – Pesticides are found in various parts of the food matrices in quite small concentration. Vegetables, such as potato and pepper are being consumed in increasing quantities which considered fresh production that is most susceptible to pesticide residues. Due to this, the information on the levels of pesticides residue in vegetables is usually used to assess their quality and safety. Potato and pepper are a type of vegetables that are mainly used throughout the world. The populations of Jimma town and around Kochi market were, one of the users of such vegetables. Therefore, the study was focused on the trace level determination of chlorfenvinphos and diazinon pesticides in potato and pepper. Thus, microwave-assisted extraction (MAE) for the sample preparation and High Performance Liquid Chromatography (HPLC) coupled with Ultraviolet Visible (UV) for the determination of these pesticides residues in these vegetables have been performed. The samples were collected from Kochi local market, Jimma town, Oromia region in Ethiopia. For analysis, the collected samples were washed by nitric acid, chopped, dried at room temperature, ground and kept in refrigerator at (-20°C) till analysis. The extraction was performed by using methanol and petroleum ether (1:2 v/v) as the microwave extraction solvents. The powders of the samples were digested using micro wave oven under the standard procedure conditions and subjected to HPLC-UV detection after cleanup for analysis. From the standard calibration curve the LOD of the two compounds (Diazinon and chlorfenvinphos) were calculated 1.263 and 2.926 ppm respectively. Under this study, the determined amounts of Diazinon in potato were 1.40 ppm and while that of the chlorfenvinphos was 0.55 ppm, which were below their detection limits.

**Keywords** – microwave assisted extraction, clean-up, residue, limit of detection, limit of quantification, linearity, recovery, calibration curve and chromatograms.

## I. INTRODUCTION

Pesticides are chemically diverse group of compounds widely use for the control of pests in agriculture practices to enhances harvest productivity (Alloway, B.J. and Ayers, D.C., 1997). Nowadays, more than 800 different kinds of pesticides are used for the control of insects, rodents, fungi, and unwanted plant in the process of agricultural production (Lijin.Z, et al 2012). However, it has the drawback of pesticide residues which remain on fruits and vegetables, consisting possible risk to consumers (Asplin, A.L 1997). Some of pesticides are known to cause birth defects, affect the functioning of central nervous system, respiratory system, endocrine system, and long term exposure to pesticides beyond tolerance limits are reported to induce cancer (Crestana, S. et al 2005). Therefore, governments and international organizations have established Maximum Residue Levels (MRLs), limiting the amount of pesticides in foods, for

example in the United States, Japan, European Union, and Food and Agriculture Organization (FAO., 2006). These legislative limits have become stricter than ever due to the concerns of food safety and the demand of trade barriers, driving the demand for more sensitive and reliable analysis methods for pesticide residues (Ledoux, M., 2011).

Sample preparation is normally required to isolate and concentrate target analytes from the sample matrix prior to chromatographic analysis (Helfrich, LA et al., 1996). Analysis of multiple pesticide residues in vegetables sample is often a time consuming, labor intensive and expensive process due to the complexity of the many analytes and matrices involved. Therefore, multi-residual methodologies capable to determine a large number of pesticides simultaneously with satisfactory sensitivity and selectivity are highly required (Abaul, J, 2008). In recent years, several alternative analytical procedures were developed for the determination of pesticide residues levels including liquid liquid extraction (LLE), solid-

phase extraction (SPE), (Eleni .B. 2003), accelerated solvent extraction (ASE), microwave-assisted extraction (MAE), matrix solid phase dispersion (MSPD) ,super critical fluid extraction (SFE) (Fenoll J, et al., 2007) and dispersive liquid-liquid micro extraction (DLLME) (Xiangyun. L, et al., 2011). Some of the procedures reported for fruits and vegetables required the additional clean-up steps to remove interferences and also to improve detection limits (Gilden RC., 2010).

