EGE UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE

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CHEMOMETRICAL APPROACH TO DETERMINATION OF PESTICIDE RESIDUES FROM PLANT SAMPLES BY ACCELERATED SOLVENT EXTRACTION (ASE)

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ÖZET

KİMİ BİTKİ ÖRNEKLERİNDE BULUNAN PESTİSİTLERİN ASE YÖNTEMİYLE ÖZÜTLENMESİNE KEMOMETRİK YAKLAŞIM

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Tütün ve ürünlerinin insan sağlığına zararları bilinmesine karşın, bu alanda piyasa arzı devam etmekte ve önemli bir tütün üreticisi olan ülkemiz için tütün analizleri önemini korumaktadır. Tütün üretimi sırasında veya çevresel faktörlerle tütünde kalıntı olarak bulunan pestisit düzeylerinin belirlenmesi de bu analizlerin başında gelmektedir.

Bu tez çalışmasında öncelikle tayini amaçlanan 10 ayrı pestisit için alıkonma zamanları belirlenmiş ve 50-1000 ng/mL derişim aralıklarında kalibrasyon grafikleri oluşturulmuştur. Daha sonra tütün gibi zor bir matrikste pestisit analizlerinin sağlıklı yürütülebilmesi için yöntem geliştirme amacıyla kromatografik analiz öncesi hızlandırılmış çözücüye özütleme (ASE) tekniğinin kullanımı esas alınmıştır. Bu teknikte özütleme verimine etkiyen parametreler seçilmiş ve Plackett-Burman tasarımı ile öne çıkan parametrelerin belirlenmesi yoluna gidilmiştir. Ardından bu örneklerin pestisit içerikleri GC-ECD sistemi ile analizlenmiştir. Alınan verilerin işlenmesi sonucunda analiz için optimum koşullar belirlenmiştir.

Anahtar Kelimeler: Pestisit, hızlandırılmış solvent ekstraksiyon, gaz kromatografi, tütün

ABSTRACT

CHEMOMETRICAL APPROACH TO DETERMINATION OF PESTICIDE RESIDUES FROM PLANT SAMPLES BY ACCELERATED SOLVENT EXTRACTION (ASE)

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Turkey is one of the major tobacco producers in the world. Even though the tobacco is known to harm human health, the demand in the world market necessitates several analyses in tobacco samples. One of the most important analysis types is the determination of pesticide by means of their production step and environmental factors.

In this thesis study the retention times of ten pesticides were determined and calibration graps were constructed between 50 - 1000 ng/mL. In order to work more reliable the accelerated solvent extraction method was choosen prior to chromatographic analysis for tobacco samples which is very difficult to work. In this technique the parameters which affect the extraction efficiency were choosen and followed by selection of main parameters were done with the help of the Plackett-Burmann design. Then the pesticide contents of samples were determined by GC-ECD system. By means of data optimum conditions were determined for an analysis.

Key Words: Pesticide, accelerated solvent extraction, gas chromatography, tobacco

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ABBREVIATIONS

ASE	: accelerated solvent extraction
b_0	: an intercept or average
b_1x_1 , b_2x_2 , b_3x_3	: linear terms depending on each of the three factors
$b_{11}x_{11}$, $b_{22}x_{22}$, $b_{33}x_{33}$	: quadratic terms depending on each of the three factors
$b_{12}x_1x_2$, $b_{13}x_1x_3$, $b_{23}x_2x_3$	: interaction terms between the factors
C18	: octadecylsilane
CORESTA	: Agro-Chemical Advisory Committee
D	: square matrix,
ECD	: Electron Capture Detector
EPA	: Environmental Protection Agency
FID	: Flame Ionization Detector
FPD	: Flame photometric detector
FSOT	: Fused Silica Open Tubular
GAP	: Good Agricultural Practice
GC	: Gas Chromatography
GCB	: Graphitized carbon black
GLC	:Gas-liquid chromatography
GRL	:Guidance Residue Levels
GPC	: Gel permeation chromatography
GSC	:Gas-solid chromatography
GC-MS	: Gas chromatography-Mass Spectrometry

LLE : Liquid-liquid extraction

LOD : Limit of detection

LOQ : Limit of quantification

MAE : Microwave-assisted extraction

MLR : Multiple linear regression

N : Number of experiments

P : Number of parameters

NPD : Nitrogen Phosphorous Detector

OCPs : Organochlorine pesticides

PAH : Polycyclic aromatic hydrocarbons

PFE : Pressurized fluid extraction

PLE :Pressurized liquid extraction

PSA : Primary secondary amine

PTV : Programmed Temperature Vaporising

QUECHERS : Quick, easy, cheap, effective, rugged and safe

RSD : Relative standard deviation

SCOT : Support-coated open tubular

S_{resid} : Standard deviation of residuals

SFE : Supercritical fluid extraction

SPE : Solid-phase extraction

t : t value for t test

TCD: Thermal Conductivity Detector

TFE	: Tetrafluoroethylene
v	: Variance of coefficient
WCOT	:Wall-coated open tubular
$\hat{y}$	: Estimated response
y_i	: Response
$\hat{y}_i$	: Average response

1. INTRODUCTION

1.1 Tobacco and Its Importance

The influence of tobacco on social and economic issues dates back several centuries. In 1492, Christopher Columbus was offered dried tobacco leaves as a gift from the American Indians that he encountered. Soon after, sailors brought tobacco back to Europe, and the plant was being grown all over Europe. The major reason for tobacco's growing popularity in Europe was its supposed healing properties. Europeans believed that tobacco could cure almost anything, from bad breath to cancer. (Randall,1999)

Tobacco cultivation is similar to the other agricultural products. Seeds are sown in cold frames or hotbeds to prevent attacks from insects, and then transplanted into the fields, and it is usually harvested in a large single-piece farm equipment. After harvesting, tobacco is stored to allow curing, which allows the slow oxidation and degradation of carotenoids. This also allows for the agricultural product to take on properties that are usually related to the ‘‘smoothness’’ of the smoke. Finally tobacco is packed and delivered to the markets. China is the world's leading tobacco grower, India is a distant second, followed by United States, Brazil, Turkey, Indonesia. (Liemt, 2002)

The importance of tobacco also comes from its hazardous effects to human body. The lung cancer was confirmed to be caused by cigarette smoking over 50 years ago. The ratio of lung cancer deaths in the United States (US) and around the world which were caused by cigarette smoking was stated as 87% and 90% respectively (The American Cancer Society, 2005; WHO, 2000).

According to the WHO and American Cancer Society database smoking causes 5 million deaths per year, and if present trends continue, 10 million smokers per year are projected to die by 2025.

Although there is a decrement of tobacco consumption in developed countries, a recent study carried out in our country has revealed that tobacco consumption was tripled according to selling rates of tobacco in markets. This shows that how the development is so important for consumption of tobacco.

Therefore, there must be regulatory procedure and control mechanisms that were accepted by the international areas and universities. Taking high quality desired yield depends on the appropriate measures to protect the growing plant in tobacco cultivation. The undesired residues may be remained on commercial tobacco and in finished tobacco products.

In order to regulate the usages of pesticides on tobacco growth CORESTA (Agro-Chemical Advisory Committee) developed the Guidance Residue Levels (GRL) and also provides technical support to the tobacco growers and tobacco industry workers interested in agrochemical application and the implementation of Good Agricultural Practice (GAP) in tobacco production. GRLs do not replace requirements to comply with regulations, neither on the use of agrochemicals, nor with regard to residue levels that may be detected. GRLs are designed to emphasize the importance of GAP for growing quality tobacco (CORESTA GUIDE 2008).

1.2 Pesticides

A pesticide is a substance or a mixture intended for preventing, destroying, repelling, or mitigating any pest. It is not refer only to insecticides, the term pesticide also applies to herbicides, fungicides, and various other substances used to control pests (http://www.epa.gov).

Pests are living organisms. They occur in undesirable places or that cause damage to crops or humans or other animals. There are many examples such as insects, mice and other animals, unwanted plants (weeds), fungi, microorganisms like bacteria and viruses. Pesticides can enter your drinking water through several avenues. Some pesticides do not break down easily in water and can remain in the groundwater for a long period of time. The insecticide DDT, though banned for nearly 20 years, can still be found in trace levels in some groundwater

Because of their nature, some risk of harm is occurred depending upon to most of pesticides. Pesticides are designed to kill or otherwise adversely affect living organisms so they can cause harm to humans, animals, or the environment. Pesticides are also useful to society. They can kill organisms that may cause

disease and control insects, weeds, and other pests. Pesticides can be classified on the basis of type of pest control and chemical composition.

On the basis of pest control they can be classified as **insecticides** (against insect pest), **herbicides** (for killing and controlling weeds), **and fungicides** (against fungal diseases). Insecticides can be classified as (this classification also shows that classification on the basis of chemical composition of pesticides) (Waxman, 1998).

Organophosphate pesticides**:** Most organophosphates are insecticides. They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. Some are very poisonous and were used in World War II as nerve agents.

Organophosphorus insecticides are normally esters, amides, or thiol derivatives of phosphoric, phosphonic, phosphorothioic or phosphonothioic acids. Most of them are stable in the pH range that may be encountered in the environment (pH 3-6) than at neutral pH. These pesticides affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter.

Carbamate pesticides: These pesticides affect the nervous system by disrupting an enzyme that regulates acetylcholine, a neurotransmitter. The enzyme effects are usually reversible.

Organochlorine insecticides; they contain carbon, chlorine and hydrogen. Their Cl-C bonds are very strong which means that they do not break down easily. They are highly insoluble in water, but are lipophilic. Organochlorine insecticides can be also classified as diphenylaliphatics, hexachlorocyclohexane (HCH), cyclodiens, polychloroterpens.

Diphenylaliphatics cause damaging of nerve sense transmission system by damaging of equilibrium of Na-K ion and also HCH has similar effect as diphenylaliphatics.

The mechanism of effect that belongs to cyclodiens has not been known exactly yet, but it is known that, they affect as an inhibitor to GABA receptors (γ-aminobutyric acid). Polychloroterpens have lower environment stableness according to others but have similarity with cyclodiens.

Pyrethroid pesticides; were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. In order to be able to increase their stability in the environment, they have been arranged. Some synthetic pyrethroids are toxic to the nervous system.

Biopesticides; they are derived from natural materials like animals, plants, bacteria and certain minerals. They can be classified as microbial pesticides, plant-incorporated-protectants (PIPS) and bio chemical pesticides. Biopesticides are usually inherently less toxic than conventional pesticides and also generally affect only the target pest. They are effective in very small quantities and can decompose quickly.

Herbicides are pesticides used to kill or inhibit the growth of forbs, grasses, woody plants and their seeds. Mechanical methods lost their popularity during forty years by the herbicide usage. Herbicides can be classified by two ways, according to their effective mechanism and chemical structures. Selective and non-selective types are in the group that is constructed as their effective mechanisms and benzonitriles, phenoxy derivatives, triazine, amide, dinitroaniline and thiocarbamates belong to group made by chemical structures.

Fungicides are extensively used in industry, agriculture, and the home and garden for a number of purposes, including: protection of mature crops, berries, seedlings, flowers, and grasses in the field, in storage, and during shipment; suppression of mildews that attack painted surfaces; control of slime in paper pulps; and protection of carpet and fabrics in the home.

1.3 Pesticides in Tobacco

Because of many applications such as insect-killers, weed-killers, plant growth regulators as well as other types of pesticides, tobacco is also a chemical-intensive crop. Pesticides are used both on tobacco and regularly on food crops.

As with food crops, trace amounts of pesticides remain on tobacco leaves after treatment; typically, residue levels decline during the drying and manufacturing process, although additional pesticides may be applied to the finished product.

Pesticides increase production of tobacco and food crops, however they exposure may harm humans; thus, regulatory agencies such as the U.S. EPA set limits on the amount of pesticide residue permitted in or on food and tobacco and establish standards for workers handling pesticides. Active and passive smokers are exposed to pyrolyzed pesticide residues due to burning of tobacco and the smoke inhaled. The U.S. EPA has concluded that this exposure poses no short-term risk, but little is known about the long-term health effects. Because of the importance of regulatory in tobacco growth the guidance residue levels (GRL) have been developed by CORESTA Agro-Chemical Advisory Committee and the list was given in Table 1.1.

Table 1.1 The guidance residue levels (GRLS) of pesticides used in tobacco

No	Agrochemical	GRL(ppm)	Residue definition
1	2,4,5-T	0,05	2,4,5-T
2	2,4-D	0,20	2,4-D
3	Acephate	0,20	Acephate
4	Acetamiprid	2,50	Acetamiprid
5	Acibenzolar-S-methyl	5,00	Acibenzolar-S-methyl
6	Alachlor	0,10	Alachlor
7	Aldicarb(Σ)	0,50	sum of aldicarb,aldicarb sulfoxide and aldicarb sulfone,expressed as aldicarb
8	Aldrin+Dieldrin	0,05	Aldrin+Dieldrin
9	Azinphos-ethyl	0,30	Azinphos-ethyl
10	Azinphos-methyl	0,30	Azinphos-methyl
11	Benalaxyl	2,00	Benalaxyl
12	Benfluarin	0,06	Benfluarin
13	Benomyl		sum of benomyl,carbendazim, and thiophanate-methyl expressed as carbendazim
14	Bifenthrin	2,50	Bifenthrin
15	Bromophos	0,20	Bromophos
16	Butralin	5,00	Butralin
17	Camphechlor (Σ) (Toxaphene)	0,10	Camphechlor (mixture of chlorinated camphenes)
18	Captan	0,70	Captan
19	Carbaryl	0,50	Carbaryl
20	Carbendazim	2,00	sum of benomyl,carbendazim, and thiophanate-methyl expressed as carbendazim
21	Carbofuran (Σ)	0,50	sum of carbofuran and 3-hydroxycarbofuran expressed as carbofuran
22	Chinomethionate	0,20	Chinomethionate
23	Chlordane (Σ)	0,10	sum of cis-chlordane and trans-chlordane
24	Chlorfenvinphos (Σ)	0,05	sum of (E)-chlorfenvinphos and (Z)-chlorfenvinphos
25	Chlorothalonil	2,00	Chlorothalonil
26	Chlorpyrifos	0,50	Chlorpyrifos
27	Chlorpyrifos-methyl	0,20	Chlorpyrifos-methyl
28	Chlorthal-dimethyl	0,50	Chlorthal-dimethyl
29	Clomazone	0,20	Clomazone
30	Cyfluthrin(Σ)	0,50	Cyfluthrin(sum of all isomers)

Table 1.1 (continue)

No	Agrochemical	GRL(ppm)	Residue definition
31	Cyhalothrin(Σ)	0,40	Cyhalothrin(sum of all isomers)
32	Cymoxanil	0,10	Cymoxanil
33	Cypermethrin(Σ)	1,00	Cypermethrin (sum of all isomers)
34	DBCP	0,05	DBCP(1,2-dibromo-3-chloropropane)
35	DDT(Σ)	0,20	sum of o,p'- and p,p'-DDT,o,p'- and p,p'-DDD(TDE), o,p'-and p,p'-DDE expressed as DDT
36	Deltamethrin	1,00	sum of deltamethrin and tralomethrin expressed as deltamethrin
37	Demeton-S-methyl(Σ)	0,10	sum of demeton-S-methyl,oxydemeton-methyl, (demeton-S-methyl sulfoxide) and demeton-S-methyl sulfone expressed as demeton-S-methyl
38	Diazinon	0,10	Diazinon
39	Dicamba	0,20	Dicamba
40	Dichlorvos (c)	0,10	sum of dichlorvos,naled and trichlorfon expressed as dichlorvos
41	Dicloran	1,00	Dicloran
42	Diflubenzuron	0,10	Diflubenzuron
43	Dimefox	0,01	Dimefox
44	Dimethoate(d)	0,50	sum of dimethoate and omethoate expressed as dimethoate
45	Dimethomorph(Σ)	2,00	sum of (E)-dimethomorph and (Z)-dimethomorph
46	Dinocap(Σ)	0,10	sum of dinocap isomers and dinocap phenols expressed as dinocap
47	Diphenamid	0,25	Diphenamid
48	Disulfoton(Σ)	0,10	sum of disulfoton,disulfoton sulfoxide, and disulfoton sulfone expressed as disulfoton
49	Dithiocarbamates (as CS_2) (e)	5,00	Dithiocarbamates expressed as CS_2
50	Endosulfans(Σ)	1,00	sum of alpha- and beta-isomers and endosulfan-sulphate expressed as endosulfan
51	Endrin	0,05	Endrin
52	Ethoprophos	0,10	Ethoprophos
53	Ethylene dibromide	0,05	Ethylene dibromide
54	Famoxadone	5,00	Famoxadone

