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Chromatographic and Spectrophotometric Analysis of Endosulfan in Vegetables

Khalid Mohamed El-Hassan
Omdurman Islamic University, the Islamic World Researches and Studies Institution, Omdurman, Sudan

Abstract
A High Pressure Liquid Chromatography (HPLC) and Spectroscopic methodologies have been validated for the determination of **α** Endosulfan**, β** Endosulfan and Endosulfan sulphate in tomato "*Lycopersicon esculentum Mill"*. The chromatographic separation method was based on liquid-liquid extraction (LLE) using two immiscible solvents. Acetonitrile/water mixture (70/30, v/v %) was used as a mobile phase. The HPLC was equipped with UV/visible detector, the separation was achieved on µ-Bondapak C18 column with dimensions, 4.0 x 125 mm and 5-micron particle diameters using activated florisil to remove the interferences from the extract. The chromatographic experiments were carried out in triplicate at three fortifications levels 1, 2 and 3 ppm. HPLC's detector was linear for the determination of pesticides with **(r)** values > 0.99. The limit of detection **(LOD)** values was0.020071, 0.002352 and 0.01619 ng/ml. The limit of quantification values **(LOQ)** were 0.066903, 0.0078414 and 0.05396 ng/ml. The recoveries percentage of the pesticides studied ranged from 88.13% to 98.77%, 84.60% to 96.86% and 82.90% to94.85%and the coefficient of variation **(CV)** values ranged from 0.003 to 0.005, 0.005 to 0.0057 and 0.004 to 0.007 for **α** Endosulfan, **β** Endosulfan and Endosulfan sulphate, respectively. The spectrophotometric method was based on the liberation of sulphur dioxide after addition of alcoholic potassium hydroxide, the liberated sulpher dioxide was passed through hydrogen peroxide and diphenylamine and the light violet color of diphenyl benzidine was measured at 605 nm using spectrophotometer. The Spectrophotometric experiments were carried out in triplicate at three fortifications levels 3, 5 and 7 ppm. The spectrophotometer showed a linear response for the detection of pesticide with **(r)** value >0.99. The **(LOD)** value was 0.089953ng/ml, the **(LOQ)** value was 0.299843ng/ml. The recoveries values ranged from 89.23%to 99.02% and the coefficient of variation **(CV)** values ranged from 0.01407 to 0.02820. The levels of Endosulfan recovered in both methodologies were above the Maximum Residue Level **(MRL)** of Endosulfan in tomato (0.5 ppm).
Keywords: HPLC, Endosulfan, chromatographic, spectrophotometric, pesticides

Introduction

The use of pesticides to control pest and diseases are a common practice in the fields to increase crop yield. However, these chemicals can reach plant tissues, leaving residues that can be detected in the vegetables for example: Endosulfan. This may become a significant route to human exposure to these toxic compounds. In order to protect consumer's health, maximum residue levels (MRLs) in these vegetables have been established in different countries and internationally by Codex Alimentarius. The high number of pesticides to be monitored in those matrices, along with the typically low concentrations of the MRLs, requires highly sensitive and selective methods. Consequently, sample preparation becomes a key step of the analytical procedure. In recent times, extensive efforts have been made to the development of new sample preparation techniques that save time, labor money and solvent consumption to improve the analytical performance of the procedure. Analytical instrument are needed to determine, quantify and confirm pesticide residues in vegetables for both research and regulatory purposes. The pesticides are generally analyzed by spectrophotometry [5, 9], thin layer chromatography (TLC) [13, 14] high performance liquid chromatography (HPLC) [4, 8], gas chromatography (GC) [1, 3], and GC-MS [2, 16]. The present study describe method of extraction, cleanup and determination of a pesticides by using high performance liquid chromatography and spectrophotometry for the separation, identification and quantification of endosulfan on tomato were developed and validated. Finally, the method was applied to the determination of these pesticides in commercial samples collected from the local markets. Therefore, the purpose of this study was to develop an analysis scheme for determination of this pesticide in tomato HPLC and spectrophotometry.

Research Issues

1- How to avoid the environmental effects like moisture, and heat etc., from contacting with test sample.
2- How to control and optimize the physical parameters e.g. temperature, pressure
2- To find an appropriate method to collect the samples fairly, or how to collect a representative sample.
3- Sample Validation, considering the type of samples was used in this experiment.
4-Thermal degradation and decomposition which causes High temperatures in injector and column, residence time, mainly injector related.
5- How to find the most efficient, reproductive, accurate and precise chromatographic and spectroscopic procedures to be applied for analysis.

6- Matrix effects which cause deactivation of HPLC system by co-extracts, waxes, colorants, Creation of active sites, catalytic processes, Both Injector/column related, and disturbance of the color reaction in spectrophotometric analysis.
7- Most problems in the application of UV/Vis spectrophotometry result from the user choosing the wrong cuvette for the sample at hand.

Experimental
Chromatography
HPLC System
Model Analytical Technologies 3000 series HPLC having a pump, an auto sampler, column oven, and UV/visible detector was used for identification and quantification of pesticides. Reversed phase liquid chromatography with 4.0 x 125 mm and 5 micron particle diameters of µ-Bondapak C18 column was employed. The working condition of HPLC was binary gradient, The optimized HPLC mobile phase using the mentioned column was a mixture of acetonitrile /water (70:30), respectively, flow rate was 0.8ml/min, injection volume 20 µl, pressure 6-7 MPa and the wavelength of the detector was fixed at 254 nm.
Apparatus
1- Blender, high speed; explosion-proof Waring Blendor, 1 qtr. jar
2- Buchner funnels (Buchner), porcelain12 cm diameter
3- Filter paper, Shark Skin, to fit Buchner
4- Graduated cylinders (graduates), glass-stopper (g-s), 100 mL, and plain, 250 ml
5- Separatory funnel (separator), 1 L
6- Vacuum filtration flask, 500 ml
7- Volumetric flasks 250 ml
8- Pipettes10 ml
9- Beakers 250 ml
Chemicals and Reagents
1- Pure (α, β) Endosulfan and Endosulfan Sulphate Powder
2- Acetonitrile
3- Petroleum ether
4-Sodium sulfate Anhydrous
5- Distilled Water
6- Sodium Chloride
Preparation of Standard: Stock standard solutions of **α**-Endosulfan, **β**- Endosulfan and Endosulfan Sulphate (5 ppm) were prepared by dissolving the appropriate quantity of the pesticide in acetonitrile, then a volume of 5 µL of each standard was injected into HPLC and both of retention time and peak area were recorded, working standard solutions having different concentrations of each pesticide were then prepared from the stock solution by dilution using mobile phase as diluent. The following series of diluted concentrations were prepared immediately before injection: (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1ppm). A volume of 5µL of each of these solutions was
injected into the HPLC and the chromatography was recorded in the conditions described above and peak areas were recorded and plotted versus the concentration of the pesticide, then the Linearity of the system was confirmed.
Spiking Scheme [*] **:** A three standard solutions of (1, 2 and 3 ppm) were prepared in acetonitrile for each of (α, β) endosulfan and endosulfan sulphate, they were used for spiking tomato samples, after extraction and clean up, 3 replicates were taken (5µL injection) of each pesticide at each spiking concentration and the corresponding retention times and peak areas were recorded after they compared with those of the pesticides standards.
Extraction Procedure: According to (*Mills, P.A., (1963) and Porter, M., (1967))* [11] A (100 g) representative sample was weighed and placed into blender jar, a volume of 200 ml of acetonitrile were added, the mixture was blended for 2 minutes at high speed, then it was filtered with suction through Buchner fitted with filtration paper into vacuum filtration flask. The filtrate was transferred into a 1L seperatory funnel, after that a volume of 100 ml of petroleum ether was measured carefully using graduated cylinders then it poured into seperatory funnel and the whole mixture was shacked vigorously for 2 minutes. A volume of 10 ml of saturated sodium chloride was added followed with 600 ml of distilled water, the seperotary funnel was held in a horizontal position and shacked vigorously for 2 minutes, after the 2 immiscible layers were separated, the aqueous layer was discarded and the solvent layer was washed gently with two portions of 100 ml of distilled water, then the washings were discarded and the solvent layer was transferred into 100 ml graduate, a weight of 15 g of sodium sulphate was added and the mixture shacked vigorously, finally, after about 1h the solution was transferred into floirsil