Among these extraction methods, MAE is popular in recent years (Sandra Y.et al., 2012). MAE is successfully used as an alternate to the traditional LLE technique to determine pesticide residues in various matrices. It directly heats the solvent by microwave to partition the analytes from matrix to solvent, resulting in reduced extraction time (Miller GT., 2004). This keeps the temperature gradient a minimum and accelerates the speed of heating besides; consumes less volume of organic solvents, enables batch process and can be automated for the simultaneous extraction of organic pollutants at high pressure and temperature in a secured closed vessel system (Gouri. S, et al., 2011). Food and Agriculture Organization of the United Nations (FAO) has established MRL for different pesticides in many vegetables. Up to now, there is no MRL set for potato in Jimma town due to the lack of information about residue studies, and there was no paper published to report the residue determination of Diazinon, and Chlorfenvinphos in potato and green pepper.

Hence, the main objective of this study was to analyze the trace levels of two pesticides (Diazinon, and Chlorfenvinphos) in vegetable potato and green pepper using MA followed by HPLC-UV detection. The method is based on microwave-assisted extraction with partition of organophosphorus pesticides between methanol and petroleum ether mixture. The cleanup step for the analysis of pesticide in this study was performed with distilled water, saturated sodium sulfate solution and anhydrous sodium sulfate. Finally the clean organic extractant were injected and run on the HPLC\_UV detection (Marek .G. 2001).

## II. MATERIALS AND METHODS

### 1. Chemicals and Reagents

HPLC grade solvents (acetonitrile and methanol) were purchased from (Sigma Aldrich, Germany), petroleum ether, methanol, sodium sulfate, distilled water, deionized water, nitric acid, acetone, Analytical standards Assay (HPLC) 97.7% diazinon and chlorfenvinphos were used. Instruments and Apparatus

Measuring cylinders (100 mL), analytical digital balance (Baling Germany), funnel, separatory funnel (250 mL), filter paper (Schleicher and Germany), spatula, knife, pestle and mortar, stand clamp, medical syringe, micropipette, dropper, wash bottle, plastic bags, beakers,

vials, aluminum foil and volumetric flasks (100 mL) were used. Sample preparation was performed by closed Microwave oven (speed wave) equipped with 12 extraction vessels of Teflon PFA (perfluoroalkoxy) with capacity of 50 ml volume was purchased from Spain. Chromatographic analysis were performed using Perkin Elmer HPLC quaternary solvent system equipped with Flexar Solvent manager, Flexar LC Auto sampler, Flexar LC pump, and Flexar Column and Flexar UV/VIS Detector all purchased from Perkin Elmer (Waltham USA). Data acquisitions and processing were accomplished with LC Chromera Software (Kamrin, M.A., 1997).

### 2. Standard Preparation

Standard solutions were prepared from stock solution of 1000 ppm of chlorfenvinphos and diazinon for calibration curve at concentration of 40 and 100 ppm respectively using methanol. The calibration curve concentration were prepared at (1, 10, 30, 60 and 100 ppm) for diazinon and (0.4, 4, 12, 24 and 40 ppm) for chlorfenvinphos. (Lotti M. et al., 2001).

### 3. Sample Collection Technique

The samples of potato and pepper were randomly collected from Jimma town in local Kochi market.

### 4. Sample Preparation Procedure

According to (Peter. J, et al., 2012) the collected samples were washed with nitric acid to remove contaminants. The edible parts of the collected vegetable (potatoes) were cut into small pieces with knife and dried at room temperature by wrapping with clean polyethene. The dried samples were ground and kept in a clean polyethene.

### 5. Microwave Assisted Extraction procedure

The powders of samples were prepared for pesticide analysis using MAE procedure (Harry M. et al., 1997). After grinding, 1 g of sample powder was placed into the microwave vessel together adding 3 mL of methanol and 6 mL of petroleum ether. Two of the extraction vessels were spiked with standard solution of mixed pesticides and one vessel with the sample was unspiked. The vessels were sealed and heated using microwave parameters programmed as the following.