Table 1.1 (continue)

No	Agrochemical	GRL(ppm)	Residue definition
55	Fenamiphos(Σ)	0,50	sum of fenamiphos,fenamiphos sulfoxide and fenamiphos sulfone expressed as fenamiphos
56	Fenchlorphos	0,10	Fenchlorphos
57	Fenitrothion	0,10	Fenitrothion
58	Fensulfothion	0,05	Fensulfothion
59	Fenthion (Σ)	0,10	sum of fenthion,fenthion sulfoxide and fenthion sulfone expressed as fenthion
60	Fenvalerate (Σ)	1,00	Fenvalerate (sum of all isomers including esfenvalerate)
61	Fluazifop-butyl (Σ)	1,00	Fluazifop-butyl (sum of all isomers)
62	Flucythrinate (Σ)	0,50	Flucythrinate (sum of all isomers)
63	Flumetralin	5,00	Flumetralin
64	Folpet	0,20	Folpet
65	Fonofos (Σ)	0,10	Fonofos (sum of all isomers)
66	Formothion	0,10	Formothion
67	HCH (α-,β-,δ-)	0,07	HCH (α-,β-,δ-)
68	HCH (γ-) Lindane	0,05	HCH (γ-) Lindane
69	Heptachlor (Σ)	0,05	sum of heptachlor and two heptachlor epoxides (cis- and trans-) expressed as heptachlor
70	Hexachlorobenzene	0,03	Hexachlorobenzene
71	Imidacloprid	5,00	Imidacloprid
72	Iprodione(Σ)	0,25	sum of iprodione and N-3,5-dichlorophenyl-3-isopropyl-2,4,dioxoimidazolyzin-1-carboxamide expressed as iprodione
73	Isopropalin	0,10	Isopropalin
74	Malathion	0,50	Malathion
75	Maleic hydrazide	80,00	Maleic hydrazide (free and bounded form)
76	Metalaxyl(Σ)	2,00	sum of all isomers including metalaxyl-M/mefenoxam
77	Methamidophos	1,00	Methamidophos
78	Methidathion	0,10	Methidathion
79	Methiocarb(Σ)	0,20	sum of methiocarb,methiocarb sulfoxide, and methiocarb sulfone expressed as methiocarb
80	Methomyl	1,00	sum of methomyl,methomyl-oxim, and thiodicarb expressed as methomyl
81	Methoprene	1,00	Methoprene
82	Methoxychlor	0,05	Methoxychlor

Table 1.1 (continue)

No	Agrochemical	GRL(ppm)	Residue definition
83	Mevinphos(Σ)	0,10	Mevinphos (sum E and Z isomers)
84	Mirex	0,10	Mirex
85	Monocrotophos	0,30	Monocrotophos
86	Naled		sum of dichlorvos,naled and trichlorfon expressed as dichlorvos
87	Nitrofen	0,02	Nitrofen
88	Omethoate		sum of dimethoate and omethoate expressed as dimethoate
89	Oxadixyl	0,10	Oxadixyl
90	Oxamyl	0,5	Oxamyl
91	Parathion(-ethyl)	0,10	Parathion
92	Parathion-methyl	0,10	Parathion-methyl
93	Pebulate	0,50	Pebulate
94	Penconazole	2,00	Penconazole
95	Pendimethalin	5,00	Pendimethalin
96	Permethrin(Σ)	0,50	Permethrin (sum of all isomers)
97	Phorate	0,10	Phorate
98	Phosalone	0,10	Phosalone
99	Phosphamidon (Σ)	0,10	Phosphamidon (sum of E and Z isomers)
100	Phoxim	0,50	Phoxim
101	Piperonyl butoxide	3,00	Piperonyl butoxide
102	Pirimicarb	0,50	Pirimicarb
103	Pirimiphos-methyl	0,10	Pirimiphos-methyl
104	Profenofos	0,10	Profenofos
105	Propoxur	0,20	Propoxur
106	Pymetrozine	1,00	Pymetrozine
107	Pyrethrins (Σ)	0,50	sum of pyrethrins 1,pyrethrins 2, cinerins 1, cinerins 2, jasmolins 1, jasmolins 2
108	Tefluthrin	0,10	Tefluthrin
109	Terbufos (Σ)	0,05	sum of terbufos,terbufos sulfoxide and terbufos sulfone expressed as terbufos
110	Tetrachlorvinphos	0,10	Tetrachlorvinphos
111	Thiamethoxam	5,00	Thiamethoxam
112	Thiodicarb		sum of methomyl, methomyl-oxim, and thiodicarb expressed as methomyl
113	Thionazin	0,05	Thionazin
114	Thiophanate-methyl		sum of benomyl,carbendazim, and thiophanate-methyl expressed as carbendazim

Table 1.1 (continue)

No	Agrochemical	GRL(ppm)	Residue definition
115	Tralomethrin		sum of deltamethrin and tralomethrin expressed as deltamethrin
116	Trichlorfon		sum of dichlorvos,naled and trichlorfon expressed as dichlorvos
117	Trifluarin	0,10	Trifluarin
118	Vamidothion (Σ)	0,1	sum of vamidothion,vamidothion sulfoxide and vamidothion sulfone expressed as vamidothion

1.4 Methods for Pesticide Residue Analysis

In the study of Schreck et. al, seven pesticides (flazasulfuron, metalaxyl, chlorpyrifos, folpet, myclobutanil, λ-cyhalothrin, flumioxazin) were investigated with the help of PLE (pressurized liquid extraction) and GC-MS (Gas chromatography-Mass Spectrometry) analysis in soil samples. The ASE (accelerated solvent extraction) system and gas chromatograph-ion trap mass spectrophotometer with an RTX-5 column were used for quantitative analysis and the mass spectrometer was operated in electron-impact mode. It was seen that the limit of detection (LOD) is between 1.4 and 4.6 μg/kg for the five analytes in dry soil samples. Also the effects of PLE variables (the extraction solvent, extraction temperature and the number of cycles) were investigated (Schreck et. al, 2008).

The acetone, hexane, dichloromethane, ethyl acetate and acetonitrile were used while the effect of extraction solvent was investigated. They were selected according to their polarity and pesticide solubility efficiency. The acetonitrile and acetone were the most efficient for the analytes. Dichloromethane and ethyl acetate have average recoveries. Hexane is efficient for chlorpyrifos and λ-cyhalothrin.

The effect of temperature was investigated at 80°C, 100°C and 120°C. The 100°C is the best temperature for the optimal pesticide extraction. At the 80°C, the solute-matrix interactions have not been broken. Also the pesticides were damaged at 120°C which causes decrement in efficiency. In order to improve the extraction of two compounds (metalaxyl and flazasulfuron) the third cycle was chosen for this study (Schreck, 2008).

In the study of Jeong-Min Lee et. al. (2008) the sample preparation methods for analysis of analytes in tobacco were compared which were liquid-liquid extraction (LLE), pressurized liquid extraction (PLE) and QUECHERS (quick, easy, cheap, effective, rugged and safe). For the LLE analysis acetonitrile was used, for the PLE acetone and followed by solid-phase extraction (SPE) was used and for QUECHERS method the effects of sorbents were investigated which were primary secondary amine (PSA), octadecylsilane (C18) and graphitized carbon black GCB).

The matrix of analytes in tobacco was also investigated in flue-cured, burley and oriental tobacco samples. The forty-nine pesticides were investigated and it was seen that LOQ values of these samples were similar and below according to Agrochemical Advisory Committee of CORESTA when LLE, PLE and QUECHERS method were used. The recoveries and RSD values were 42.9-123.4% and 1.1-21.1% for LLE, 39.0-136.8% and 1.8-21.1% for PLE, 70.9-120.1% and 0.7-18.9% for QUECHERS respectively.

Mayer-Helm et al. have developed a method for the quantitative determination of alachlor, benelaxyl, clomazone, diflubenzuron, dimethomorph, diphenamid, ethofumesate, metalaxyl, methoprene, metobromuron and piperonyl butoxide on tobacco. The methanol and water were used for the extraction of pesticides. The extracts were purified by partition on an extraction cartridge containing diatomaceous earth. The purified extracts were analyzed by reversed-phase high performance liquid chromatography connected to an atmospheric pressure ionization-electrospray-triple quadrupole mass spectrometer which was operated in positive ion mode. The recovery range for all pesticides was between 35% and 110%. The LOD values were equal or below the guidance residue levels which were proposed by the Agrochemical Advisory Committee of CORESTA (Helm, 2008).

In the study of Wang et. al., the PAHs (polycyclic aromatic hydrocarbons), organochlorine pesticides (OCPs) which are hexachlororocyclohexane isomers (HCHs) and dichloro diphenyltrichloroethane plus its metabolites (DDTs) in soils were compared according to Soxhlet extraction, microwave-assisted extraction (MAE) and accelerated solvent extraction techniques.

The recoveries for OCPs obtained from Soxhlet extraction, MAE and ASE were in the range of 87-105%, 85-104% and 83-105% respectively and for PAHs 69-116%, 61-126% and 70-112% (Wang, 2007).

Another study used accelerated solvent extraction as the sample preparation method for the determination of 33 pesticides in tea samples (Beizhen et. al., 2008). Acetone/dichloromethane (1:1 v/v) was used as the extraction solvent for ASE. After extraction, the extracts were purified by using gel permeation chromatography (GPC) which allows eliminating the co-extracts, such as pigments, lipids and waxes. Followed by, they were purified by using Carb-NH_2 and Florosil SPE cartridges before the GC-MS analysis.

The flame photometric detector (FPD) was used for organophosphorous pesticides and for organochlorine and pyrethroid pesticides the electron capture detector was used. The recoveries were between 70%-120% and the relative standard deviations were less than 20% for the most of samples which were spiked at 0.05 mg/kg level. All these studies were summarized in Table 1.2;

Table 1.2 Summary of the studies

Topic	Aim	Method	Result	Reference
Pesticides in dry soil samples	Investigation of pesticides& ASE parameters	ASE&GC-MS	LOD range is 1.4-4.6 µg/kg	Schreck et.al
Analytes in tobacco sample	Comparison of sample preparation methods; ASE, SPE, QUECHERS	ASE, SPE, QUECHERS	The recoveries and RSD values were 42.9-123.4% and 1.1-21.1% for LLE, 39.0-136.8% and 1.8-21.1% for PLE, 70.9-120.1% and 0.7-18.9% for QUECHERS respectively.	Jeong-Min Lee et. al
Pesticides in tobacco sample	Method development for the determination of pesticides in tobacco	Reversed phase HPLC	The recovery range for all pesticides was between 35% and 110%	Mayer-Helm et al

Table 1.2 (continue)

Topic	Aim	Method	Result	Reference
Determination of PAHs and organochlorine pesticides (OCPs) in soils	Comparison of preparation techniques	Soxhlet extraction, (MAE) and accelerated solvent extraction	The recoveries for OCPs obtained from Soxhlet extraction, MAE and ASE were in the range of 87-105%, 85-104% and 83-105% respectively and for PAHs 69-116%, 61-126% and 70-112%	Wang et. al
Pesticides in tea samples	Determination of pesticides in tea samples	ASE & GC-MS	The recoveries were between 70%-120% and the relative standard deviations were less than 20%	Beizhen et. al

It is very difficult to work with tobacco samples since it has a rather stiff structure. Stiff matrix prevents the solvent penetration to interact deeper sites of the matrix. Conventional solvents can be poor when we work with tobacco samples. In order to overcome this problem, solvents can be used at elevated temperatures and at high pressures in ASE. This property of ASE makes this method become more efficient and useful among other sample preparation methods.

Next section gives fundamental knowledge about the conventional analysis method, gas chromatography and sample preparation techniques applied prior to GC analysis.

1.5 Gas Chromatography

Gas chromatography based on the vaporization of sample and injection onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase.

There are two types of gas chromatographic method that can be applied to the samples; gas-solid chromatography (GSC) and gas-liquid chromatography (GLC).

Gas-solid chromatography is a separation technique in which the mobile phase is a gas and the stationary phase is a suitable adsorbent such as silica gel, alumina or carbon. This technique based on a solid stationary phase in which retention of analytes is the result of physical adsorption. Nonlinear character of the adsorption process causes tailing of elution peaks so that this technique is not widely used except for the separation of low-molecular-weight gaseous species (Skoog et.al., 1997).

Gas-liquid chromatography is based on the partition of an analyte between the gaseous mobile phase and a liquid phase immobilized on the surface of an inert solid such as celite (a diatomaceous earth) or calcined celite (a form of brick dust). The instruments for gas chromatography contain carrier gas source, inlet, column and detector. Nitrogen, argon, helium, and carbon dioxide and hydrogen are the inert gases that can be used as the carrier gas. The column inlet (or injector) supply introducing of the sample into a continuous flow of carrier gas. There are three types of inlet; Split/Splitless injector and PTV (Programmed Temperature Vaporizing). The most common injection method is where a micro syringe is used to inject sample through a rubber septum into a flash vaporizer port at the head of the column.

The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample.

The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vaporizes to form a mixture of carrier gas, vaporized solvent and vaporized solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column (A.Braithwaite &F.J.Smith, 1995).

PTV injector; temperature-programmed sample introduction was developed as a method for the introduction of large sample volumes in capillary GC. The temperature of the liner was chosen slightly below the boiling point of the solvent.

The low-boiling solvent was continuously evaporated and vented through the split line. By introducing the sample at a low initial liner temperature many of the disadvantages of the classic hot injection techniques could be overcome (Poole, 1991).

There are two general types of column, packed and capillary (also known as open tubular). Packed columns are filled with a granular column material which can be the adsorbent itself (GSC), or an inactive granular support impregnated with the liquid stationary phase (GLC).

The support can be porous material like diatomaceous earth or compact material like glass microbeads. The length of packed column is usually 1-3 m, but lengths up to 30m. Standard packed columns are constructed of metal or glass tubing and have diameter 2-9 mm (inside diameter). (Leslie et.al, 1967) Capillary columns have an inner diameter of 0.10-0.53 mm and the length range 10-100m There are three types of capillary columns available; *wall-coated open tubular (WCOT)* or *support-coated open tubular (SCOT)* and porous layer open tubular (PLOT).

WCOT columns consist of a capillary tube whose walls are coated with liquid stationary phase. In *SCOT* columns liquid phase is coated to the solid support which is bonded to the inner walls of the capillary column. *SCOT* columns are generally less efficient than *WCOT* columns. Both types of capillary column are more efficient than packed columns. In PLOT columns solid stationary phase is coated to the inner wall of the column. (Henden et al , 2001).

There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property and a specific detector responds to a single chemical compound. The ideal GC detector should have adequate sensitivity, good stability and reproducibility, a linear response to solutes that extends over several orders of magnitude, a temperature range from room temperature to at least 400°C, a quick response time that is independent of flow rate, high reliability and ease of use, similarity in response toward all solutes or alternatively a highly predictable and selective response

toward one or more classes of solutes, nondestructive of sample (Skoog, Holler, Neiman, 1997).

Flame Ionization Detector (FID) The sample and the carrier gas are burned at the end of the column by mixing the hydrogen and then air. When the most of the organic compounds are burned, the ions, electrons and carbon particles occur and they supply the flame to become conductive.

Surrounding the flame is a cylindrical electrode and a relatively high voltage is applied between the jet and the electrode to collect the ions that are formed in the flame. The resulting current is amplified by a high impedance amplifier and the output fed to data acquisition system or a recorder. The detector usually requires three separate gas supplies together with their precision flow regulators. The gases normally used are hydrogen for combustion, helium or nitrogen for the carrier gas and oxygen or air as the combustion agent. The detector is normally thermostatted in a separate oven; this is not because the response of the FID is particularly temperature sensitive but to ensure that no solutes condense in the connecting tubes (Yıldız, 1993).

Thermal Conductivity Detector (TCD) This detector senses changes in the thermal conductivity of the column effluent and compares it to a reference flow of carrier gas. Since most compounds have a thermal conductivity much less than that of the common carrier gases of helium or hydrogen, when an analyte elutes from the column, the effluent thermal conductivity is reduced and produces a detectable signal.