[*] The addition of a known amount of analyte (known as a *spike*) to the sample, which is then analysed as normal.

column for cleanup.

Clean up by Column Chromatography: According to *(Luke, M.A., (1975))* [12] the extract was cleaned up by column chromatography using activated florisil (4 g) was placed in a 22 mm column, 0.5 g anhydrous sodium sulphate was added to column and then it was washed with 40-50 ml petroleum ether. The extract was diluted to 10 ml with acetone and transferred to 100 ml graduated cylinder, and then it was diluted with 100 ml with petroleum ether. The extract was introduced to the column letting it pass through a flow rate of 5 ml/min. The column was eluted at about 5 ml/min with 200 ml of 15% ethyl ether/petroleum ether eluant, finally and the eluate was concentrated by rotary evaporator and left at room temperature to dryness.

Sectrophotometry:

Spectrophotometer System: A Double beam UV 1800 ultraviolet-visible spectrophotometer model Shimadzu 1800, with quartz cells of 1 cm optical path length**.**

PH Meter: PH meter model pH 211(HANNA Italy) was used for pH measurements.

Apparatus:

1- Conical Flasks 250 ml
2- Volumetric flasks 250 ml
3- Pipettes 10 ml
4- Beakers 250 ml
5- Water Path
6- Blender, high speed; explosion-proof Waring Blendor, 1 qtr. jar
7- Buchner funnels (Buchner), porcelain, and 12 cm diameter
8- Filter paper, Shark Skin, to fit Buchner

Chemicals and Reagents:

1- Ethanol
2- Hydrogen Peroxide, (0.1N)
3- Potassium Hydroxide
4- Diphenyl Amine
5- Pure Endosulfan Powder

Preparation of Standard: Stock solution of endosulfan (5ppm) was prepared by dissolving the appropriate amount of endosulfan in ethanol. The stock solution was used to prepare the working standard solutions by serial dilution (2, 4, 6, 8 and 10 ppm) into five of 250 ml volumetric flasks, into each working standard solution,1 ml of alcoholic potassium hydroxide, 10 ml of 0.1N hydrogen peroxide and 0.1 ml of diphenyl amine were added to give a light violet color, into another 250 volumetric flask the same amounts of alcoholic potassium hydroxide, 0.1 N hydrogen peroxide and diphenyl amine were added and then filled to the mark with ethanol as a blank. The solutions were kept aside for 5 min before taking absorbance then absorbance was measured and recorded at 605 nm against reagent blank. The absorbance corresponding to the bleached color which in turn corresponds to the analyte endosulfan concentration was obtained by subtracting the absorbance of the blank solution from that of test solution.

Spiking Scheme: A three standard solutions of endosulfan (3, 5 and 7 ppm) were prepared in ethanol and were used for spiking tomato samples, after extraction, 3 replicates of each concentration were taken and the absorbance was measured and recorded at 605 nm against reagent blank.

Procedure: According to *(N.V.S. Venugopal and B. Sumalatha, 2011)* [12] a (5 g) representative sample was weighed and placed into blender jar, blended for 2 minutes at high speed, it was filtered with suction through Buchner fitted with filtration paper into vacuum filtration flask. The filtrate was placed in a water path for 2 minutes and then it was transferred into a beaker, a volume of 1ml of alcoholic potassium hydroxide was added carefully using graduated cylinder then 5 ml of 0.1N hydrogen peroxide was added followed by 0.1 ml diphenyl amine and the violet color was obtained**,** finally, the absorbencies of the solutions were taken and recorded at 605 nm against the reagent blank.

Results:

Chromatography:

Table (1): Retention times and peak areas of the standard solutions of pesticides (5ppm)

Pesticide	Retention Time (R_t) (min)	Peak Area
α-Endosulfan	13.142	175381.39
β-Endosulfan	10.374	150695.50
Endosulfan sulphate	7.598	132046.64

Source: Results of the experiment conducted by the researcher.

Table (2): Obtained peak areas for each pesticide at all standard series concentration

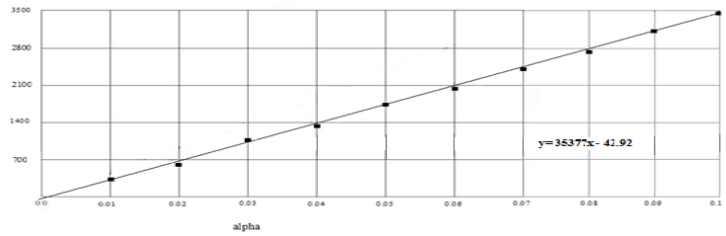
Series	Concentration (ppm)	peak area of α Endosulfan	peak area of β Endosulfan	peak area of Endosulfan Sulphate
1	0.01	307.20	287.43	270.87
2	0.02	642.12	594.86	533.65
3	0.03	1014.28	895.14	802.23
4	0.04	1371.54	1196.76	1064.94
5	0.05	1722.49	1493.24	1330.59
6	0.06	2078.33	1803.65	1594.61
7	0.07	2424.67	2104.36	1859.52
8	0.08	2781.58	2405.28	2124.46
9	0.09	3141.88	2701.73	2396.37
10	0.1	3487.78	3013.91	2660.93

Source: Results of the experiment conducted by the researcher.