Table -I: Microwave parameters for extraction (Marek. B and Jolanta. S, 2015).

Temperature	Pressure	Ramp	Time	Power
90	30	2	1	80
100	35	2	10	80
50	25	1	0	80

After heating, the vessel was cooled to room temperature and filtered by using what man No# 42 filter paper. Then,

the aliquot was transferred to a 250 mL separatory funnel for the clean-up

### 6. Cleanup Procedures

Ledoux. M, 2011 to clean the interferences from the analyte, 15 mL of distilled water was added to the concentrated methanol: petroleum ether extractant obtained from MAE as described above in the separatory funnel. The separatory funnel was capped, vented, and gently swirled for 1 min. The funnel was then allowed to stand for 10 min, to allow the water and organic solvent layers to separate. After separation, the water layer was drained off and discarded. The organic layer was washed, with gentle agitation of the separatory funnel, three times, each time with 15 mL of distilled water without added sodium sulfate. After the third water wash was drained, 10 mL of distilled water and 1 mL of saturated sodium sulfate solution were added to the funnel and the separatory funnel was swirled for a few seconds. This final wash served to break any emulsions formed from the previous water washes. The final wash was discarded, and the organic solvent extract was transferred to a 15 mL vial that contained 1 g of anhydrous sodium sulfate. The vial was sealed with a Teflon-lined cap, shaken for 1 min, and allowed to settle for 10 min. This step was repeated two additional times. The extract was allowed to stand undisturbed over the sodium sulfate for 1 hr. to allow any particulates to settle and followed by filtration with 0.25  $\mu$ L. Finally, the clean organic layers were taken by medical syringe and collected in the vials. Finally, the clean extractant was kept in the refrigerator till to analysis by HPLC\_UV detection.

#### HPLC Condition

Cunha S. and M. B. (2011) after clean-up process 60 mL of the clean extractant was taken in vials by Micropipette and mixed with 50 mL of HPLC grade Methanol. Finally, the clean organic extractant mixed with Methanol were ejected and run on HPLC under the mobile phase composition of water: acetonitrile (40:60), flow rate was (1 mL min<sup>-1</sup>), injection volume (10 $\mu$ L), retention time of (16 min) and wavelength of the UV/visible detector fixed at (230 nm) for the determination of the selected pesticides. Separation was performed on reversed phase C-8 column and the detector was connected to the computer for data processing.

## III. RESULTS AND DISCUSSIONS

### 1. Analytical Performance Study

The analytical method was performed on parameters of linearity, recovery, limit of detection (LOD), and limit of quantification (LOQ). The linearity of the method was obtained by least- squares linear regression analysis of the peak area versus analyte concentration, using five concentration levels at (1, 10, 30, 60 and 100 ppm) and (0.4, 4, 12, 24, and 40 ppm) in triplicates for both Chlorfenvinphos and Diazinon respectively. It can be

expressed in a model such as  $y=a+bx$ . This model is used to compute the sensitivity  $b$  and the LOD and LOQ.

$$\text{LOD}=3\text{SD}/b \ \& \ \text{LOQ}=10\text{SD}/b$$

Where SD is the standard deviation of the response and  $b$  is the slope of the calibration curve. The HPLC detector response was give linear calibration curve for Chlorfenvinphos  $y= 12817x + 614.46$  and Diazinon  $y= 7141.8x + 834.11$  at these levels. The correlation coefficients ( $R^2$ ) are shown in (Table 4.1) and the value were not less than ( $R^2 =0.9995$ ) for both standards. For a linear calibration curve, it is assumed that the instrument response  $y$  is linearly related to the standard concentration  $x$  for a limited range of concentration. A five -point linear calibration curve of standards were constructed in Microsoft Excel, 2013 as (Figure 1).