The ***Nitrogen Phosphorous Detector (NPD)*** is a very sensitive, specific mass flow dependant detector. It is a very sensitive but specific detector that responds almost exclusively to nitrogen and phosphorous compounds. It is based on the flame ionization detector but differs in that it contains a rubidium or cesium silicate (glass) bead situated in a heater coil, a little distance from the hydrogen flame.

Electron Capture Detector (ECD) this detector is commonly used for environmental samples. It is possible to analyze the chlorine pesticides and polychlorobiphenyls with high sensitivity which are very important environmental.

ECD contains a low energy β-ray source which is used to produce electrons for capturing by appropriate atoms. It requires nitrogen or a mixture of argon plus 5% methane as the carrier gas and a radioactive source. Formerly tritium was used as the excitation source, but ^{63}Ni is more common now because it has higher temperature limit of 350°C.

The electrons caused by the ionization produce a large current output from the detector, but the presence of an electron-capturing analyte decreases this current as the electrons are adsorbed. The decrement in this current is proportional to amount of material that is reached to the detector in unit time. These detectors are very selective and sensitive and they do not damage the samples but have narrow dynamic range (Poole,1991) The components of ECD are shown in Figure 1.1.

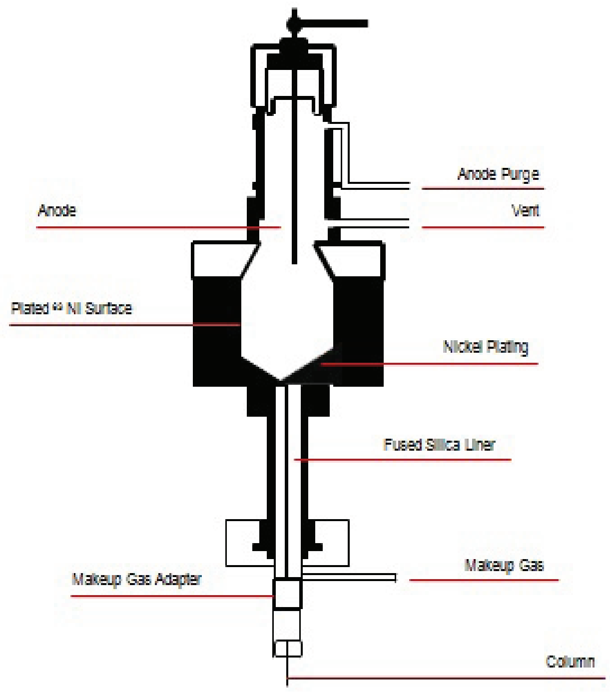


Figure 1.1 The Components of ECD

For a sensitive and accurate analysis, it is important to use reliable preparation steps prior to the analysis. Next section deals with the sample preparation methods.

1.6 Sample Preparation Techniques

There are lots of sample preparation techniques that can be applied prior to GC. ***Soxhlet extraction*** is one of them but not commonly used because of a high solvent consumption for the sample extraction, an evaporation step that is required to concentrate the sample, lack of thermal stability and volatility of some sample analytes, and interference from contaminants in the extraction thimbles.

Microwave-assisted extraction (MAE)*;** sometimes referred to as microwave-assisted solvent extraction (MWE***). The pressure generated is a few hundred psi; however, the extraction container must be microwave transparent (e.g., PTFE or quartz). The solvent used may be microwave absorbing or non-microwave absorbing.

The temperature increases the penetration of the solvent into the matrix and constituents are released into the surrounding hot solvent In place of microwaves, ultrasonic vibrations may be used to supply good contact between sample and solvent. This is a fast technique but, efficiency is not as high as with other techniques. Low concentrations of analytes in samples require multiple extractions (Vivekananda et. al, 2007).

In ***supercritical fluid extraction*** (***SFE***), the extractant is in its supercritical state, which means that both pressure and temperature are above their critical values. Supercritical fluids (***SFs***) are dense gases above their critical temperature and pressure. It can diffuse through solids like a gas, and dissolve materials like a liquid. Thus, SFs possess properties which resemble both liquids and gases.

Analytes are more soluble in SFs when they are in their liquid state and also some organic modifiers may be added to the fluid in order to improve its solvating properties; thus, analyte melting points and solubility in the SF are important properties to consider (Camel, 2001).

Another sample preparation technique is ***solid-phase extraction (SPE)***. SPE uses the affinity of solutes dissolved or suspended in a liquid for a solid through which the sample is to separate a mixture into desired and undesired components.

SPE is designed for rapid, selective sample preparation and purification prior to chromatographic analysis. Using liquid chromatography principles to control selectivity, SPE provides the sample clean-up, recovery, and concentration

necessary for accurate quantitative analysis. SPE is a technique referring to a non-equilibrium exhaustive removal of analytes from a liquid sample by retention on a sorbent and then subsequent removal of selected analytes by solvent elution. Particulate matter in the sample can interfere with the analysis. Thus, particulate matter may sorbs some analytes of interest and cause low analytical recoveries. Particulates must be removed, by filtration, prior to SPE analysis.

This technique can be applied by two ways: Interferences may be retained on column and the analytes are passed through the column (this technique is preferred when the analyte concentration is high) or the analytes may be retained on column and the interferences are passed through the column (Yavuz, 2006).

Accelerated solvent extraction (***ASE***) is another sample preparation method which is also known as pressurized liquid extraction (***PLE***) and pressurized fluid extraction (***PFE***). ASE is a fully automated technique that uses common solvents to rapidly extract solid and semisolid samples. ASE operates at temperatures above the normal boiling point of most solvents, using pressure to keep the solvents in liquid form during the extraction process. Typically, ASE methods are completed in 15–25 min, while consuming only 15–50 mL of solvent. ASE was introduced in 1995 by Dionex Corporation and is recommended under US EPA Methods 3545 and 3545A for extraction of organophosphorus pesticides (Mitra, 2003).

The sample, solvent and the interactions between them are affected under the elevated temperature and pressure. The high temperature causes the increment at solvent boiling point which allows the extraction applicability at higher temperatures. As the temperature increases the viscosity and surface tension of the solvent decreases and extraction becomes easier. Because vapor remains in the cell and the pressure in it increases in the extraction period of the sample and high pressure allows the solvents to be kept constant in liquid form and boil at higher temperatures.

The role of the temperature is to increase the analyte diffusion to the medium surface and to break the bonds between analyte and matrix due to van der Waals forces, hydrogen bonding and dipole attractions. The ability of solvent movement through the deeper sides of the sample matrix increases by applying high pressure.

The ASE system contains solvent tank(s), solvent pump, an extraction cell, a heating oven, a collection vial, and a nitrogen tank (Figure 1.2).

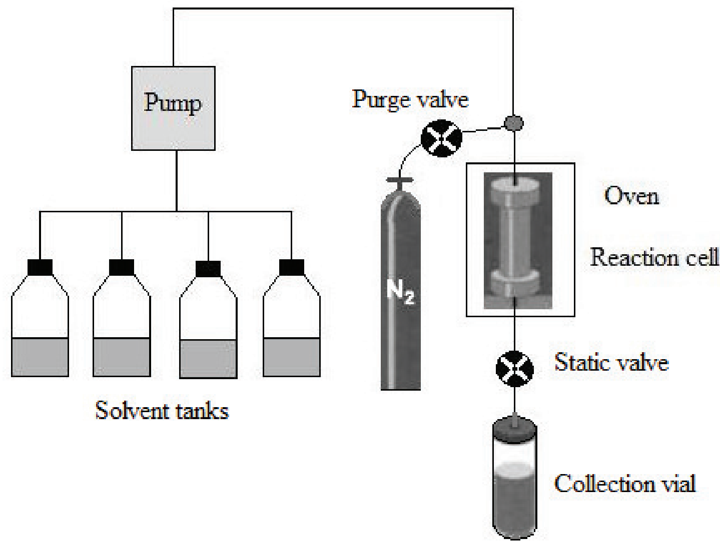


Figure 1.2 Schematic representation of ASE system

The sample is loaded to the reaction cells which are made of stainless steel that can resist to high pressure and temperature. The cells have two removable finger-tight caps on the ends and they supply easy sample loading and cleaning. The filter is placed into the two sides of the caps (one is placed before sample loading to the cell and the other one is placed after sample loading to the cell). The auto seal actuator moves the cell from the carousel into the heating oven.

The solvent is delivered from one or more solvent bottles into the extraction cell by a pump. The temperature and the pressure in the cell rise by the heated oven. When the pressure reaches 200 psi above the preset value, the static valve opens and releases the excessive pressure and then closes again. Then, the pump delivers fresh solvent to the cell to bring the pressure back to the preset value.

The addition of fresh solvent increases the concentration gradient and increases both mass transfer and extraction efficiency. The extracts are collected in 40 or 60 mL of collections vials which are placed on a removable vial tray. The vial caps have TFE (tetrafluoroethylene)-coated solvent-resistant septa. When the extracts are collected in the collection vial there is no need to additional cooling

due to the enough heat loss when the extracts are transferring from reaction cell to the collection vials.

The sample is loaded into the reaction cell, and then the solvent is pumped in. Then the cell is heated to the desired temperature and pressure. The heat-up time can be between 5 to 9 minutes (for up to 200^oC). This method is referred as prefill method. And also the sample can be heated before adding the solvent which is referred as preheat method.

When the preheat method is applied the volatile analytes can be lost so that generally the prefill method is preferred. After heating, the extraction can continue dynamically, statically or as a combination of both of them.

Dynamic ASE might be expected to yield faster extractions by continuously providing fresh extraction solvent to the sample, but this technique requires more solvent than static ASE, especially when large samples are extracted (less suited for trace analysis). Therefore, a combination of static and dynamic extraction will often be the best choice in practice.

In static ASE, the sample is extracted with a solvent at elevated temperature and pressure conditions without any outflow of solvent. When the extraction has reached equilibrium, the analytes are collected by rapidly flushing the extraction cell with solvent and inert gas (N_2).

On the other hand the static ASE may lead to incomplete extraction due to the limited volume of solvent used. Static extraction time can be between 5-99 minutes. After extraction, the extract is flushed into the collection vial with fresh solvents. The flush volume can be 5 to 150% of the cell volume, but generally 60% is the chosen. Five static cycles are possible to choose but commonly single cycle is chosen. The total flush volume is divided by the number of cycles, and an equal portion is used in each cycle. After the final solvent flush, the solvent is purged into the collection vial with nitrogen.

Solvent selection is very important parameter when working with ASE. The analytes that will be investigated should be soluble but sample matrix should not be soluble itself in the selected solvent. The solvents that commonly used are acetone, hexane, and petroleum ether.

The sample size is also a parameter that should be considered before an ASE extraction is performed. The sample size should be large enough to supply sample homogeneity and obtain sufficient sensitivity for trace analysis.

However, larger samples require large amounts of solvent for quantitative extraction. Generally, a small sample size is preferred provided that requirements for sample homogeneity and sensitivity are satisfied. When it is examined the ASE system has many advantages among the other sample preparation methods.

Low solvent consumption, short extraction time, easy to use and develop method, no additional filtration is needed, possibility to work with high temperature and pressure, work with common organic solvents are the advantages of the ASE system.

1.7 Chemometrics: Experimental Design

Chemometrics is a chemical discipline that uses mathematics, statistics, and formal logic:

- ✓ to design or to select optimal experimental procedures;
- ✓ to provide maximum relevant chemical information by analyzing chemical data; and,
- ✓ to obtain knowledge about chemical systems

In recent years several chemometric tools have been used for resolving overlapping signals, calibration and model identification. Soft-modeling and multivariate-analysis techniques have been shown to be very powerful for several aims, especially for multianalyte calibration, and modeling in multicomponent dynamic systems (Brereton, 2002).

Design Matrices and Modeling: An experimental design may consist of a series of experiments performed under different conditions. Experimental data can be described as a mathematical relationship between the factors or independent variables. A typical equation for three factors might be of the following form including the term; $\hat{y}$ (Table 1.2) since the equation estimates its value, and is unlikely to give an exact value that agrees experimentally due to the error.

Table 1.3 Terms used in the equation of experimental design

$\hat{y}$	Estimated response
b_0	an intercept or average
$b_1x_1+b_2x_2+b_3x_3$	linear terms depending on each of the three factors
$b_{11}x_{11}+b_{22}x_{22}+b_{33}x_{33}$	quadratic terms depending on each of the three factors
$b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_2x_3$	interaction terms between the factors

The intercept is an average in certain circumstances. It is an important term because the average response is not normally achieved when the factors are at their average values. This term can be ignored if it is known that there are no baseline problems or interferents. The linear terms allow for a direct relationship between the response and a given factor. For some experimental data, there are only linear terms.

On the other hand, quadratic terms are important in many situations. This allows curvature, and is one way of obtaining a maximum or minimum. Interaction terms arise due to the influence of two factors on the response is rarely independent. In advance of experimentation it is often hard to predict which factors are important. Many chemometricians find it convenient to work using matrices. The design matrix is simply one in which the rows refer to experiments and the columns refer to individual parameters in the mathematical model or equation linking the response to the values of the individual factors. There are two considerations required when computing a design matrix, namely; the number and arrangement of the experiments, including replication and the mathematical model to be tested.

The relationship between the response, the coefficients and the experimental conditions can be expressed in matrix form given below. It is easy to calculate b (or the coefficients in the model) knowing D and y using MLR (multiple linear regression).

$$\hat{\boldsymbol{y}} = \boldsymbol{Db} \qquad (1.1)$$

If $\boldsymbol{D}$ is a square matrix, then there is exactly the same number of experiments as coefficients in the model. If $\boldsymbol{D}$ is not a square matrix pseudo-inverse form is used with an easy calculation with all matrix based software.

Once ***b*** has been determined, it is then possible to predict ***y*** and so calculate the sums of squares and other statistics. The statistical significance can be obtained from a two-tailed *t*-distribution (student's t-test) and alternatively with *F*-test.

An alternative, statistical indicator, based on Student's *t* -test, can be used, provided that more experiments are performed than there are parameters in the model. Whereas this and related statistical indicators have a long and venerated history, it is always important to back up the statistics by simple graphs and considerations about the data. There are many diverse applications of a *t* -test, but in the context of analyzing the significance of factors on designed experiments, the following the main steps are used.

✓ Calculate the matrix $(D'D)^{-1}$. This will be a square matrix with dimensions equal to the number of parameters in the model

✓ Calculate the error sum of squares between the predicted and observed data:

$$S_{resid} = \sum_{i=1}^{I} (y_i - \hat{y}_i)^2 \qquad (1.2)$$

✓ Take the mean the error sum of squares (divided by the number of degrees of freedom available for testing for regression):

$$s = S_{resid}/(N-P) \qquad (1.3)$$

✓ For each of the *P* parameters (=10 in this case), take the appropriate number from the diagonal of the matrix obtained instep a above.

✓ For each coefficient, ***b***, calculate

$t = b/\sqrt{sv}$ (v, the variance of coefficient). (1.4)

The higher this ratio, the more significant is the coefficient. This ratio is used for the *t* -test.

✓ The statistical significance can then be obtained from a two tailed *t*-distribution, or most packages such as Excel have simple functions for the *t* -test. Take the absolute value of the ratio calculated above. Normally, fairly high probabilities are expected if a factor is significant, often in excess of 95%.

The size of the coefficients reflects their significance; however, the physical scale for each variable is different. It is useful to put each variable on a comparable scale in order to have a better idea of the significance. Coding the experimental data is commonly preferred for this purpose. Each variable is placed on a common scale, often with the highest coded value of each variable equal to +1 and the lowest to −1.

Factorial Designs are often used for screening or when there are a large number of possible factors. Full factorial designs at two levels are mainly used for screening and are also called as saturated designs.

The first step is to choose a high and low level for each factor. Then a standard design is used where the value of each factor is usually coded. Following the experiments the data obtained are analyzed by setting up a design matrix. Here an interaction term must be taken into account, and a design matrix based on the below model is set up.

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 \qquad (1.5)$$

This can be expressed either as a function of the true or coded concentrations. It should also be noted that each of the columns in the set up matrix is different. This is an important and crucial property and allows each of the possible terms to be distinguished uniquely from one another, and is called ***orthogonality***. Final next step is to calculate and then interpret the coefficients.