Calibrations Curves:

α - Endosulfan:

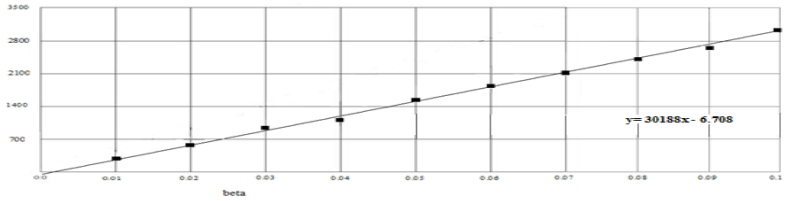
Figure (1): Calibration curve of 10 series of endosulfan α



Source: Results of the experiment conducted by the researcher.

β -Endosulfan:

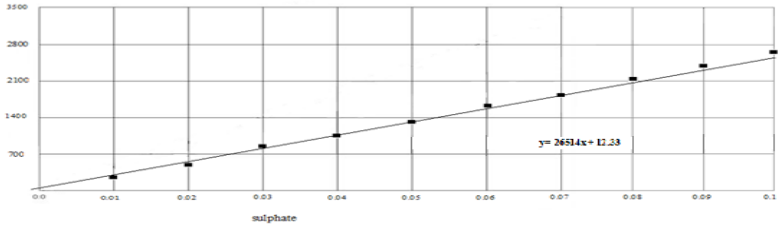
Figure (2): Calibration curve of 10 series of endosulfan β



Source: Results of the experiment conducted by the researcher.

Endosulfan Sulphate:

Figure (3): Calibration curve of 10 series of endosulfan sulphate



Source: Results of the experiment conducted by the researcher.

Table (3): Data on calibration curves for each pesticide series

Pesticide	Regression equation	Correlation coefficient (R)
α- Endosulfan	y= 35377x - 42.92	0.999978
β –Endosulfan	y= 30188x - 6.708	0.999997
Endosulfan Sulphate	y= 26514x + 12.33	0.999996

Source: Results of the experiment conducted by the researcher.

- The resulted retention times for each pesticide`s triplicate were compared with the corresponding of those of standards and the resulted peak areas were used to obtain the recovered concentrations for each

pesticide`s triplicate at the three Spike concentrations by using the regression equations illustrated in table (3).

Table (4): Retention times and peak areas of the triplicate injection of the (1 ppm) spiked samples

Replicate	α-Endosulfan Peak Area	α-Endosulfan Retention Time	β-Endosulfan Peak Area	β-Endosulfan Retention Time	Endosulfan sulphate Peak Area	Endosulfan sulphate Retention Time
1	31137.29	10.246	25786.28	13.082	22157.09	7.576
2	30843.35	10.324	25757.46	13.140	22306.18	7.506
3	30886.69	10.221	25533.88	13.096	21993.56	7.412

Source: Results of the experiment conducted by the researcher.

Table (5): The recovered concentrations of each pesticide at 1(ppm)

	Concentration (ppm)		
Replicate	α-Endosulfan	β-Endosulfan	Endosulfan sulphate
1	0.8813	0.8544	0.8352
2	0.8730	0.8534	0.8408
3	0.8742	0.8460	0.8290

Source: Results of the experiment conducted by the researcher.

Table (6): Retention times and peak areas of the triplicate injection of the (2 ppm) spiked samples

Replicate	α-Endosulfan Peak Area	α-Endosulfan Retention Time	β-Endosulfan Peak Area	β-Endosulfan Retention Time	Endosulfan sulphate Peak Area	Endosulfan sulphate Retention Time
1	67523.78	10.336	56233.66	13.102	48373.86	7.568
2	67468.03	10.348	56293.26	13.134	48819.10	7.597
3	67138.12	10.368	56752.74	12.936	48158.53	7.557

Source: Results of the experiment conducted by the researcher.

Table (7): The recovered concentrations of each pesticide at 2 (ppm)

	Concentration (ppm)		
Replicate	α-Endosulfan	β-Endosulfan	Endosulfan sulphate
1	1.9099	1.8630	1.8239
2	1.9083	1.8649	1.8407
3	1.8990	1.8801	1.8158

Source: Results of the experiment conducted by the researcher.

Table (8): Retention times and peak areas of the triplicate injection of the (3 ppm) spiked samples

Replicate	α-Endosulfan Peak Area	α-Endosulfan Retention Time	β-Endosulfan Peak Area	β-Endosulfan Retention Time	Endosulfan sulphate Peak Area	Endosulfan sulphate Retention Time
1	104788.81	10.315	86727.21	13.110	75071.58	7.526
2	104465.34	10.353	87722.53	13.078	75464.63	7.463
3	103943.76	10.311	87209.23	13.121	7475977	7.433

Source: Results of the experiment conducted by the researcher.

Table (9): The recovered concentrations of each pesticide at 3 (ppm)

Replicate	Concentration (ppm)		
	α-Endosulfan	β-Endosulfan	Endosulfan sulphate
1	2.9632	2.8731	2.8309
2	2.9541	2.9060	2.8457
3	2.9393	2.8890	2.8191

Source: Results of the experiment conducted by the researcher.

The data in tales (4 to 9) were used to construct (Spike Concentration Vs. Peak Area mean) curves for each pesticide and to calculate standard deviation, recovery (R%), limit of detection (LOD), limit of quantification (LOQ) and coefficient of variation[*].

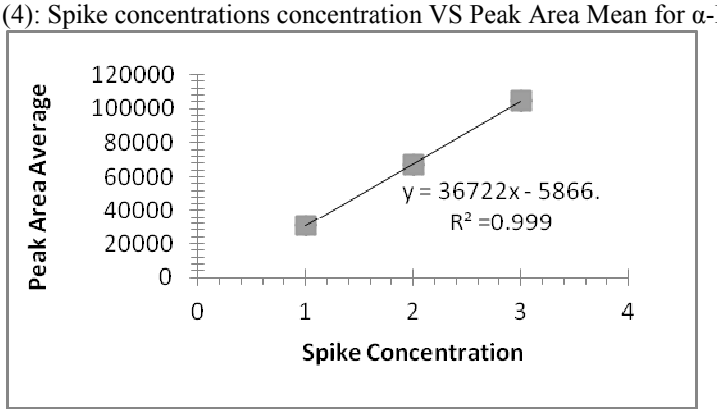
α –Endosulfan:

Table (10): Mean, standard deviation and the coefficient of variation of α-endosulfan peak areas at the three Spike concentrations

Spike concentration	Peak Area			Peak Area Mean	Standard Deviation	Coefficient of Variation
	Replicate (1)	Replicate (2)	Replicate (3)			
1 ppm	31137.29	30843.35	30886.69	30955.78	158.6818	0.005126
2 ppm	67523.78	67468.03	67138.12	67376.64	208.4396	0.003094
3 ppm	104788.81	104465.34	103943.76	104399.30	426.3778	0.004084

Source: Results of the experiment conducted by the researcher.