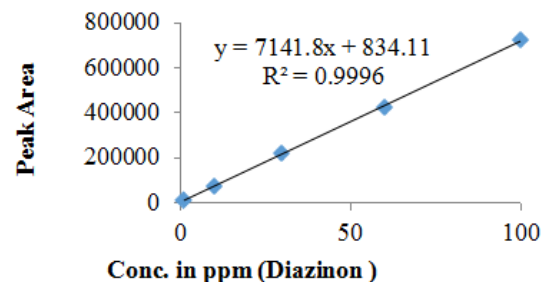
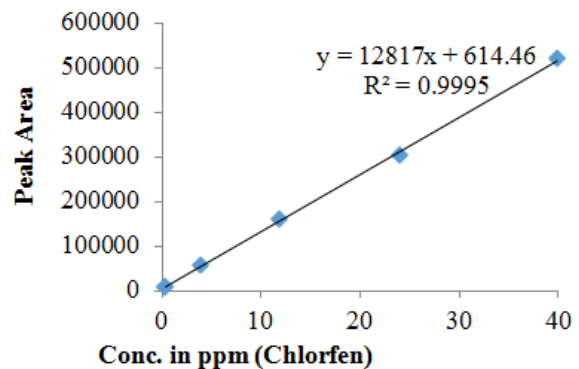


Fig.1. a) Calibration curve for the Chlorfenvinphos b) Calibration curve for the Diazinon.

Recovery studies were performed to examine the efficacy of extraction and clean up method. Recoveries were conducted by spiking Chlorfenvinphos and Diazinon at level of 28, 70 ppm respectively. The samples (potato) were spiked with known concentration of the pure standard solution of each type of pesticide and extraction and clean-up were performed as described earlier.

The obtained percent recovery of Chlorfenvinphos and Diazinon after MAE and clean up procedures in the

potato samples were 40.89% and 31.15% within 17.5% RSD respectively.

Table -II. Data on performance of the method

Compounds	Peak area	Retention time (min)	R <sup>2</sup>	SD	RSD	LOD (ppm)	LOQ (ppm)	Recovery
Chlorfenvinphos	7746	6.9	0.9995	5397.39	17.6	1.263	4.211	40.89%
Diazinon	10861	9.7	0.9996	6966.68	17.5	2.926	9.754	31.15%

SD=standard deviation, LOD=limit of detection, LOQ=limit of quantification, RSD= relative standard deviation. The LOD is the lowest concentration of the analyte in a sample, which can be detected but not necessarily quantified. LOQ is the lowest concentration of the analyte in a sample, which can be quantified with an acceptable degree of accuracy and precision under the stated conditions of test. Limit of Detection (LOD) of unspiked potato was calculated at the peak area 7746 and 10865 ppm for the chlorfenvinphos and diazinon respectively. The LOD of chlorfenvinphos and diazinon were 1.263 and 2.926 ppm respectively. According to the results shown in Table 4.1 Chlorfenvinphos and Diazinon insecticides were not detected in the samples because they were present in the samples below our detection level. From the result obtained chlorfenvinphos and diazinon pesticide residues was not detectable by the MAE method in the potato samples collected from market of Jimma city.

## 2. HPLC Chromatograms

### • HPLC Chromatograms' of Standards

The blank methanol was run as seen from the figure 4.2 to check the solvent in which the standar pesticides prepared is free of any interfering peaks on our retention time and it is exactly blank.

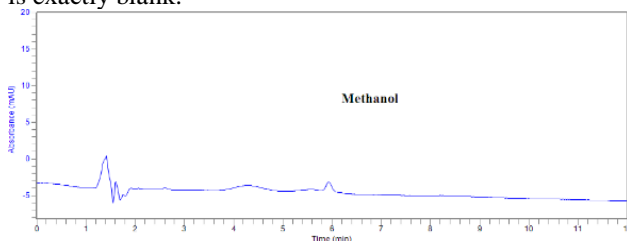


Fig. 2. Chromatogram of blank methanol.