Major drawback of full factorial design is that large number of experiments must be performed. Two level fractional factorial designs are used to reduce the number of experiments by ½, ¼, ⅛ and so on. Such fractional factorial designs only exist when the number of experiments equals a power of 2. Where the number of factors is fairly large, this situation can be rather restrictive. Figure 1.6 shows the difference between two kinds of factorial designs

Plackett and Burman Design has proposed a number of two level factorial designs, where the number of experiments is a multiple of four. Hence design exist for 4, 8, 12, 16, 20, 24, etc. experiments. The number of experiments exceeds the number of factors, k, by one ($k+1$). Generators for Plackett-Burman designs are given in Table 1.3

Table 1.4 Generators used in Plackett-Burman design

Factors	Generator
7	+ + + - + - -
11	+ + - + + + - - - + -
15	+ + + + - + - + + - - + - - -
19	+ + - + + + + - + - + - - - - + + -
23	+ + + + + - + - + + - - + + - - + - + - - - -

Central Composite Design is used for optimization and to produce a detailed quantitative model. The main effects and interaction terms were included in this design. This design is composed of a full factorial design, a star design and replicates (Figure 1.3).

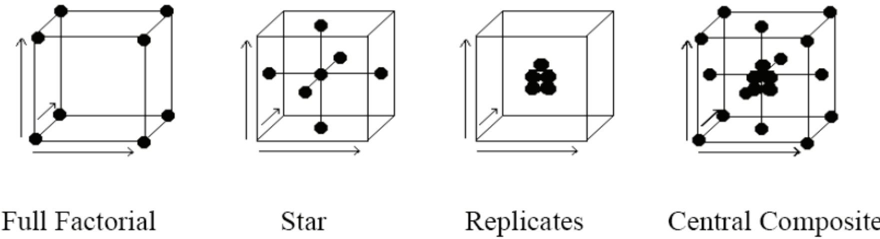


Figure 1.3 Elements of a central composite design

Number of experiments is calculated from (*2k + 2k + 1*) where the *k* is number of factors. *2k* is the number of experiments in full factorial design (*Nf*) in the model and all interaction terms are included. The levels of factors are expressed as +1 and -1. *2k + 1* term represents the number of experiments in star design of this model. The levels are expressed as ±α 1 level, 0 being in the center.

α levels take different values for rotational and orthogonal designs. For rotational design; N being the total number of experiments;

$$\alpha = \pm\sqrt[4]{2^k} \quad (1.6)$$

For orthogonal design

$$\alpha = \pm\sqrt{\frac{\sqrt{NN_f} - N_f}{2}} \quad (1.7)$$

Replicates in central composite design can be used to provide a mean response and an estimate of pure experimental uncertainty. These runs are generally at center level and at least five replicates are proposed ±α values for the factors were calculated as follows,

$$\pm\alpha = \frac{X_1 - Med.value}{Max.value - Med.value} \; or \; \pm\alpha = \frac{X_1 - Med.value}{Min.value - Med.value} \quad (1.8)$$

Using the model design matrix (***X***) estimated b parameters can be calculated as;

$$b = (X^T X)^{-1} X^T y \quad (1.9)$$

Superscripts T and -1 denote the transpose and inverse of a matrix respectively. Estimated responses are calculated as

$$\hat{y} = Xb \quad (1.10)$$

Response surface methodology: The nature of response surface system (maximum, minimum, or saddle point) depends on the signs and magnitudes of the coefficients in the model. The second order coefficients (interaction and pure quadratic terms) play a vital role. These coefficients are estimates of the *b* coefficients of the model equation These graphs are very useful for interpreting graphically the effect on the response of each pair of independent variables. (Myers and Montgomery, 2002).

1.8 The Aim of The Thesis

In this thesis study, determination of pesticide contents of tobacco samples was aimed. For this purpose, trifluarin, fenchlorphos, penconazole, α-endosulfane, β-endosulfane, endosulfanesulfate, phosalone, cyfluthrin, cypermethrin, esfenvalerate, were chosen.

The main parameters of the ASE that effect the extraction recovery were investigated. In order to observe the effects of parameters on extraction recovery the Plackett-Burman Design was chosen. There can be lots of parameters that are insignificant or very important that are very effective in the analysis. Then, it was planned to reveal optimal conditions for the analysis of selected pesticides in tobacco samples. The chemical properties of these selected pesticides can be seen in the next section.

1.9 Properties of the Pesticides Used In Study

Table 1.5 Molecular formulas & structures of the pesticides used in the study

Pesticide	IUPAC Name	Molecular Formula	Molucular Structure
Trifluarin	α – α – α – trifluoro – 2,6 – dinitro-N,N – dipropyl – p - toluidine	$C_{13}H_{16}(NO_2)_2F_3$	
Fenchlorfos	O,O-Dimethyl-O-(2,4,5-trichlorophenyl) phosphorothioate	$C_8H_8Cl_3O_3PS$	
Penconazole	1-(2,4-dichloro-b-propylphenethyl)-1*H*-1,2,4-triazole	$C_{13}H_{15}Cl_2N_3$	
α-Endosulfane	an equimolar mixture of 2 asymmetric, twisted-chair conformers of (5a*R*,6*S*,9*R*,9a*S*)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine 3-oxide	$C_9H_6Cl_6O_3S$	
β-Endosulfane	an equimolar mixture of 2 asymmetric, twisted-chair conformers of (5a*R*,6*S*,9*R*,9a*S*)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine 3-oxide	$C_9H_6Cl_6O_3S$	
Endosulfane Sulfate	5-norbornene-2,3-dımethanol, 1,4,5,6,7,7-hexachloro-, cyclıc sulfate	$C_9H_6Cl_6O_4S$	
Phosalone	*S*-6-chloro-2,3-dihydro-2-oxo-1,3-benzoxazol-3-ylmethyl *O,O*-diethyl phosphorodithioate	$C_{12}H_{15}ClNO_4P_2$	

Table 1.5 (continue)

Pesticide	IUPAC Name	Molecular Formula	Molucular Structure
Cyfluthrin	(*RS*)-α-cyano-4-fluoro-3-phenoxybenzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate or (*RS*)-α-cyano-4-fluoro-3-phenoxybenzyl (1*RS*)-*cis-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate	$C_{22}H_{18}Cl_2FNO_3$	
Cypermethrin	(*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate or (*RS*)-α-cyano-3-phenoxybenzyl (1*RS*)-*cis-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate	$C_{22}H_{19}Cl_2NO_3$	
Esfenvalerate	(*S*)-α-cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate	$C_{25}H_{22}ClNO_3$	

(Ehrenstorfer, 2008)

2. EXPERIMENTAL

2.1 Instrumentation

The extraction part was performed by ASE 300 from Dionex.

Chromatographic part of the study was carried out with the help of the Shimadzu GC 2010 system. The column properties that was used in this study; 30 m length, 0.32 mm inner diameter, coated by 100% dimethylpolysiloxane with 0.25 µm film thickness of DB-1 quartz capillary column (Agilent Technologies, USA). The conditions of the instrument during the study were shown below;

Carrier gas: Helium
Column Flow: 1.5 ml/min
Injection Temperature: 250 ^{o}C
Detector Temperature:300 ^{o}C
Injection Mode: Splitless (1µL)
Makeup Gas: Ar
Makeup Flow: 30 mL/min

Table 2.1 Oven temperature program

Rate (^{o}C/min)	Temperature(^{o}C)	Hold Time(min)
-	70	2
25	150	0
3	200	0
8	280	15

Chemometric calculations were performed by the Microsoft Office Excel and Matlab 6.5 package program.

2.2 Chemical Reagents and Samples

HPLC grade acetone, n-hexane were obtained from Lab-Scan, Florosil (0.150-0.250 mm) was obtained from Merck (Darmstadt, Germany). Standards of pesticides were taken from Dr. Ehrenstorfer (Germany). The standard solutions were prepared by dissolving them in acetone (with respect to their purity percent) and stored at +4°C.

2.3 Procedure

First of all the retention times of the pesticides were determined and then 1 gram of blank tobacco samples were weighted and spiked so as to be 1000 ng/g and the cells were full filled with florosil, both of them were put into the ASE cell. The spiked samples were examined in eight trials. (Retention times of pesticides, chromatograms of standard solutions, areas and recovery values of each pesticide for each trial and conditions of the studies were given in results and discussion part). After that the extracts were dried under nitrogen flow and diluted to 5 mL with acetone and analyzed with gas chromatography. This procedure was also followed for thirty trials and detected optimum conditions.

Calibration graphs were prepared with five points which were 50 ng/g, 100 ng/g, 250 ng/g, 500 ng/g and 1000 ng/g , factors and Plackett-Burman design were given in table 3.5 and table 2.3.

Table 2.2 Factors and levels used for Plackett-Burman Design

Factors	Explanation	(-) Level	(+) Level
F1	Heat Time (min)	5	10
F2	Tempetarute (^{o}C)	40	120
F3	Static Time (min)	6	12
F4	Flush Volume (%)	60	90
F5	Purge time (sec)	120	200
F6	Number of Static Cycles	2	4
F7	Solvent Ratio(%)	hexane-acetone(20%-0%)	acetone-hexane(20%-80%)

Table 2.3 Plackett-Burman Design model

Method	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**
1	(-)	(-)	(-)	(-)	(-)	(-)	(-)
2	(+)	(-)	(-)	(+)	(-)	(+)	(+)
3	(+)	(+)	(-)	(-)	(+)	(-)	(+)
4	(+)	(+)	(+)	(-)	(-)	(+)	(-)
5	(-)	(+)	(+)	(+)	(-)	(-)	(+)
6	(+)	(-)	(+)	(+)	(+)	(-)	(-)
7	(-)	(+)	(-)	(+)	(+)	(+)	(-)
8	(-)	(-)	(+)	(-)	(+)	(+)	(+)

According to Plackett-Burman design three parallel studies were done and the F values were given below.

Table 2.4 F values of first study

	Trifluarin	Fenchlorfos	Penconazole	A.Endosulfane	B.Endosulfane	Endosulfane S.	Phosalone	Cyfluthrin	Cypermethrin	Esfenvalerate
F1	-15,7755	-6,00758	39,88351	-11,259	-1,06533	-0,40282	-6,16604	-4,90687	-12,5954	-2,0252
F2	24,06703	3,865815	-1,87648	16,26685	14,47307	4,019218	-17,0177	6,221931	-0,35285	18,38655
F3	11,00096	22,93381	-14,1788	33,04134	15,96967	-3,21464	10,54835	24,17773	27,54744	23,87527
F4	-24,8908	-21,7365	3,415409	-16,2576	-17,2893	-7,17146	-16,8944	-12,9166	-12,3808	-16,5301
F5	12,91687	16,2109	-6,72588	18,27039	8,56385	-8,78141	4,003216	8,490869	9,64302	2,025344
F6	-21,6403	-39,7739	-13,5882	-29,117	-34,8312	-27,7673	-29,9651	-33,9835	-22,2123	-19,185
F7	-0,82059	-8,84883	-17,1957	3,789612	0,255958	-6,03215	-31,4335	-2,85318	-29,468	-19,7055

Table 2.5 F values of second study

	Trifluarin	Fenchlorfos	Penconazole	A.Endosulfane	B.Endosulfane	Endosulfane S.	Phosalone	Cyfluthrin	Cypermethrin	Esfenvalerate
F1	-12,525	-10,95	40,475	-4,025	-5,25	-2,675	-4,4	-3,0	-6,825	-2,7
F2	15,725	2,35	-12,225	14,425	8,95	5,925	-9,15	8,5	6,075	10,4
F3	6,925	15,35	-1,475	20,925	15,35	-5,675	15,6	17,5	23,675	17,8
F4	-9,725	-12,5	-5,575	-16,225	-13,6	-4,425	-17,5	-1,35	-13,075	-13,05
F5	8,225	2,45	-9,325	17,425	8,0	-15,225	8,8	11,45	11,925	17,65
F6	-15,825	-28,4	-9,075	-26,825	-30,55	-21,175	-29,35	-6,7	-18,325	-22,7
F7	8,375	3,7	-8,825	-6,025	0,4	-10,025	-13,65	-3,75	-22,175	-8,65

Table 2.6 F values of third study

	Trifluarin	Fenchlorfos	Penconazole	A.Endosulfane	B.Endosulfane	Endosulfane S.	Phosalone	Cyfluthrin	Cypermethrin	Esfenvalerate
F1	-17,2414	-6,64319	49,71922	-11,8905	-2,65162	2,573829	-5,6121	-25,3334	-13,5729	-11,4608
F2	27,53019	6,561641	7,048053	14,84773	15,19056	4,721733	-17,7288	-9,10718	-7,34735	13,12084
F3	15,18301	25,87065	-21,3852	33,75701	18,38128	-4,99567	11,0656	20,01977	19,9969	32,41417
F4	-21,8154	-19,1183	11,55994	-16,8416	-16,2225	-7,07985	-17,6012	-28,3279	-18,7407	-28,8942
F5	12,24456	14,10929	-14,6539	18,99047	5,892235	-11,1279	3,475414	-2,55168	5,757567	-5,55664
F6	-22,2484	-41,7641	-22,298	-29,6093	-36,5858	-29,1365	-30,7002	-44,7747	-24,9423	-19,1906
F7	0,339122	-14,1468	-25,8522	3,427158	2,135043	-9,01069	-32,1782	-8,32427	-38,2957	-22,7436

3. RESULTS AND DISCUSSION

3.1 Calibration Studies with Standard Solution

Initial studies were conducted to calibrate the instrument for the determination of the pesticides selected by GC-ECD system. First of all, the retention times of the pesticides were determined by injecting the standard solution which contains ten pesticide into the GC inlet. Figure 3.1 shows the chromatographic peaks of ten pesticides obtained with 0.25 μm DB-1 column. 1μL of standard solution was injected in splitless mode. Other conditions of the instrument during the study were given in Experimental Section.

The retention times of the pesticides studied were given in the Table 3.1.

Table 3.1 Retention times (t_R) of the pesticides (min)

Pesticide	t_R	Pesticide	t_R
Trifluarin	8.5	Endosulfane sulfate	17.80
Fenchlorphos	12.32	Phosalone	19.88
Penconazole	14.4	Cyfluthrin	22.06
α-endosulfane	15.5	Cypermethrin	22.50
β-endosulfane	16.8	Esfenvalerate	24.37