Figure (4): Spike concentrations concentration VS Peak Area Mean for α-Endosulfan



Source: Results of the experiment conducted by the researcher.

Table (11): r, limit of detection, limit of quantification of the recovered α-endosulfan concentrations at the three Spike concentrations

r	LOD	LOQ
0.99949	0.020071	0.066903

Source: Results of the experiment conducted by the researcher.

[*]**SD**: Standard deviation is a measure of the dispersion of a set of data from its mean.
R: how much amount of drug recovered by proposed analytical method after the sample is spiked with standard drug.
LOD: is the lowest quantity of a substance that can be distinguished from the absence of that substance (a *blank value*) within a stated confidence limit (generally 1%).
LOQ: is the lowest concentration of the analyte that can not only be detected but can be quantified within defined limits of certainty after replicate measurements are made on the blank and known low concentration.
CV: A coefficient of variation is a statistical measure of the dispersion of data points in a data series around the mean.

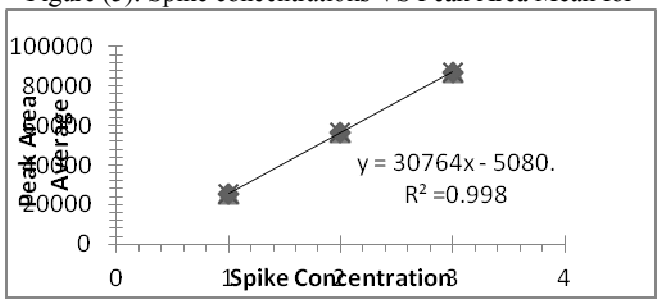
β -Endosulfan:

Table (12): Mean, standard deviation and the coefficient of variation of β-endosulfan peak areas at the three Spike concentrations

Spike concentration	Peak Area			Peak Area Mean	Standard Deviation	Coefficient of Variation
	Replicate (1)	Replicate (2)	Replicate (3)			
1 ppm	25786.28	25757.46	25533.88	25692.54	138.1571	0.005377
2 ppm	56233.66	56293.26	56752.74	56426.55	284.0534	0.005034
3 ppm	86727.21	87722.53	87209.23	87219.66	497.7419	0.005707

Source: Results of the experiment conducted by the researcher.

Figure (5): Spike concentrations VS Peak Area Mean for



Source: Results of the experiment conducted by the researcher.

Table (13): r, limit of detection, limit of quantification and standard error of the recovered β-endosulfan concentrations at the three Spike concentrations

r	LOD	LOQ
0.99899	0.002352	0.0078414

Source: Results of the experiment conducted by the researcher.

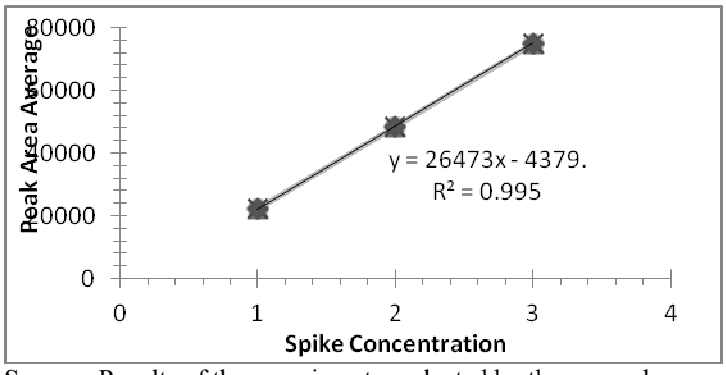
Endosulfan Sulphate:

Table (14): Mean, standard deviation and the coefficient of variation of endosulfan sulphate peak areas at the three Spike concentrations

Spike concentration	Peak Area			Peak Area Mean	Standard Deviation	Coefficient of Variation
	Replicate (1)	Replicate (2)	Replicate (3)			
1 ppm	22157.09	22306.18	21993.56	22152.27	156.3656	0.007058
2 ppm	48373.86	48819.1	48158.53	48450.49	336.8873	0.006953
3 ppm	75071.58	75464.63	7475977	75098.66	353.2094	0.004703

Source: Results of the experiment conducted by the researcher.

Figure (6): Spike concentrations VS Peak Area Mean for Endosulfan Sulphate



Source: Results of the experiment conducted by the researcher.

Table (15): r, limit of detection, limit of quantification and standard error of the recovered endosulfan sulphate concentrations at the three Spike concentrations

r	LOD	LOQ
0.99749	0.01619	0.05396

Source: Results of the experiment conducted by the researcher.

Table (16): Recovery percentages for the obtained recovered concentrations of α-endosulfan at the three Spike concentrations

Spike Concentration	Replicate (1)	Replicate (2)	Replicate (3)	Average
1 ppm	88.13%	87.30%	87.42%	87.61%
2 ppm	95.49%	95.41%	94.95%	95.28%
3 ppm	98.77%	98.47%	97.97%	98.40%

Source: Results of the experiment conducted by the researcher.

Table (17): Recovery percentages for the obtained recovered concentrations of β-endosulfan at the three Spike concentrations

Spike Concentration	Replicate (1)	Replicate (2)	Replicate (3)	Average
1 ppm	85.44%	85.34%	84.60%	85.12%
2 ppm	93.15%	93.24%	94.00%	93.46%
3 ppm	95.77%	96.86%	96.30%	96.31%

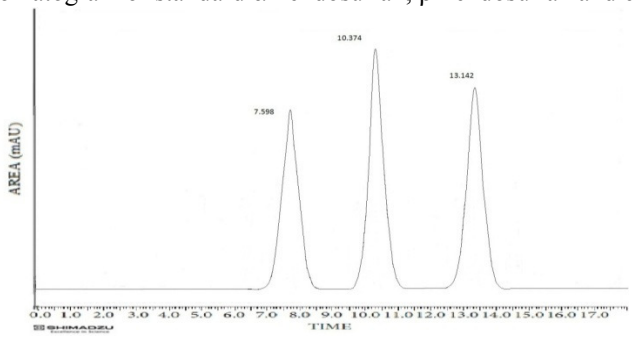
Source: Results of the experiment conducted by the researcher.

Table (18): Recovery percentages for the obtained recovered concentrations of endosulfan sulphate at the three Spike concentrations

Spike Concentration	Replicate (1)	Replicate (2)	Replicate (3)	Average
1 ppm	83.52%	84.08%	82.90%	83.50%
2 ppm	91.19%	92.03%	90.79%	90.83%
3 ppm	94.36%	94.85%	93.97%	94.39%

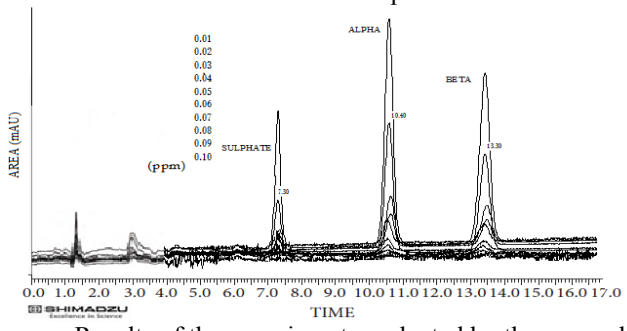
Source: Results of the experiment conducted by the researcher.