Figure 4.3 below shows chromatograms of the reference standards. The HPLC retention times for Chlorfenvinphos and Diazinon were 6.9 and 9.7 min, respectively. The elution time of the chlorfenvinphos is shorter than the elution time of diazinon. This is due to that chlorfenvinphos is more likely the mobile phase composition of RHPLC or more polar than diazinon. The maximum absorption wavelength for both chlorfenvinphos and diazinon was evaluated with detection at 254 nm. This simplified the data handling, and very few interfering peaks at the retention times of the pesticides were observed for the quantification of the two pesticides at this wavelength.

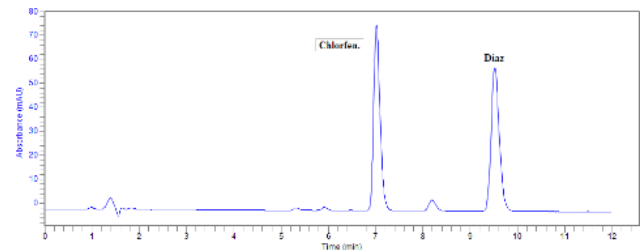


Fig.3. HPLC chromatograms of the reference standards of Chlorfenvinphos and Diazinon.

### • Chromatogram of unspiked potato

Table -III. Residues levels of Chlorfenvinphos and diazinon analyte in unspiked potato

Compounds name	Analyte concentration (ppm)	Retention time (min.)	Average peak area
Chlorfenvinphos	0.55	6.9	7746
Diazinon	1.40	9.7	10861

The concentration of the Chlorfenvinphos and Diazinon pesticide residues in the extract were determined by comparing their peak area with that of reference standards. Table 4.2 shows the amount of chlorfenvinphos and diazinon in the potato sample. The concentration of chlorfenvinphos and diazinon in the potato were 0.55 and 1.40 ppm respectively. Both of these compounds were not detected, because they are present below the detection limit determined from the linear calibration curve of the standards.

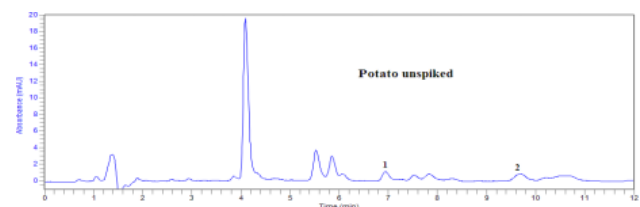


Fig. 4. HPLC chromatograms unspiked potato.

The HPLC chromatograms of unspiked potato Figure 4.4, shows the presence of chlorfenvinphos and diazinon pesticides in the potato collected from the Kochi market area. In the unspiked potato, the peak area of chlorfenvinphos was 7746 while of the diazinon was 10861 (Table 3); which is directly related to the concentration of each compound in the sample. This is why the amount diazinon in the potato is higher than chlorfenvinphos. The retention times for the two pesticides in the unspiked potato were the same with their standards; which is 6.9 and 9.7 min for both chlorfenvinphos and diazinon respectively.

### • Chromatograms of spiked potato

The compound was identified by comparing its retention time with respect to technical grade reference standard. When comparing the retention time of spiked sample

(Figure 4.5) with that of standards (Figure 4.3) they are the same. This shows that the presence of the two pesticides (chlorfenvinphos and diazinon) in the spiked potato. The standard solutions for the calibration curves were prepared in control matrix because samples may possess coextractant in the matrix which may affect the peak area of the unknown samples.

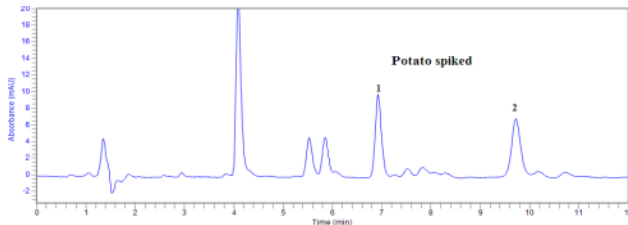


Fig.5. HPLC chromatograms of spiked potato.