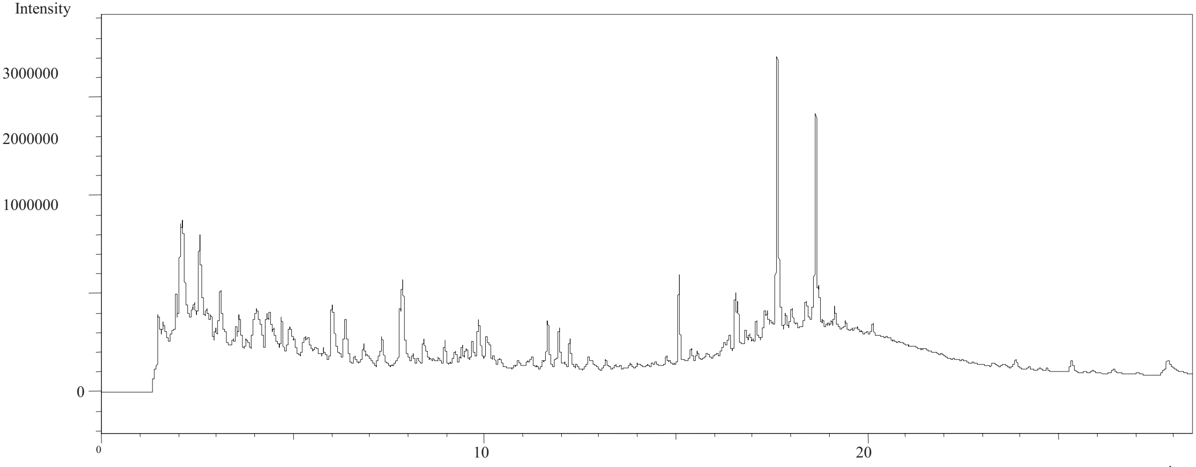
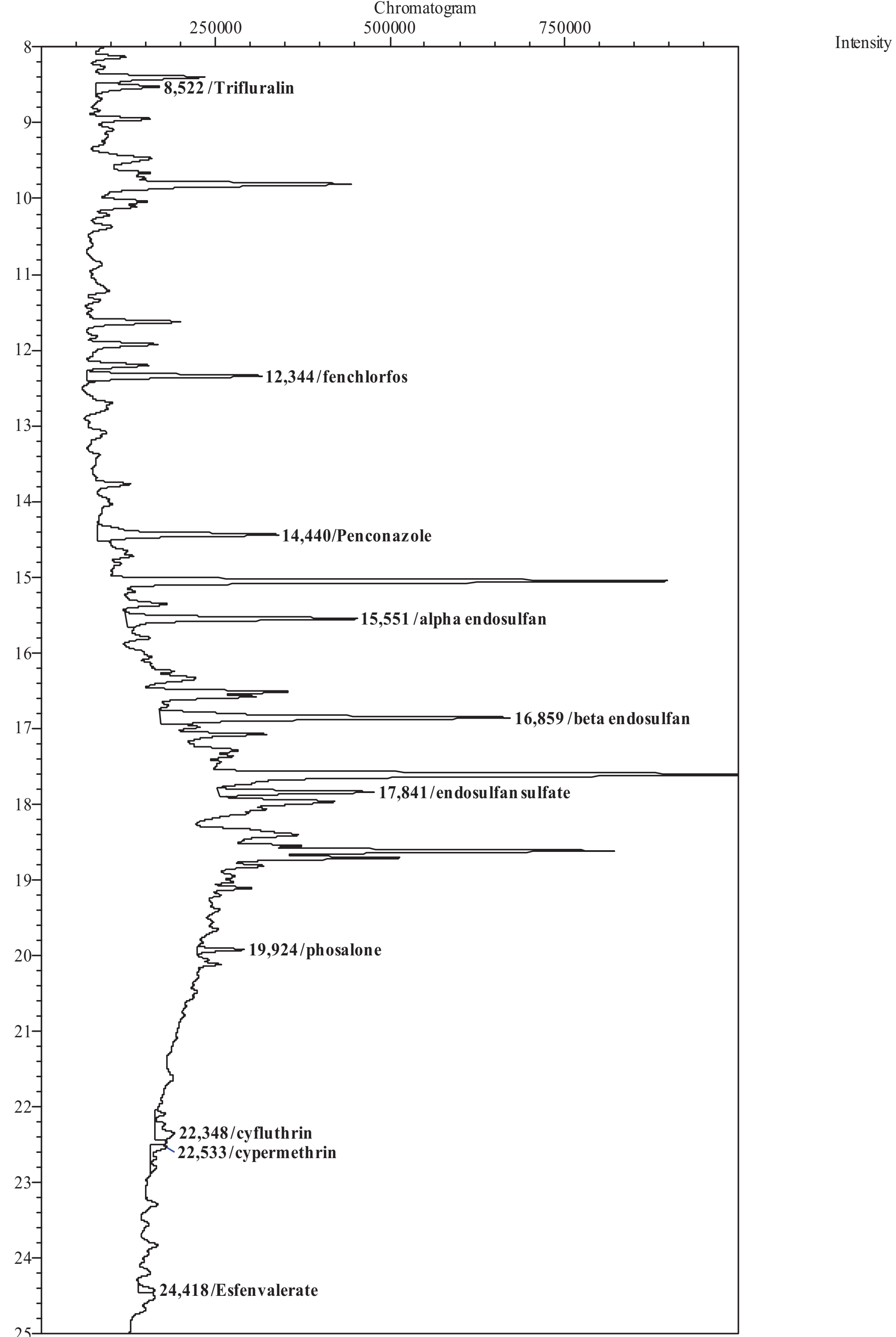


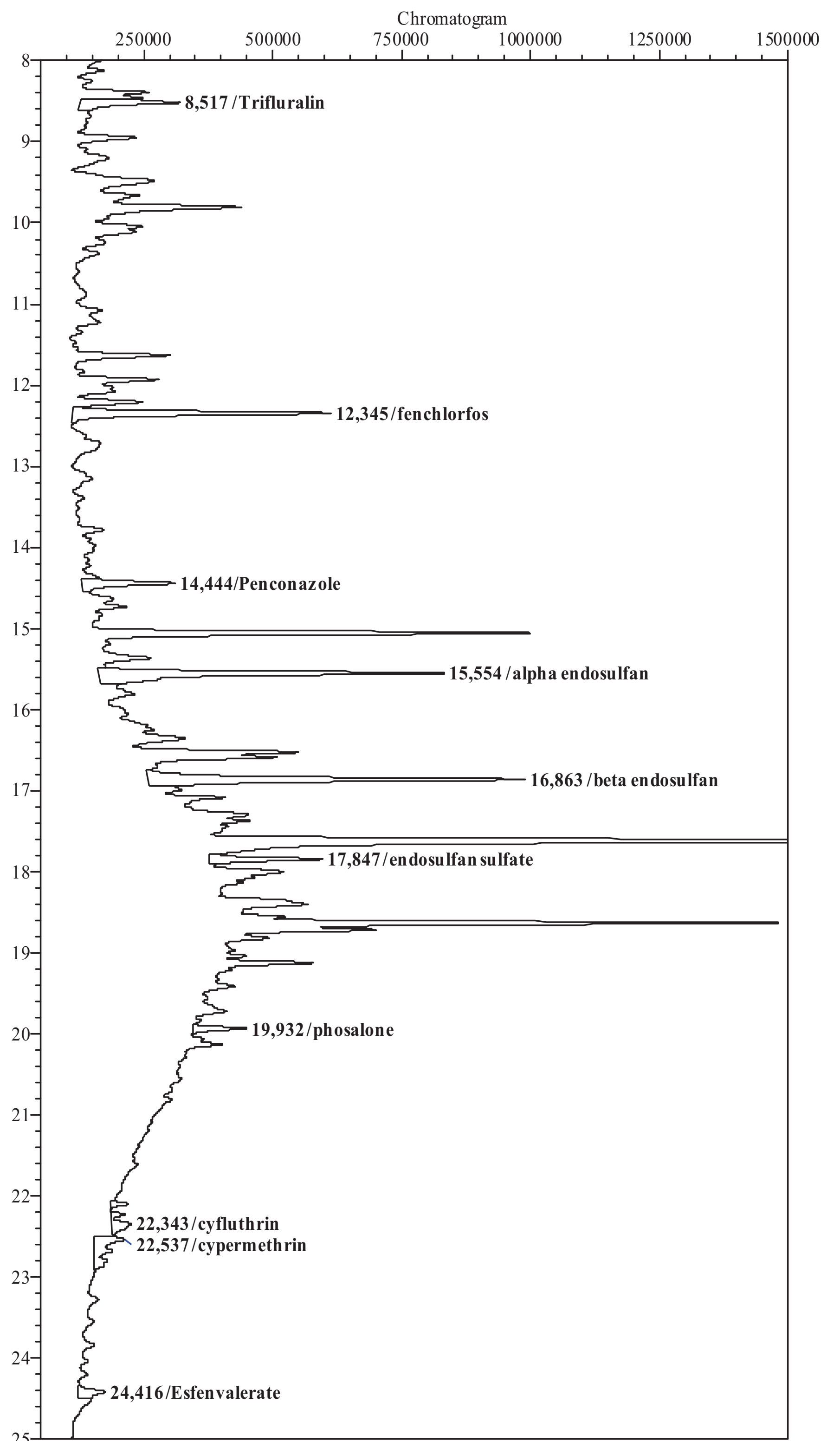
Figure 3.1 The chromatogram for blank tobacco sample

Figure 3.2 The chromatograms for A) 50 , ng / mL B) 100 ng / mL,C) 250 ng / mL, D) 500 ng / mL, E) 1000 ng / mL pesticide standard solutions obtained with GC-ECD system



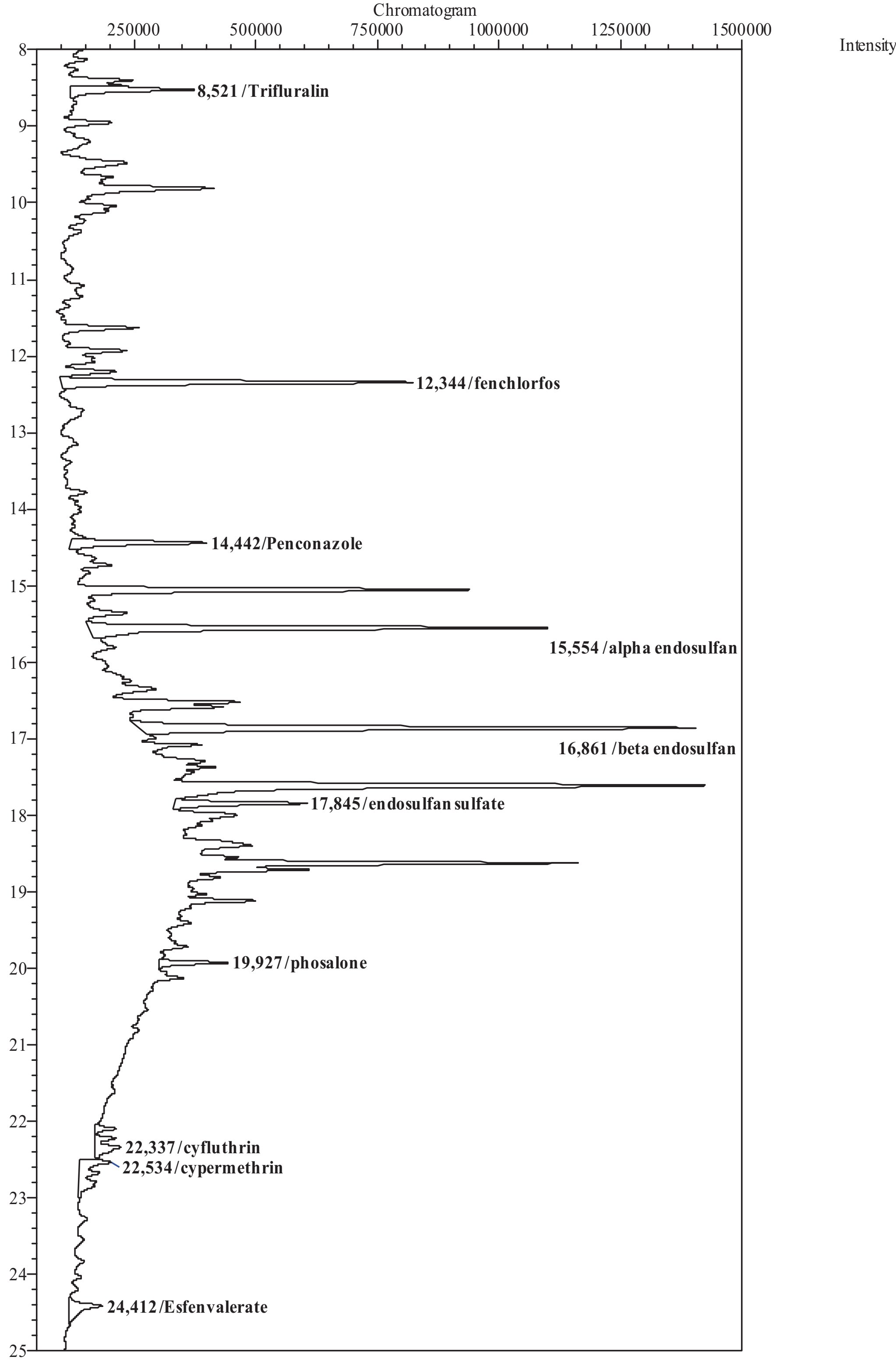
A) 50 ng / mL pesticide standard solution

Figure 3.2 (continue)



B) 100 ng / mL pesticide standard solution

Figure 3.2 (continue)



C) 250 ng / mL pesticide standard solution

Figure 3.2 (continue)

Chromatogram

250000 500000 750000 1000000 1250000

Intensity

8,522 /Trifluralin

12,347/fenchlorfos

14,443/Penconazole

15,556 /alpha endosulfan

16,867 /beta endosulfan

17,844/endosulfan sulfate

19,924/phosalone

22,337/cyfluthrin

22,535/cypermethrin

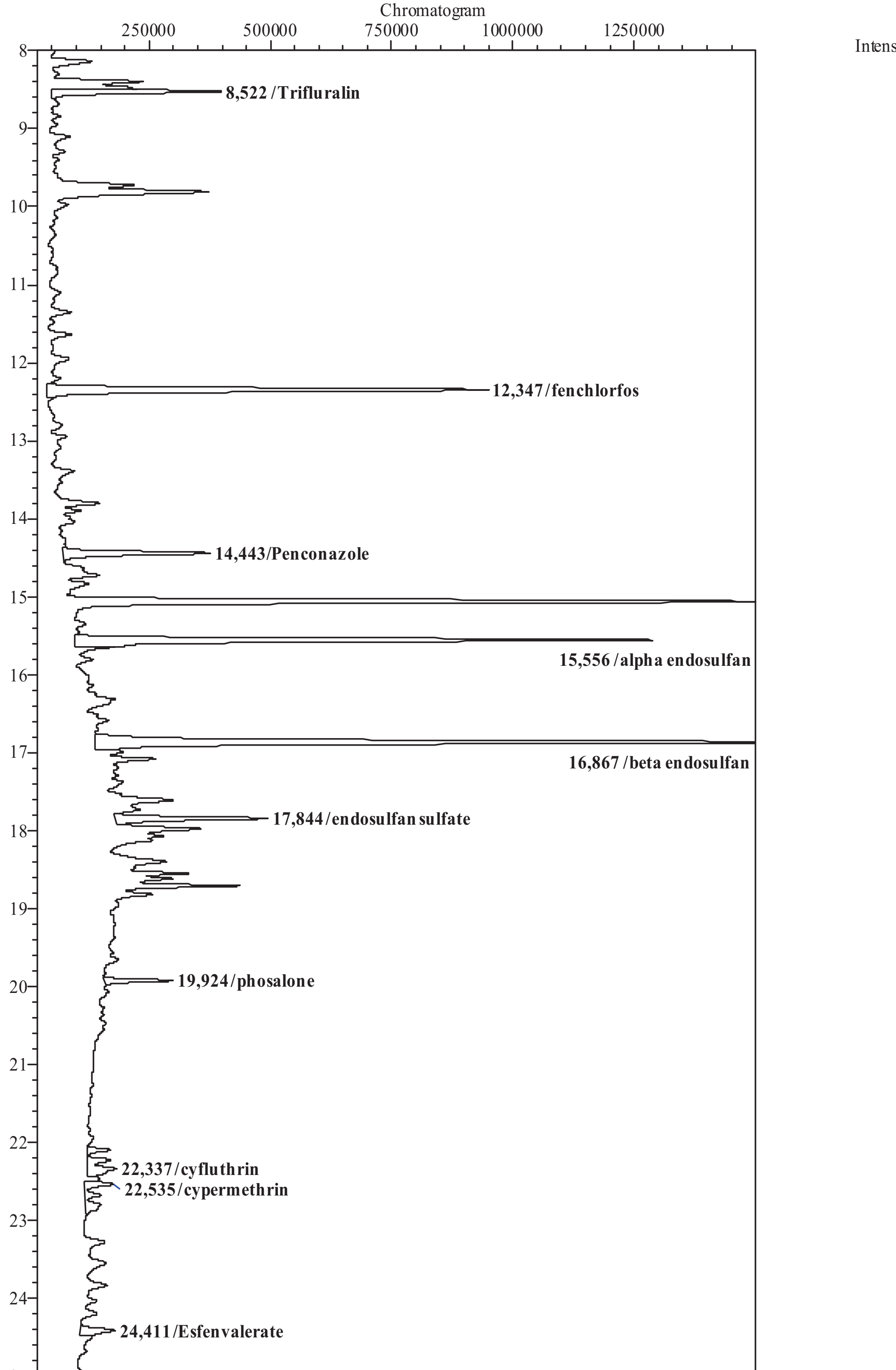
24,411/Esfenvalerate

8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

min

D) 500 ng / mL pesticide standard solution

Figure 3.2 (continue)