Figure (7): HPLC Chromatogram of standard α - endosulfan, β - endosulfan and endosulfan sulphate



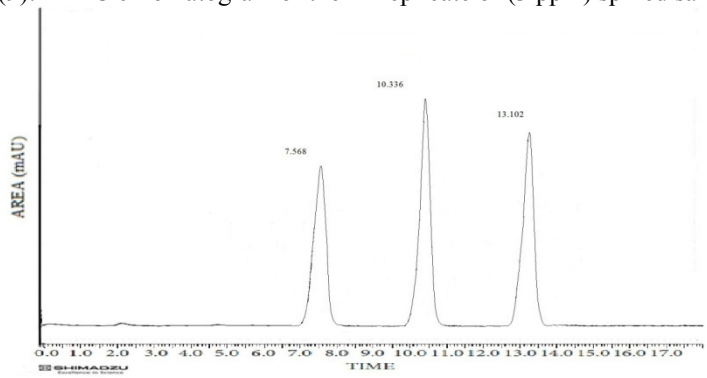
Source: Results of the experiment conducted by the researcher.

Figure (8): HPLC chromatograms (superimposed) of different series of standard α - endosulfan, β - endosulfan and endosulfan sulphate



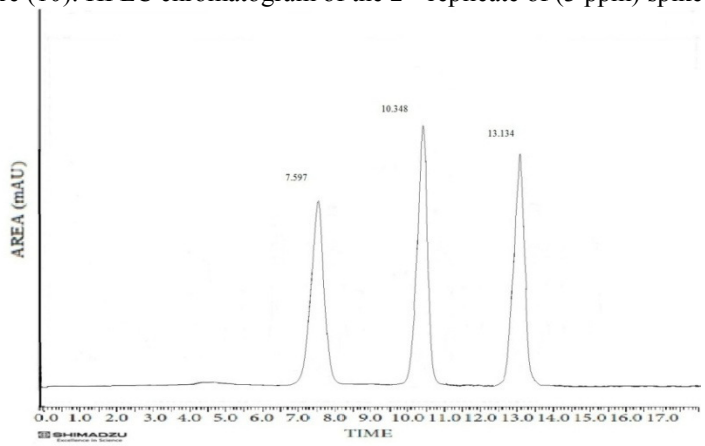
Source: Results of the experiment conducted by the researcher.

Figure (9): HPLC chromatogram of the 1st replicate of (3 ppm) spiked sample



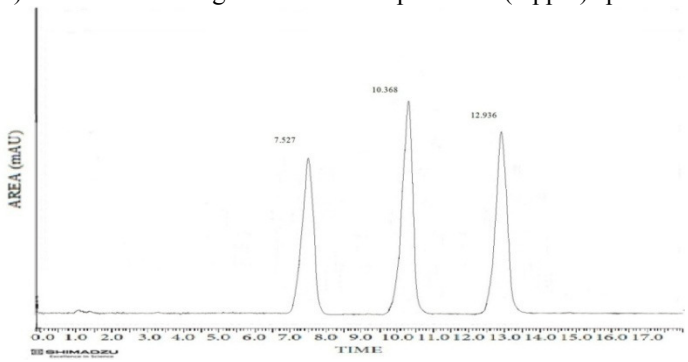
Source: Results of the experiment conducted by the researcher.

Figure (10): HPLC chromatogram of the 2nd replicate of (3 ppm) spiked



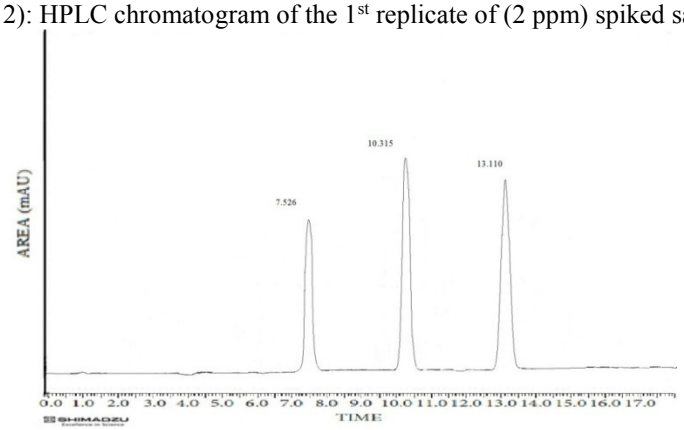
Source: Results of the experiment conducted by the researcher.

Figure (11): HPLC chromatogram of the 3rd replicate of (3 ppm) spiked sample



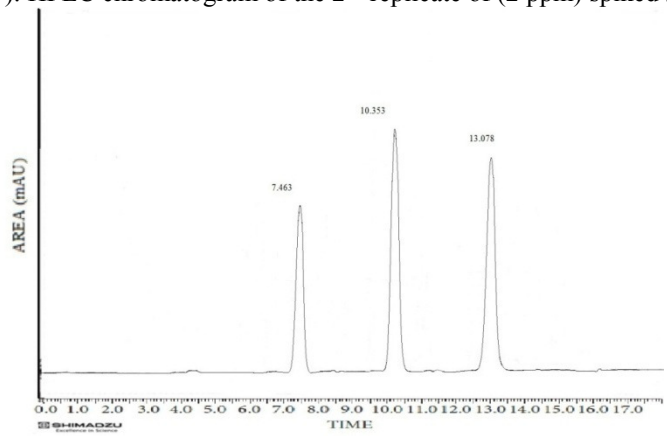
Source: Results of the experiment conducted by the researcher.

Figure (12): HPLC chromatogram of the 1st replicate of (2 ppm) spiked sample



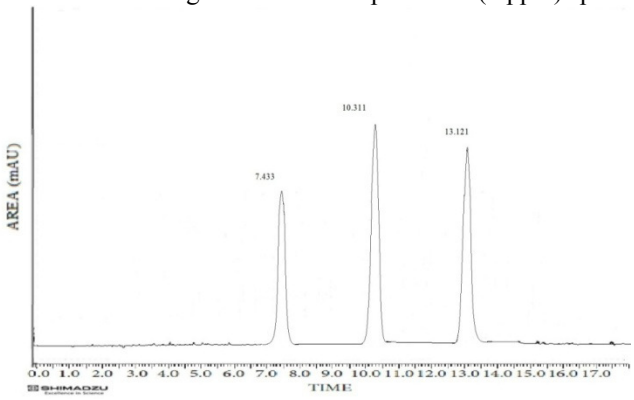
Source: Results of the experiment conducted by the researcher.