From this chromatogram, no more interfering peaks were observed. In the case of MAE extracts of potatoes, a peak was always found at an early retention time, but as such, it did not affect the pesticide peaks used in detection. Also, the result obtained shows the performance of microwave assisted extraction method for the determination of these pesticides at the trace level. As indicated in the table 4.3, the amount of chlorfenvinphos in the spiked sample with 28 ppm was 12.003 ppm and the diazinon with the spiking level at 70 ppm was 23.221 ppm.

Table -IV: residues of Chlorfenvinphos and Diazinon in the spiked potato samples.

Component Name	Retention time (min)	Average peak area	Analyte concentration (ppm)
	Chlorfenvinphos	6.9	154449.5
Diazinon	9.7	166602.5	23.211

• **Chromatograms of spiked and unspiked pepper**

Similarly the procedure was performed for the extraction of chlorfenvinphos and diazinon in peppers. However, highly interfering peaks were observed for the detection and quantification of the two pesticides from the pepper.

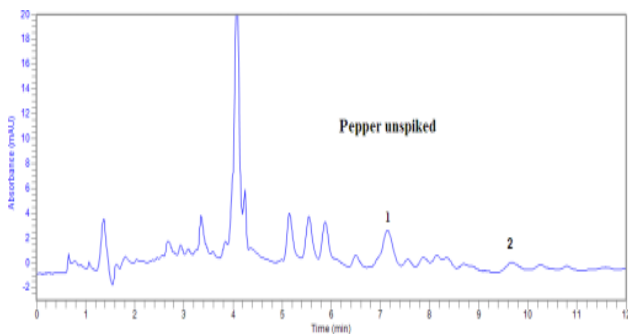


Fig.6. chromatogram of unspiked pepper.

The (Figure 4.6) shows the chromatogram of unspiked pepper. From this the peak of both chlorfenvinphos and diazinon were comes at the retention time of 6.9 and 9.7 min respectively. However, their peaks were highly dominated by the interference peaks come before and after the analytes peaks. Due to this interferences, the observed peak area of the target analytes were not good. Therefore, it was difficult to determine the amounts of chlorfenvinphos and diazinon in the samples of pepper.



Fig.7. chromatogram of spiked pepper.

The peak area of the above pepper was not good; it might be the interference comes due to the lack of clean-up procedures or MAE method. The MAE extraction procedure and clean up steps described above was not good for the extraction of chlorfenvinphos and diazinon in the peppers. This is because of that the matrixes exist in potatoes and peppers are not similar. So, for the extraction of these pesticides in pepper other extraction method is required.

**IV. CONCLUSION**

In general, MAE was carried out for the determination of the organophosphate pesticides diazinon and chlorfenvinphos in potato samples. The method presented here demonstrates that MAE is an efficient tool for simultaneous extraction of pesticides residues from potato without showing strong matrix effects. However, the extraction method was not performed for the pepper, due to its highly interfering peaks. Extraction was made by MAE using methanol: petroleum ether (1:2) as the extraction solvents. The selected extraction solvents were based on their good microwave absorbing property and high selectivity towards the analytes of interest excluding unwanted matrix. Vegetables are very complex, so a cleanup step is required to decrease the presences of interferent in the final extract and also to avoid the deterioration of chromatographic column. Finally, the amount of chlorfenvinphos and diazinon in the potato were determined by injecting the clean organic extractant to the HPLC\_UV and the result were displayed on the chromera data acquisition software. The amount of the chlorfenvinphos and diazinon determined in the potato sample were 0.55 and 1.40 ppm respectively. Both of these standards were not detected because they were

present below the calculated detection limit. Good linearity was obtained with this method for all the investigated pesticides. The regression coefficients in the linearity were better than 0.9995 in all cases with the relative standard deviation (RSD) value less than 17.5%. The amount of chlorfenvinphos and diazinon pesticides in potato obtained from the investigation tell us the importance of further analysis of vegetables found in kochi local market.

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