E) 1000 ng / mL pesticide standard solution

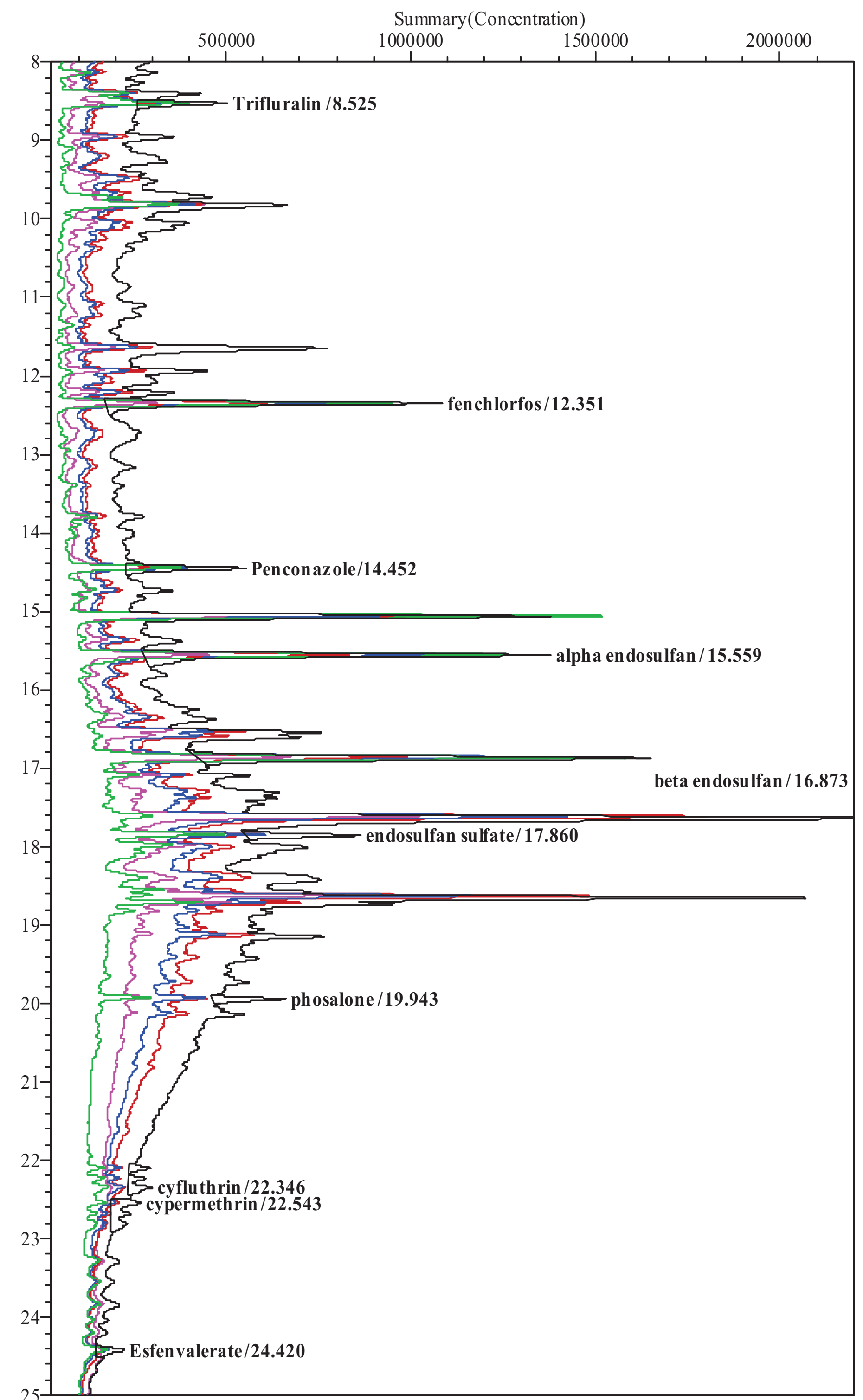


Figure 3.3 Chromatograms for standard solutions in tobacco matrix

- Chromatogram for 1000 ng/ml standart solution in tobacco matrix
- Chromatogram for 500 ng/ml standart solution in tobacco matrix
- Chromatogram for 250 ng/ml standart solution in tobacco matrix
- Chromatogram for 100 ng/ml standart solution in tobacco matrix
- Chromatogram for 50 ng/ml standart solution in tobacco matrix

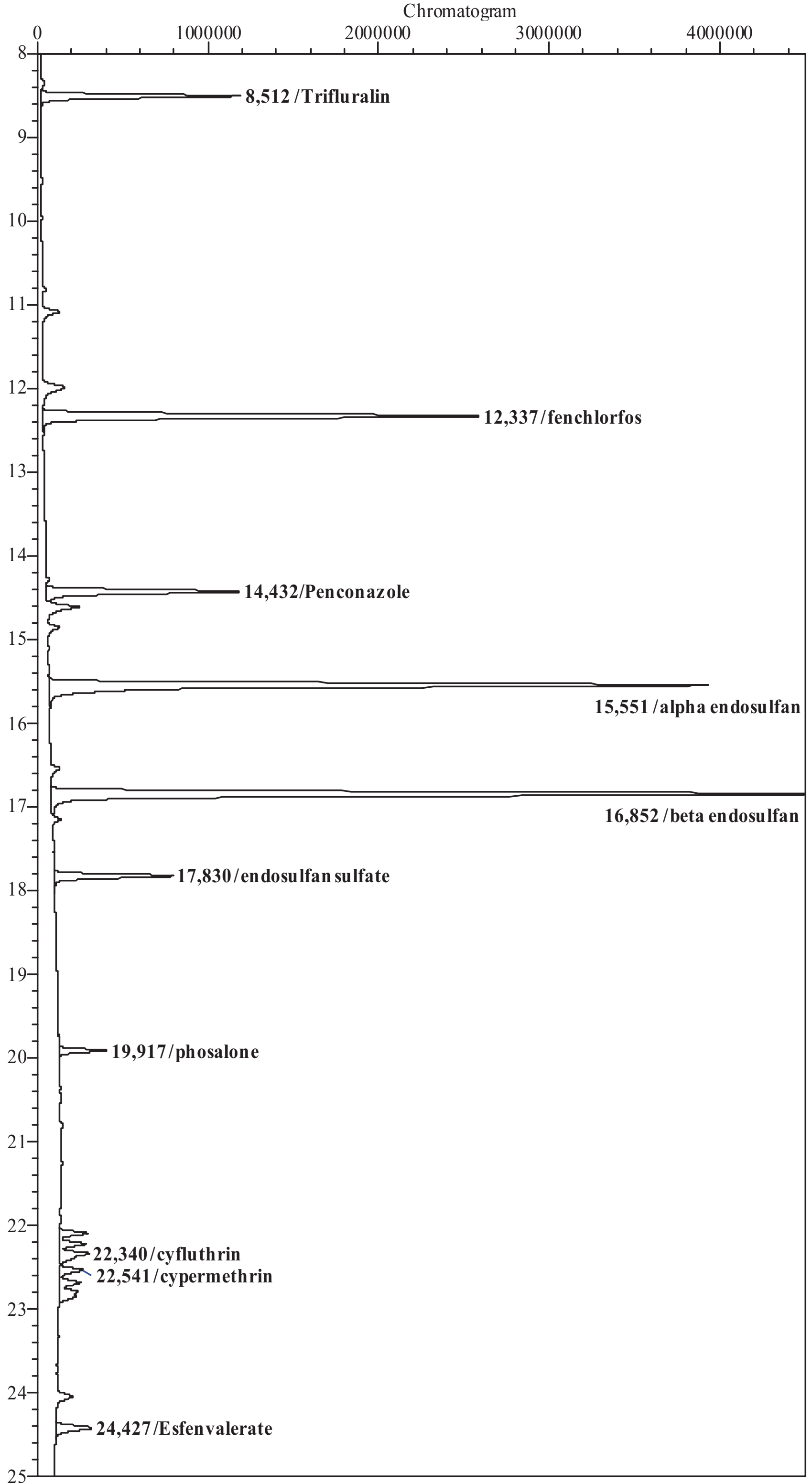
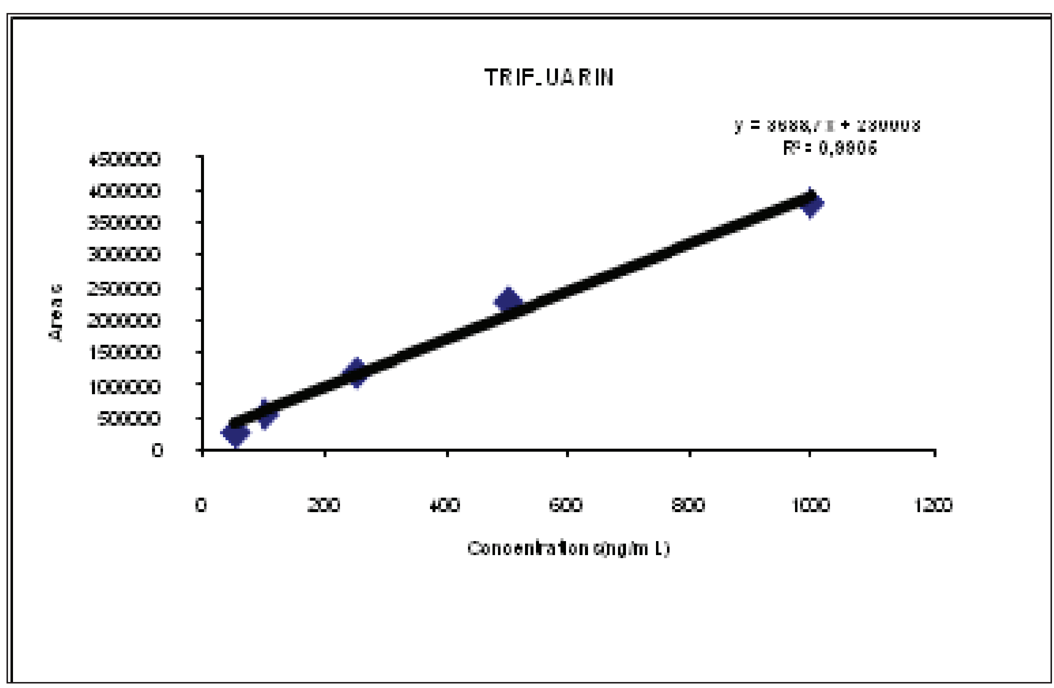
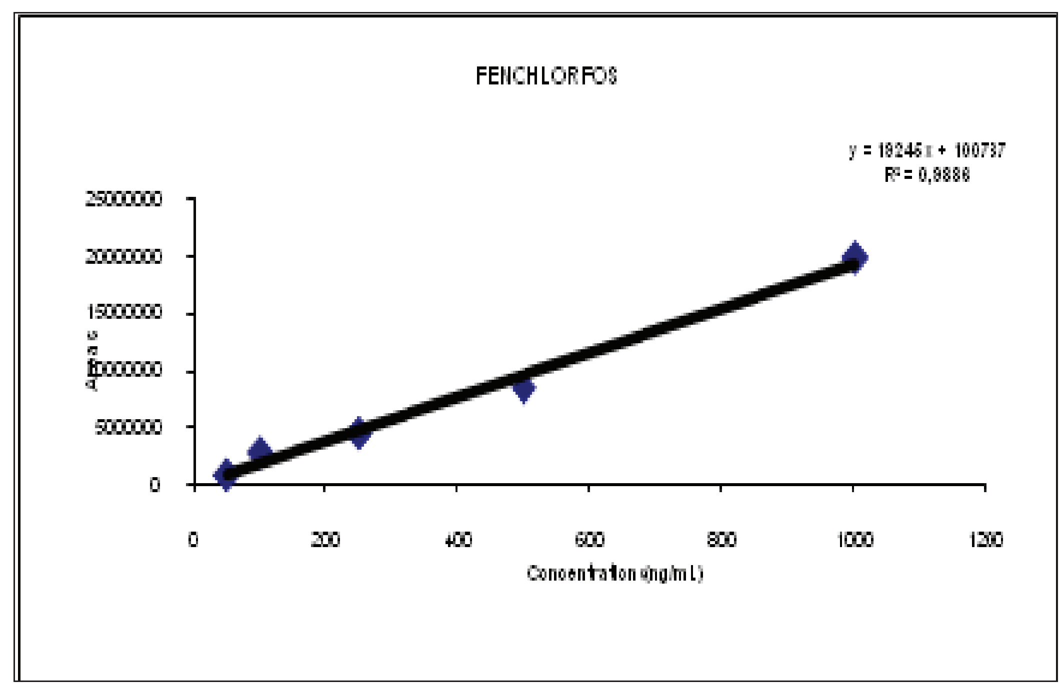


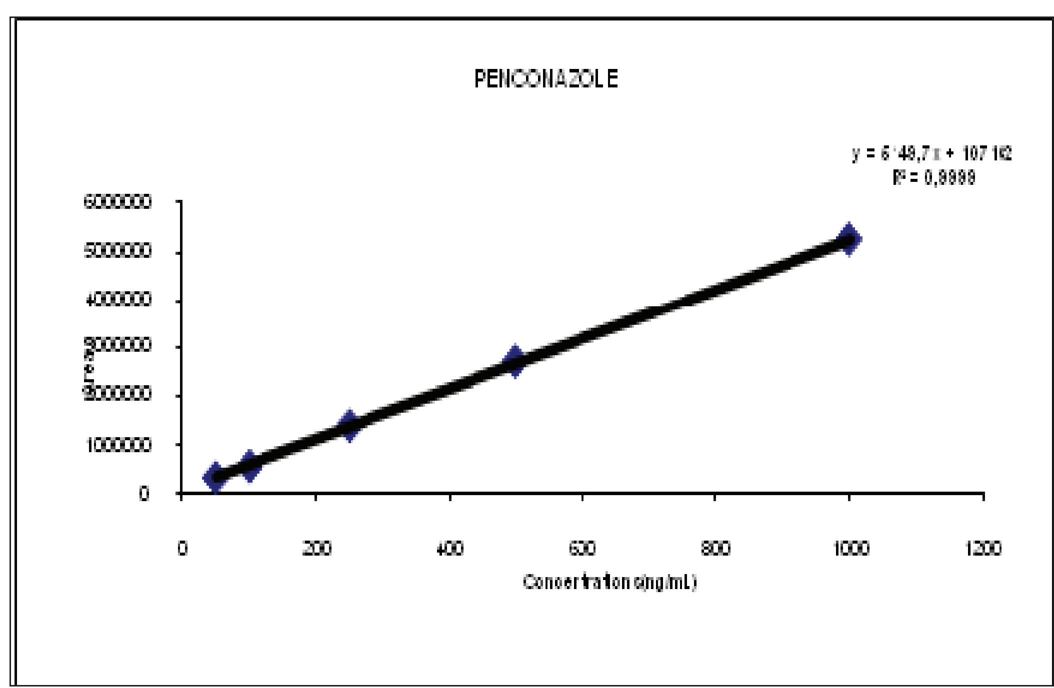
Figure 3.4 Chromatogram for 1000 ng/mLstandard solution in acetone matrix

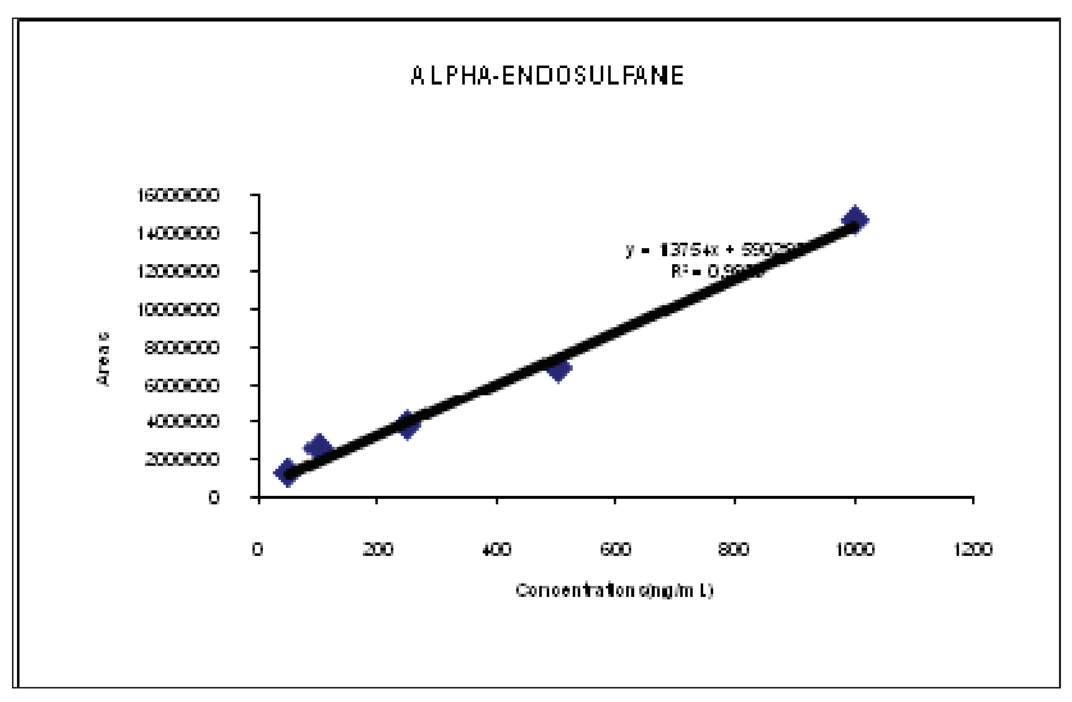
Figure 3.5 The calibration graphs for Pesticides