Figure (13): HPLC chromatogram of the 2nd replicate of (2 ppm) spiked sample



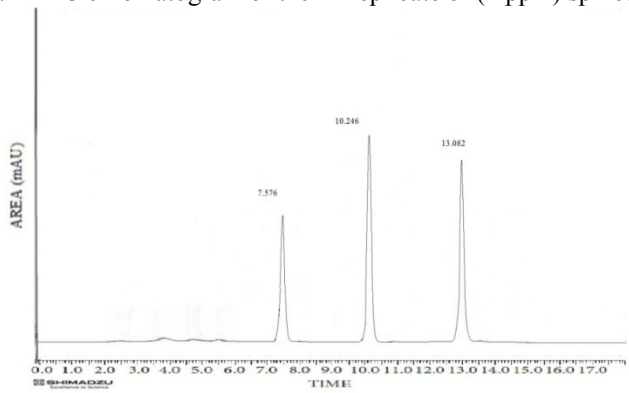
Source: Results of the experiment conducted by the researcher.

Figure (14): HPLC chromatogram of the 3rd replicate of (2 ppm) spiked sample



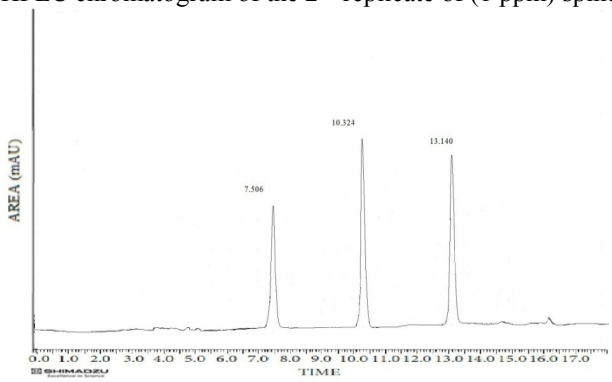
Source: Results of the experiment conducted by the researcher.

Figure (15): HPLC chromatogram of the 1st replicate of (1 ppm) spiked sample



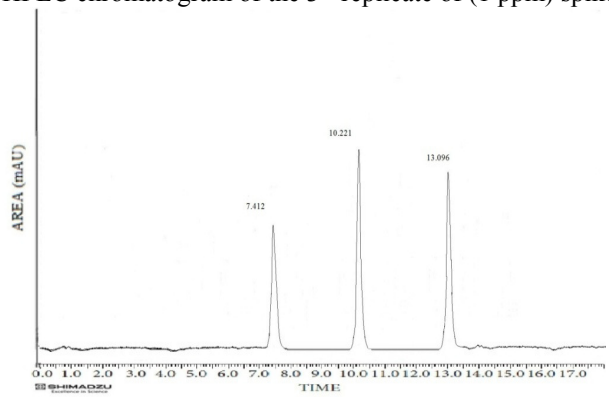
Source: Results of the experiment conducted by the researcher.

Figure (16): HPLC chromatogram of the 2nd replicate of (1 ppm) spiked sample



Source: Results of the experiment conducted by the researcher.

Figure (17): HPLC chromatogram of the 3rd replicate of (1 ppm) spiked sample



Source: Results of the experiment conducted by the researcher.

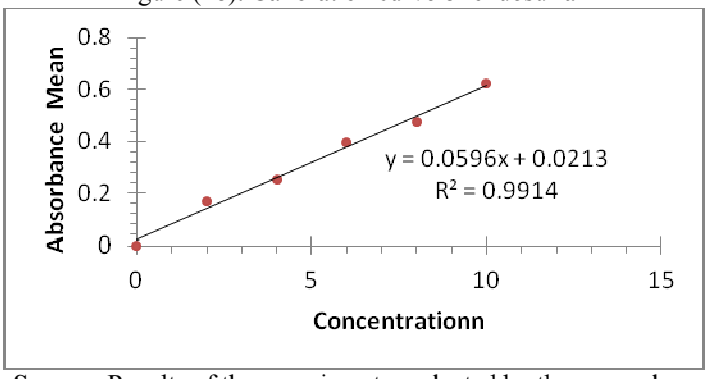
Spectrophotometry:

Table (19): Obtained absorbencies of endosulfan at each concentration

Absorbance	Concentration (ppm)
0	0
0.169	2
0.254	4
0.396	6
0.475	8
0.623	10

Source: Results of the experiment conducted by the researcher.

Figure (18): Calibration curve of endosulfan



Source: Results of the experiment conducted by the researcher.

Table (20): The obtained absorbencies and mean of endosulfan at the three spike concentration

Spike Concentration(ppm)	Replicate 1	Replicate 2	Replicate 3	Mean
3	0.188	0.185	0.179	0.184
5	0.310	0.302	0.306	0.306
7	0.423	0.430	0.418	0.423667

Source: Results of the experiment conducted by the researcher.

- The resulted absorbencies were used to calculate recovered concentration by using regression equation of the calibration curve.

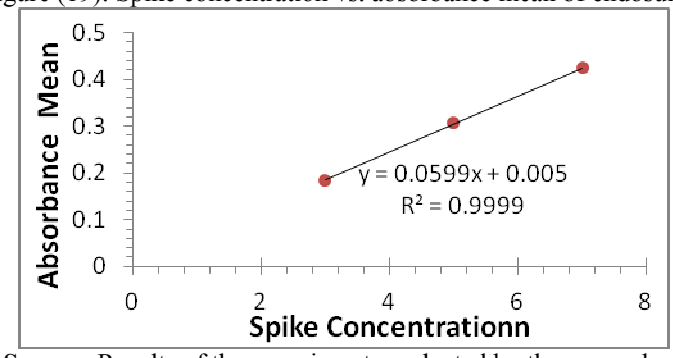
Table (21): The recovered concentrations mean and standard deviation of endosulfan replicates at the three spike concentration

Spike Concentration(ppm)	Replicate 1	Replicate 2	Replicate 3	Mean	Standard Deviation	CV
3	2.83	2.779	2.677	2.762	0.077904	0.02820
5	4.898	4.762	4.830	4.83	0.068	0.01407
7	6.813	6.932	6.728	6.824333	0.102471	0.01501

Source: Results of the experiment conducted by the researcher.

- The data on table (21) were used to construct (spike concentration vs. absorbance mean) curve in order to calculate LOD, LOQ and r values.

Figure (19): Spike concentration vs. absorbance mean of endosulfan



Source: Results of the experiment conducted by the researcher.

Table (22): r, limit of detection and limit of quantification values

LOD	0.089953
LOQ	0.299843
r	0.99994

Source: Results of the experiment conducted by the researcher.

Table (23): Recovery percentages for the obtained recovered concentrations of endosulfan at the three spike concentrations

Spike Concentration(ppm)	Replicate 1	Replicate 2	Replicate 3	Average
3	94.33%	92.63%	89.23%	92.06%
5	97.96%	95.24%	96.60%	96.6%
7	97.32%	99.02%	96.11%	97.48%

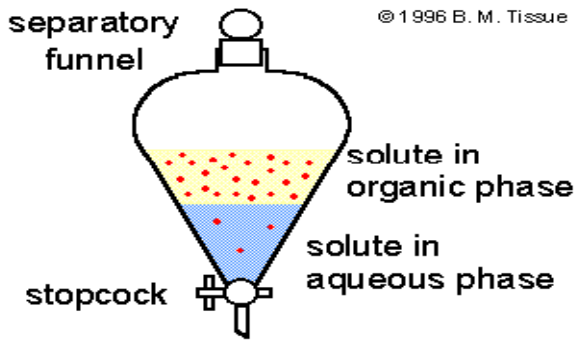
Source: Results of the experiment conducted by the researcher.

Discussion:

Chromatography:

Principle: Liquid–liquid extraction (LLE), also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid into another liquid phase. It consists of transferring one (or more) solute contained in a feed solution to another immiscible liquid (solvent). The solvent that is enriched in solute is called extract. The feed solution that is depleted in solute is called the raffinate.

Figure (20): Liquid - Liquid Extraction Concept



Source: Results of the experiment conducted by the researcher.

Drying Agent Addition: After an organic solvent has been in contact with an aqueous solution, it will be "wet", i.e. it will contain some dissolved water even though the organic solvent will typically have a very low miscibility with water. The amount of water dissolved varies from solvent to solvent. In order to remove the dissolved water a drying agent is used (and here we used sodium chloride and sodium sulphate.)

Linearity: A linear regression analysis was carried out by plotting the chromatographic response (Peak Area) for each pesticide (y-axis) versus the final concentrations of pesticides (x-axis). For the evaluation of the standards curves a stock solution of each pesticide was prepared and a 10 several dilutions of each stock was prepared for each pesticide (0.01, 0.02, 0,03, 0,04, 0,05, 0,06, 0,07, 0,08, 0,09, 0,1 ppm), then those series were injected in the HPLC, and the chromatographic data were used to assess linearity. Regression analysis yielded an excellent coefficients of correlations (r) for each pesticide (figures 1, 2 and 3), (table 3), and for the quantification of the pesticides, tomato samples were spiked with 3 concentrations (1, 2 and 3 ppm) for each pesticide and were analyzed in triplicate, and the regression analysis showed good correlation coefficients (r) (figures 4, 5 and 6), (tables 4, 6 and 8). According to *(Eurachem 1998)* [6], the analytical response was linear over certain concentration ranges if r obtained is higher than 0.995. Therefore, it can be stated that the used method was linear for analysis of selected pesticides at specified linear ranges.

Precision: Precision of analytical method is generally evaluated by calculating coefficient of variation (CV) of a set of data. Precision of HPLC method was checked to assess the reproducibility of instrument response to target of analyte. In order to assess the analytical method precision, measurements were done under conditions of repeatability. Repeatability was evaluated by measuring 3 tomato samples after they spiked with three different endosulfan concentrations (1, 2 and 3 ppm) under similar conditions (day, analyst, instrument and sample).The CV values obtained in (tables 10, 12 and 14). According to Horwitz, as cited from *(Gonzalez and Herrador 2007)* [7], the maximum CV value acceptable for the analyte level of 1 ppm is 16 % Therefore, it can be stated that the developed method exhibited a good precision. The coefficient of variation was calculated as (CV = σ/M, where: σ is the standard deviation and M is mean).

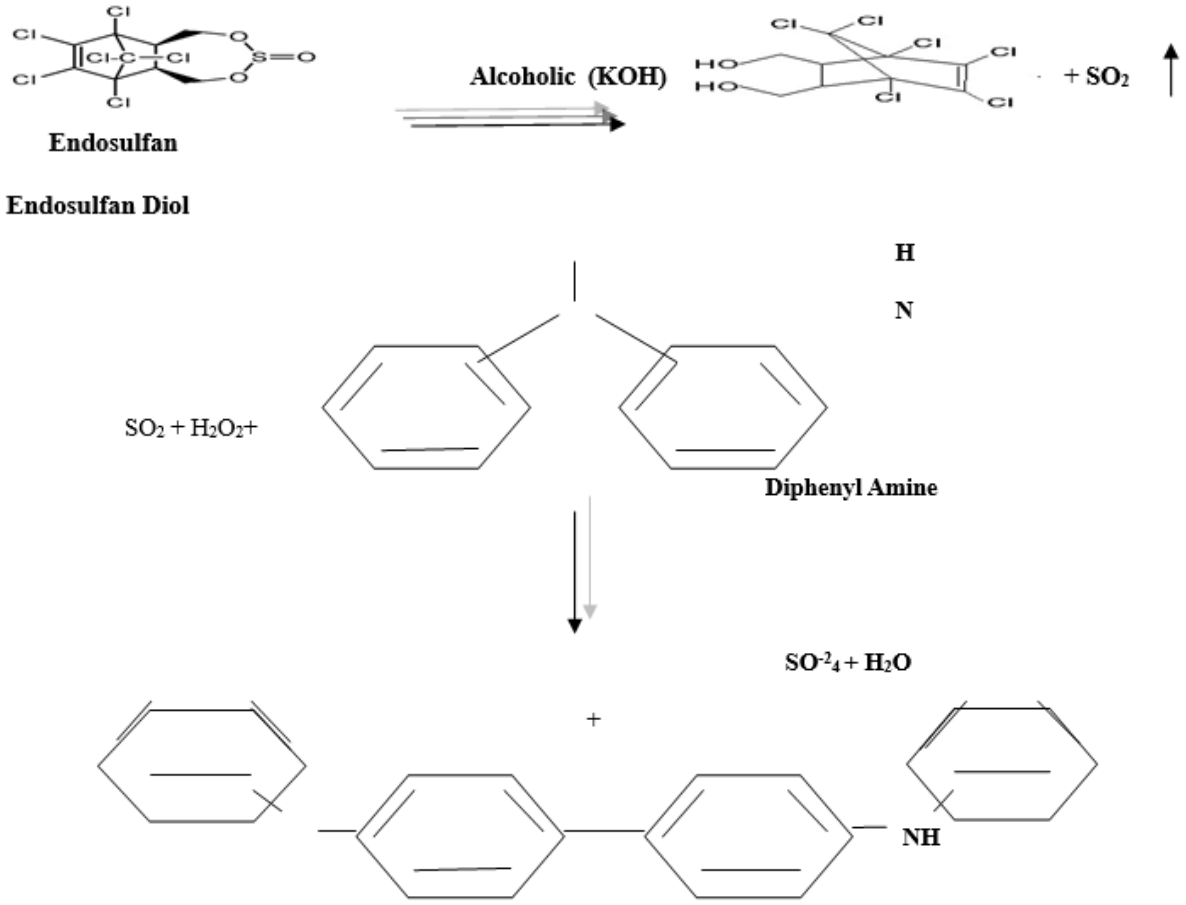
Recovery: In this study, the accuracy of analytical method was assessed using standard addition method by calculating the recovery values for each pesticide. These studies were carried out to confirm the lack of analyte losses during sample preparation and matrix interferences during the measurement step required *(Eurachem, 1998).* That accuracy studies was performed using three different levels of spiked standards here were- namely- (1, 2 and 3 ppm). All analytical steps were performed in three replicates. The recovery percentage values for accuracy studies were shown in (tables 16, 17 and 18). According to *(Codex Alimientarius commission 2003)* [15],