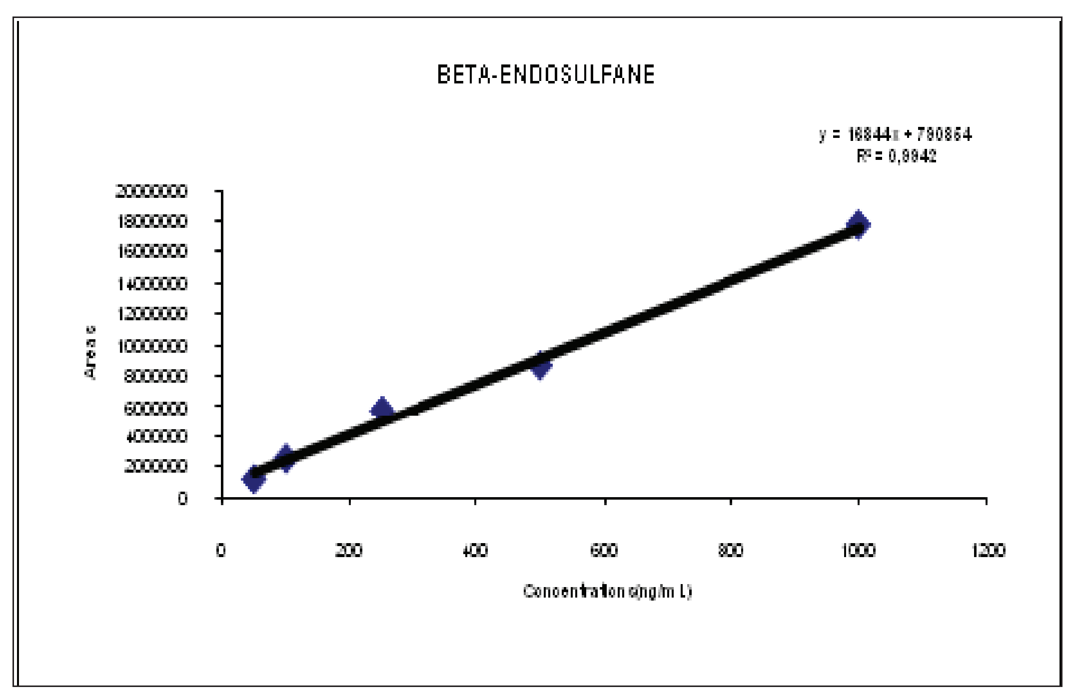


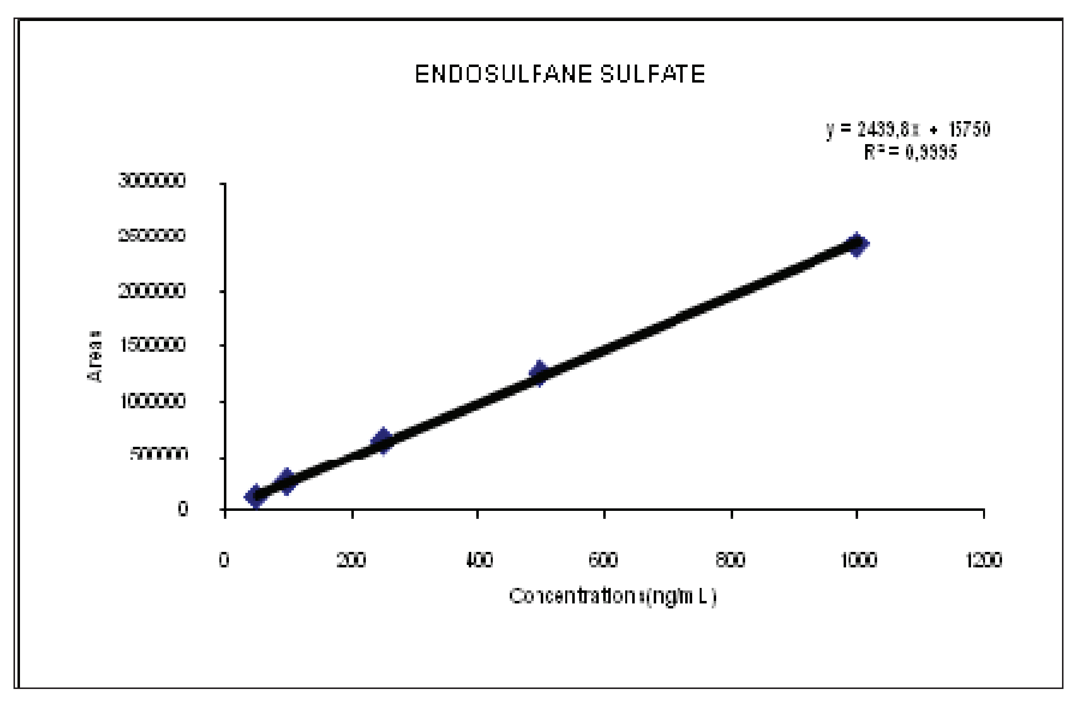
Figue 3.5 (continue)



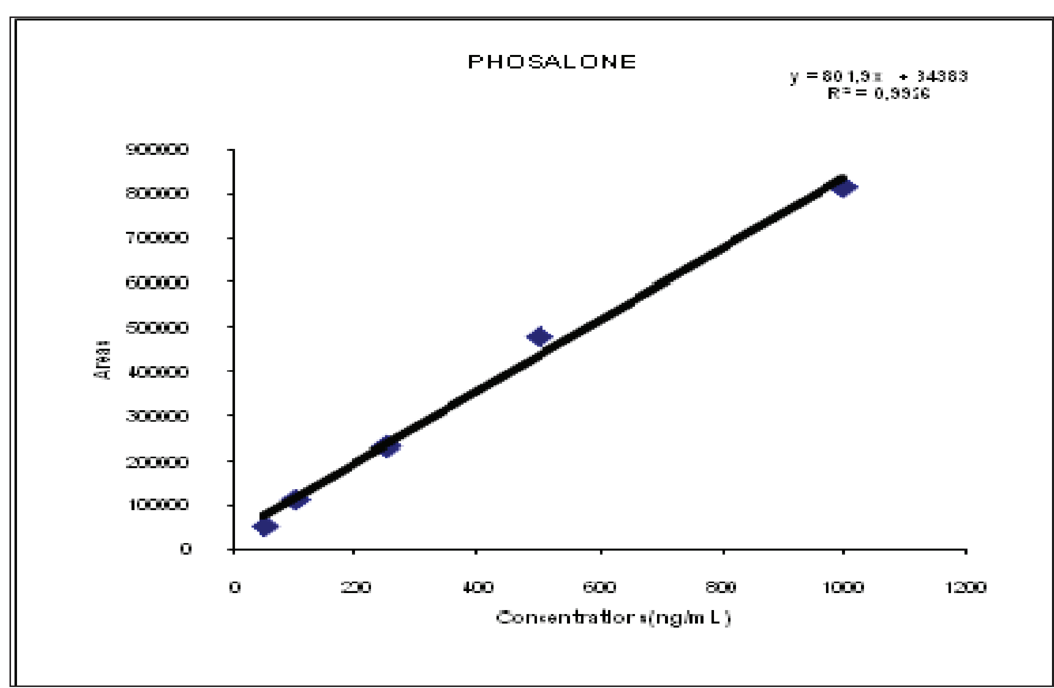


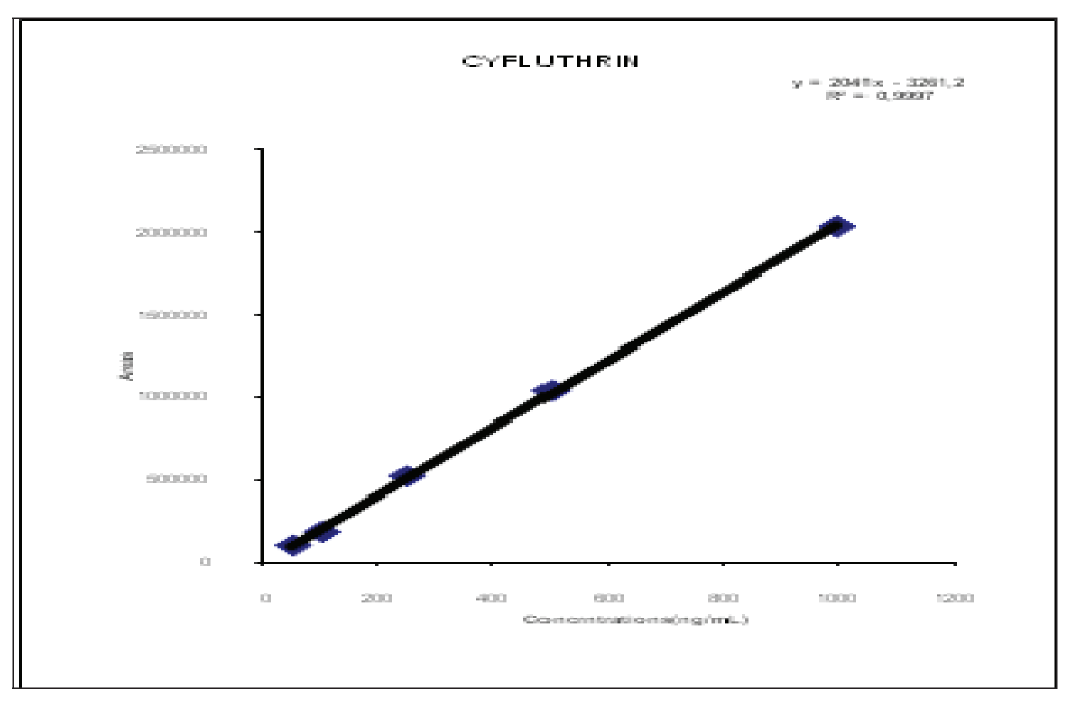
Figue 3.5 (continue)



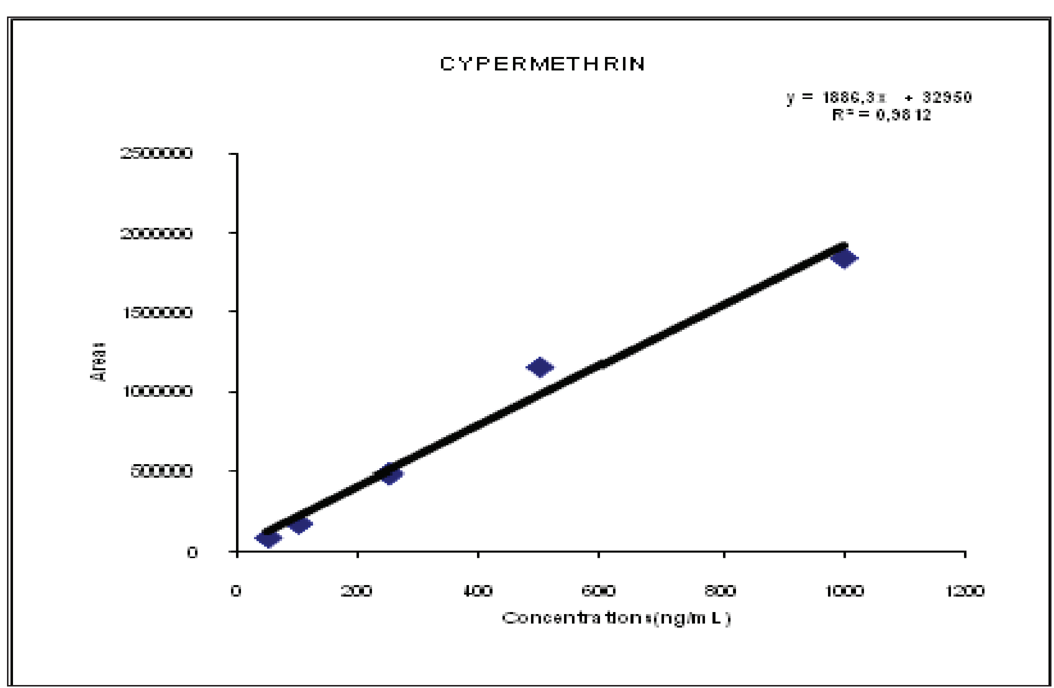


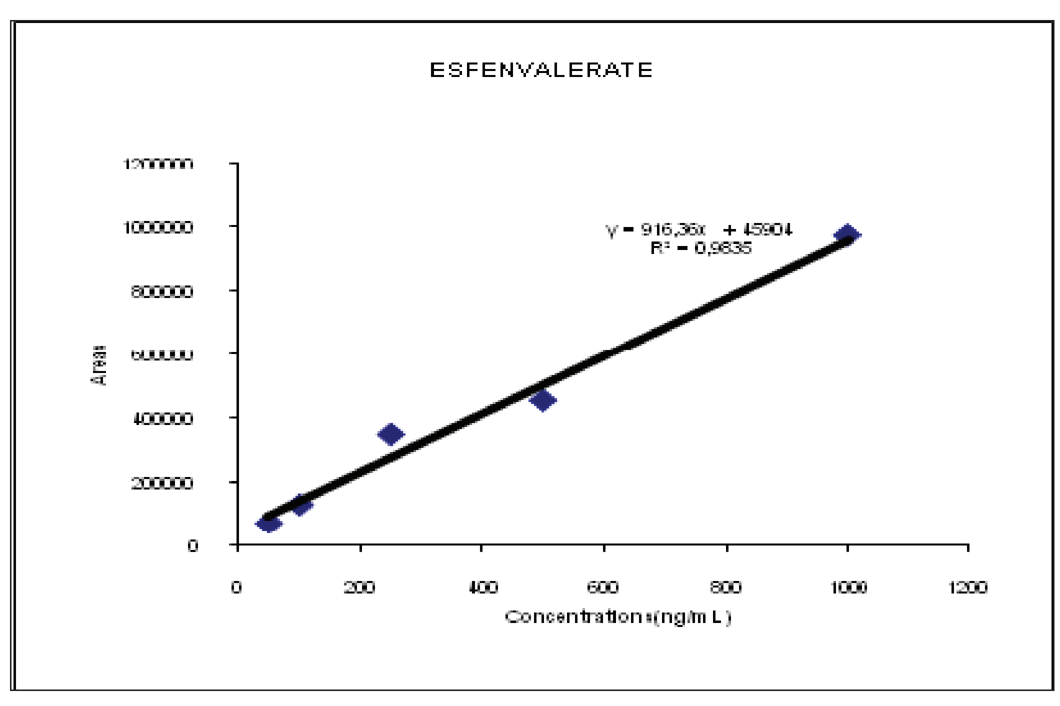
Figue 3.5 (continue)





Figue 3.5 (continue)





The calibration studies were carried out by using the standard pesticide solutions in concentration levels of 50, 100, 250, 500 and 1000 ng / mL. Rather high concentration range was selected since they present at high level in tobacco samples. Resulting calibration graphs can be seen in Table 3.2. As can be followed from the correlation coefficients, the calibration graphs are linear in the range studied (50 - 1000 ng / mL).

Table 3.2 The characteristics of the calibration graphs obtained with standard pesticide solutions

Name of Pesticide	Equation	R^2
Trifluarin	y = 3688.7x + 230003	R^2 = 0.9905
Fenchlorfos	y = 19245x + 100787	R^2 = 0.9886
Penconazole	y = 5149.7x + 107162	R^2 = 0.9999
α-Endosulfane	y = 13754x + 590293	R^2 = 0.9929
β-Endosulfane	y = 16844x + 790854	R^2 = 0.9942
Endosulfane Sulfate	y = 2439.8x + 15750	R^2 = 0.9995
Phosalone	y = 801.9x + 34383	R^2 = 0.9926
Cyfluthrin	y = 2041x – 3261.2	R^2 = 0.9997
Cypermethrin	y = 1886.3x + 32950	R^2 = 0.9812
Esfenvalerate	y = 916.36x + 45904	R^2 = 0.9835

3.2 Optimization studies with ASE

The studies were carried out with a blank tobacco samples which is known that does not contain any of these pesticides. Blank and/or spiked samples were put into the ASE cell together with a certain amount of florosil as given in the experimental section. Then, extracts were dried under nitrogen flow and diluted to 5 mL with acetone and analyzed with GC-ECD system.