for the level of analyte of > 1 ppm to ≤ 0.01 ppm, the recovery values should be in the range of 60 – 120 %. Therefore, the developed method was accurate for quantification of these pesticides in tomato. The recovery was calculated as (R= final concentration/initial concentration*100).

Sensitivity: Sensitivity of HPLC with UV detector was evaluated by calculating the values of limit of detection (LOD) and limit of quantification (LOQ). In order to calculate the values of LOD and LOQ, they were calculated as (3*STE/b and 10*STE/b) respectively, where STE is the standard error of HPLC chromatogram area and b is the slope of calibration curve. The values of LOD and the LOQ values were obtained in (tables 11, 13 and 15).

Spectrophotometry:

Color reaction:

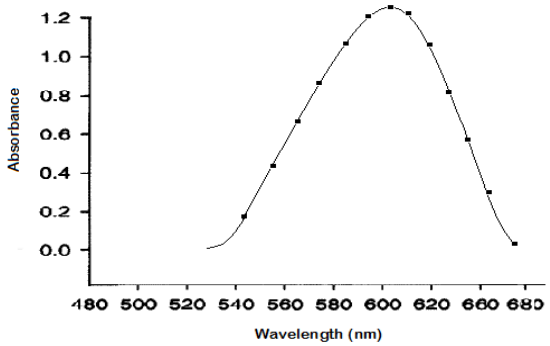


Diphenyl Benzidine (Light violet at 605 nm)

Principle: This reaction is based on the liberation of sulphur dioxide after addition of alcoholic potassium hydroxide, and then the liberated sulpher dioxide is passed through hydrogen peroxide and diphenyl amine, finally the light violet color of diphenyl benzidine is measured at 605 nm.

Spectral Characteristics: The absorption spectra of the reaction product of endosulfan with diphenyl amine shown in figure 21 with maximum absorption at 605 nm and the color was light violet, the reagent blank had negligible absorbance at this wavelength. A temperature of 25 -30 ° C was selected for the reaction. Beer's law was obeyed (figure 19) in the concentration range of (2-10 ppm) of endosulfan.

Figure (21): Absorption maximum of endosulfan



Source: Results of the experiment conducted by the researcher.

Color Producing Reagent: The rate of formation of complex in solution is generally rapid; changes in spectrum or color are associated with a transfer/interchange of electrons when such molecules undergo properly oriented collisions. The absorbance of the developed color was stable for more than 9 hours at 25 -30 ° C.

Application: The results for the determination of endosulfan are shown in tables (Tables 20, 21, 22 and 23) which reveal the sensitivity, validity and repeatability of the method. The method is also reasonably precise and accurate. Both of limit of detection (LOD) and limit of quantification (LOQ) was calculated to evaluate sensitivity of the spectrophotometer by applying the following formulas (3*STE/b and 10*STE/b) for (LOD) and (LOQ) respectively, where STE is the standard error area and b is the slope of calibration curve.

The accuracy of the experiment was achieved by calculating recovery; the recovery was calculated using three concentrations of fortification (3, 5 and 7 ppm) at the same conditions. Therefore, this method indicates its quality for quantification of this pesticide in vegetables. The recovery was calculated as (R= final concentration/initial concentration*100).

Coefficient of variation (CV) was calculated in order to assess the Precision of the analytical method of interest the calculated (CV) values show good precision of the method was used. The coefficient of variation was calculated as (CV = σ/M, where: σ is the standard deviation and M is mean).

The spectrophotometer exhibited linear behaviour, after performing regression analysis; a good (r) value was yielded. To quantify the pesticide, samples were fortified three different concentrations (3, 5 and 7 ppm), and was analyzed in triplicate and the regression analysis showed good correlation coefficient.

The method has been applied for the determination of endosulfan in various water and vegetable samples, the suggested procedure shows advantages, some of these advantages are easiness, rapidity, sensitivity and selectivity, stability of the light violet colored dye and it does not need an excessive heating, the reliability of the method to analyze real samples are checked by recovery experiments.

Conclusion

The spectrophotometric method for the determination of endosulfan is simple, reliable, rapid, sensitive and less time consuming. The color reaction is selective for endosulfan. The advantage of the present procedure is that it does not require many solvents, whereas the HPLC procedures are long, tedious, and very expensive, involving many reagents and solvent, and does not involve any stringent reaction conditions and offers the advantages of the stability of the reaction system. Contamination in the vegetables with the pesticide residues poses a significant health risk to the public from consuming contaminated fruits hence it is important to develop effective method for the detection of these residues. This HPLC method has been applied successfully within fortified samples. The method has several advantages over most traditional methods of analysis in the following ways:

(i) A good separation and high sensitivity was achieved by HPLC method for all pesticides, less time consuming and low detection limit.
(ii) The classical procedure that involves extraction with water and acetonitrile, cleanup Activated florisil, showed an efficient removal of interferences, providing a simple, rapid and reliable analysis of pesticides in all matrices.
(iii) For most of the pesticides assayed the performance characteristics obtained within validation study were acceptable, within the quality control requirements.
(iv) High recoveries are achieved of pesticides and the suggested method offers excellent linearity and sensitivity for the determination of target pesticides at trace levels.
(v) Solvent usage and waste is considerable. This method was useful for detection of pesticide residue present in tomato. It is most effective and widely acceptable in terms of accuracy and reliability.

Recommendations

The obtained results clearly indicate the actual situation of the misuse of pesticides which may affect in turn at long period the consumers health which make this a recommendable choice for the routine determination of pesticides in biological matrices. According to maximum residue levels (MRLs) data base the MRL of endosulfan in tomato is (0.5 ppm) [17] which is less than all recovered concentrations obtained here, and since pesticides -generally- cause harmful impacts to the human beings, not to mention endosulfan, and basing on this research, we highly recommend to prohibit and terminate the application, manufacturing, trading of this pesticide and apply the maximum penalties of those who deal with this toxin.

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