The spiked samples were examined in eight runs according to Plackett-Burman design. The experimental parameters which are though to be important on the chromatograms are namely, heat time, temperature, static time, flush volume, purge time and static cycles. These factors and their levels are given in Table 2.2. The pressure was kept constant as 1500 psi. The Plackett-Burman Design model applied is given in Table 2.3.

Most important factors that affect the ASE recovery values were revealed as following. The treatments were considered to be in two halves with the factors at their lower levels in one half and the higher levels in the other. By means of this division, the responses obtained for these treatments were averaged and the average at the lower level substracted from that of higher level ($\bar{y}_{+} - \bar{y}_{-}$) . The effects of these parameters for each pesticide were calculated according to three parallel Plackett-Burman design studies and given in Table 3.3.

Table 3.3. The effective factors calculated for each pesticide.

Name of Pesticides	**Effective factors**
Trifluarin	F6,F2,F1,F4
Fenchlorphos	F6,F3,F4,F1
Penconazole	F1,F2,F5,F6
α-Endosulfane	F6,F3,F5,F4
β-Endosulfane	F6,F3,F4,F2
Endosulfane Sulfate	F6,F5,F7,F2
Phosalone	F6,F4,F3,F7
Cyfluthrin	F3,F5,F2,F6
Cypermethrin	F3,F7,F6,F4
Esfenvalerate	F6,F3,F5,F4

The most frequently encountered effects are F3, F4, F5 and F6.

k=4 # of factors

# of experiments = $2^k + 2k + 1 = 16 + 8 + 1 = 25$

# of replicates at center point = 5

Total # of experiments (N) = 25 + 5 =30

Factors and their levels were given in table 3.7.

Table 3.4. Factors and coded values for central composite design

Factor	-2.0 (α)	-1	0	1	2.0(α)
X1(Static Time, min)	3	6	9	12	15
X2(Flush Volume, %)	45	60	75	90	105
X3(Purge Time, sec)	80	120	160	200	240
X4(Static Cycles)	1	2	3	4	5

Static time is the time the cell is held with the solvent at the selected temperature and pressure.

Flush volume is the amount of new solvent to flush through the sample after the static step, expressed as a percent of the cell volume.

Purge time is the time in seconds that the nitrogen gas purges the cell to expel solvent from the sample and lines.

Static cycle consists of one static heating and one flush step. When more than one cycle is specified, the flush volume is divided among the cycles.

Experimentally found b values were given in table 3.5.

Table 3.5 Experimentally found $t_{experimental}$ values for b coefficients

	Trifluarin	Fenchlorfos	Penconazole	α-endosulfane	β-endosulfane	Endosulfane sulfate	Phosalone	Cyfluthrin	Cypermethrin	Esfenvalerate
b0	63.4	29.816667	67.466667	52.2	47.25	52.016667	51.616667	70.11667	78.45	43.95
b1	-1.5041667	-4.0375	0.75833333	-3.2791667	-3.3166667	-2.8208333	1.5083333	0.675	4.9083333	-9.1708333
b2	2.9625	-0.10416667	3.1333333	-0.7125	-2.80868E-14	-1.6541667	-0.80833333	2.558333	-3.1333333	8.4958333
b3	3.9875	-0.1625	1.2583333	2.3458333	1.2916667	0.2625	-0.59166667	1.425	0.9	2.2375
b4	1.1208333	-1.0208333	-2.8083333	-6.3041667	-6.875	-4.2541667	-2.1333333	4.741667	-2.9583333	-1.2375
b11	-1.5427083	1.0427083	-0.21041667	3.4822917	4.8583333	2.1864583	0.54583333	1.6625	-4.2125	8.7989583
b22	-2.6427083	1.6052083	6.2520833	1.3947917	1.2958333	1.5489583	-0.56666667	-0.9625	-5.675	5.4614583
b33	-1.6052083	2.6052083	6.7020833	1.3572917	1.5708333	0.44895833	9.6958333	-0.0875	-3.8	9.6739583
b44	-4.2177083	0.85520833	-1.4604167	1.9072917	2.3708333	3.6739583	2.5708333	3.6125	1.9875	0.83645833
b12	-3.06875	-1.69375	2.2125	-4.40625	-5.875	-5.03125	-2.75	-0.675	-3.625	3.08125
b13	1.23125	-2.70625	-0.25	-1.85625	-3.0875	1.29375	2.9625	-0.7	0.875	-8.74375
b14	-0.23125	2.33125	4.0375	3.91875	3.3375	4.81875	-0.075	8.15	-4.525	8.93125
b23	2.58125	-0.56875	-3.625	-1.96875	-1	-0.49375	-1.325	2.775	-1.65	3.86875
b24	2.56875	2.89375	0.2625	2.95625	1.925	0.75625	4.1875	3	2.45	-1.00625
b34	2.84375	4.63125	2.55	0.98125	-0.8375	-0.21875	6.325	3.025	-3.075	8.79375

The t test was performed for testing the significance of selected parameters and the results were given in table 3.6. The values were compared with t_{table} values (for d.f. 10 , t_{table} = 2,228139 , p = 0.05).

Table 3.6 Calculated $t_{experimental}$ values for b coefficients

	Trifluarin	**Fenchlorfos**	**Penconazole**	**α-endosulfane**	**β-endosulfane**	**Endosulfane sulfate**	**Phosalone**	**Cyfluthrin**	**Cypermethrin**	**Esfenvalerate**
b0	7.551577	1.49764509	10.10684354	4.019274077	3.873591716	4.355692439	5.637273	9.11116	9.121062723	4.701175146
b1	-0.35822	-0.40547317	0.227136121	-0.504824457	-0.543642791	-0.472271653	0.3293641	0.17537	1.141001755	-1.961354624
b2	0.705515	-0.01046112	0.938496492	-0.109688667	-4.60377E-15	-0.276945129	-0.17651	0.664675	-0.728381423	1.81699322
b3	0.949618	-0.01631935	0.376896192	0.361138707	0.211720186	0.04394847	-0.1291981	0.370226	0.209215943	0.478531321
b4	0.266925	-0.10251901	-0.84115244	-0.970520202	-1.126897733	-0.712244265	-0.4658409	1.231922	-0.687700546	-0.264662574
b11	-0.39269	0.11192678	-0.067363989	0.573011891	0.851178615	0.391270632	0.1273974	0.461674	-1.046679639	2.011408028
b22	-0.6727	0.17230686	2.0015775	0.229513291	0.227029626	0.277188864	-0.1322599	-0.26728	-1.410066932	1.248468364
b33	-0.4086	0.27964923	2.145643059	0.223342658	0.275209548	0.080341898	2.2630056	-0.0243	-0.944185787	2.211429663
b44	-1.07361	0.09180009	-0.467546107	0.313845283	0.415369321	0.657461423	0.600032	1.003186	0.493834014	0.191211157
b12	-0.59695	-0.13893988	0.541299166	-0.554082004	-0.786588681	-0.688047425	-0.4905007	-0.14325	-0.688316684	0.538272608
b13	0.239509	-0.22199619	-0.061163748	-0.23342178	-0.413377456	0.176926481	0.528403	-0.14855	0.166145406	-1.527471357
b14	-0.04498	0.19123459	0.987794522	0.492779314	0.446849315	0.658987037	-0.0133773	1.729569	-0.859209102	1.560226282
b23	0.502119	-0.04665509	-0.886874339	-0.247568555	-0.133887435	-0.067522667	-0.2363321	0.588902	-0.313302767	0.675843295
b24	0.499687	0.23737699	0.064221935	0.371745798	0.257733312	0.103420793	0.7468987	0.636651	0.465207138	-0.175784767
b34	0.553181	0.37990572	0.623870225	0.123391311	-0.112130727	-0.029915105	1.1281515	0.641956	-0.583882429	1.536206004

Model equation for this design is:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 + b_{123}x_1x_2x_3 + b_{124}x_1x_2x_4 + b_{134}x_1x_3x_4 + b_{234}x_2x_3x_4 + b_{1234}x_1x_2x_3x_4$$

According to selected four factor, the central composite design was constructed for thirty trials and this design was shown in table 3.7.

Table 3.7. Four Factor Central Composite Design

Run no	X0	X1	X2	X3	X4	X11	X22	X33	X44	X12	X13	X14	X23	X24	X34	X123	X124	X134	X234	X1234
1	1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	1	1	-1	-1	-1	-1	1
2	1	1	-1	-1	-1	1	1	1	1	-1	-1	-1	1	1	1	1	1	1	-1	-1
3	1	-1	1	-1	-1	1	1	1	1	-1	1	1	-1	-1	1	1	1	-1	1	-1
4	1	1	1	-1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	-1	1	1	1
5	1	-1	-1	1	-1	1	1	1	1	1	-1	1	-1	1	-1	1	-1	1	1	-1
6	1	1	-1	1	-1	1	1	1	1	-1	1	-1	-1	1	-1	-1	1	-1	1	1
7	1	-1	1	1	-1	1	1	1	1	-1	-1	1	1	-1	-1	-1	1	1	-1	1
8	1	1	1	1	-1	1	1	1	1	1	1	-1	1	-1	-1	1	-1	-1	-1	-1
9	1	-1	-1	-1	1	1	1	1	1	1	1	-1	1	-1	-1	-1	1	1	1	-1
10	1	1	-1	-1	1	1	1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	1
11	1	-1	1	-1	1	1	1	1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1
12	1	1	1	-1	1	1	1	1	1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1
13	1	-1	-1	1	1	1	1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	1
14	1	1	-1	1	1	1	1	1	1	-1	1	1	-1	-1	1	-1	-1	1	-1	-1
15	1	-1	1	1	1	1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	-1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	1	2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	1	-2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	1	0	2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
20	1	0	-2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
21	1	0	0	2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
22	1	0	0	-2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
23	1	0	0	0	2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
24	1	0	0	0	-2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
25	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.8 Recoveries of Pesticides

Run no	Trifluralin	Fenchlorfos	Penconazole	α-endosulfane	β-endosulfane	Endosulfane sulfate	Phosalone	Cyfluthrin	Cypermethrin	Esfenvalerate
1	25.0	29.5	70.0	59.4	43.8	66.3	64.4	60.9	60.7	76.6
2	58.9	78.2	73.2	82.7	76.6	81.4	87.5	83.0	101.6	53.9
3	76.1	93.6	93.6	94.9	97.2	88.8	99.2	101.5	61.9	96.5
4	36.9	15.6	90.1	50.2	59.2	55.0	43.7	63.8	51.6	97.3
5	62.2	89.9	105.0	104.3	101.1	89.6	74.8	97.4	71.8	100.5
6	43.3	8.7	57.9	60.9	64.1	66.6	64.8	56.2	94.3	33.6
7	44.1	13.3	63.0	60.8	56.9	70.0	43.0	70.8	57.1	88.4
8	56.5	58.8	95.9	77.5	66.3	66.2	73.5	66.8	80.6	52.9
9	51.4	64.6	59.8	61.3	64.2	74.8	56.5	73.5	62.2	46.0
10	42.7	16.7	72.7	50.8	63.9	60.2	49.3	80.9	55.3	78.2
11	49.3	15.0	76.2	47.3	42.7	54.5	61.7	61.0	56.1	42.8
12	39.8	67.5	83.4	77.3	66.8	70.5	65.1	79.2	65.8	49.6
13	39.6	19.1	63.0	39.9	25.4	36.9	68.5	53.6	58.7	65.6
14	44.2	66.7	94.1	89.0	70.8	92.7	72.1	75.1	71.6	45.5
15	78.0	92.9	85.9	96.9	94.1	76.7	83.7	86.9	49.7	98.8
16	76.1	13.0	84.8	42.9	45.5	51.1	70.8	109.4	38.2	98.5
17	58.8	11.9	61.2	38.5	34.7	29.4	62.0	74.8	74.7	47.4
18	63.2	14.0	69.9	61.1	68.4	56.3	31.4	71.1	56.2	104.6
19	52.0	15.5	90.9	37.3	32.6	39.3	36.7	63.1	69.6	82.4
20	61.2	14.9	91.9	45.6	42.0	41.3	47.8	61.8	49.6	42.9
21	68.7	22.8	93.1	43.3	43.7	37.9	73.8	71.4	70.8	82.2
22	52.8	15.6	93.3	39.3	33.1	33.9	92.8	60.5	63.4	76.8
23	52.5	14.1	50.9	27.0	23.3	39.9	47.8	104.4	103.0	55.4
24	48.1	10.3	70.2	60.0	59.9	57.7	61.8	57.1	77.5	32.9
25	50.4	11.5	66.2	33.5	42.8	32.3	32.9	77.5	69.1	42.9
26	53.6	10.0	70.8	60.1	58.3	64.7	44.4	64.6	97.6	26.7
27	77.3	13.4	68.8	53.0	42.4	50.5	41.8	56.9	86.3	38.6
28	57.2	65.3	74.8	76.3	62.6	75.8	82.6	81.1	59.7	62.6
29	49.8	62.0	74.9	64.9	51.8	66.4	60.3	67.1	96.0	22.9
30	92.1	16.7	49.3	25.4	25.6	22.4	47.7	73.5	62.0	70.0

Table 3.9 Recoveries of Three Parallel Studies at Optimum Point

Pesticide	% Recovery 1	% Recovery 2	% Recovery 3
Trifluralin	75.9	73.6	70.7
fenchlorfos	90.3	85.3	81.7
Penconazole	81.5	73.9	75.9
alpha endosulfane	93.7	85.3	88.5
Beta endosulfane	82.8	76.3	77.6
Endosulfan sulfate	75.6	74.2	74.9
Phosalone	71.6	72.2	74.4
Cyfluthrin	81.8	79.5	80.8
Cypermethrin	47.2	46.0	48.0
Esfenvalerate	92.2	88.9	91.0

4. CONCLUSION

The analysis of pesticides in tobacco samples is an important task for our country being a major tobacco producer. However, the tobacco is a very difficult matrix and therefore, accelerated extraction technique was chosen as it is more effective at elevated temperatures.

Considering the steps of ASE, the parameters affecting the extraction efficiency and therefore the recovery levels can be figured out easily namely; temperature, pressure, type of solvent, preheat time, heat time, static time, flush volume, purge time and number of static cycles. However, the ASE instrument used did not have any control for the pressure therefore; it was set at 1500 psi and thought to be constant throughout the study. Type of solvent is another parameter to be optimized. The other parameters were changed according to Plackett-Burman design and the results were evaluated by treating corresponding recovery values.

Most important factors that affect the ASE recovery values were revealed by calculating the differences of the average at the lower level subtracted from that of higher level. The most frequently encountered effects were found as static time, flush volume, purge time and number of static cycles by three parallel Plackett-Burman design studies.

After determination of important parameters the central composite design was constructed with thirty trials. It was seen that optimum conditions for selected parameters are the points that all the parameters are at level one. This means that if static time is twelve minute, flush volume is ninety, purge time is two hundred second , static cycle is four we can get the good results. Maximum recoveries for each pesticide taken from the thirty trials were given in table 4.1.

Table 4.1. Maximum Recoveries of Pesticides

Pesticide	# of Experiment	% Recovery
Trifluarin	30	92.1
Fenchlorfos	3	93.6
Penconazole	5	105.0
α-Endosulfane	5	104.3
β-Endosulfane	5	101.1
Endosulfane Sulfate	14	92.7
Phosalone	3	99.2
Cyfluthrin	16	109.4
Cypermethrin	23	103
Esfenvalerate	18	104.6

The detected optimum conditions are only compatible with cyflutrin. The detected optimum conditions are at the sixteenth trial. If sixteenth trial is chosen the recoveries of trifluarin, penconazole, phosalone, cyfluthrin and esfenvalerate will not have the maximum values except cyflutrin but will be in the acceptable range in validation studies (70 % - 110 %). This shows that another design is necessary to construct. If the recovery range is restricted as 70% - 110 % some of the pesticide recoveries can decrease however can be still in acceptable range by the way some of them can increase also. By this way we can get better results for more than five pesticides simultaneously. It was also seen that if fifteenth is chosen it will give us good results except cypermethrin and three parallel studies were carried out at the optimum conditions and their results were also given in results and discussion part. There is something interesting, although three parallel studies at the optimum conditions give good results , the reproducibility of the studies at the medium part of the thirty trials were seen that very weak. This is the point which can be investigated in another studies.

This study shows that if ten pesticides that were indicated in the aim of the thesis part will be analyzed with ASE followed by GC-ECD system it is possible to analyze them successfully except cypermethrin, it can be very useful to investigate the reproducibility of the method that will be able to give an idea about the confidence of method.

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