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TRACE LEVEL SAMPLE PREPARATION TECHNIQUES FOR SELECTIVE EXTRACTION OF PESTICIDE RESIDUES IN ENVIRONMENTAL AND FOOD SAMPLES, AND THEIR QUANTITATIVE REMOVAL FROM CONTAMINATED WATERS UTILIZING *TYPHA LATIFOLIA* PLANT PARTS

BY

TESHOME TOLCHA DADI

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Trace Level Sample Preparation Techniques for Selective Extraction of Pesticide Residues in Environmental and Food Samples, and their Quantitative Removal from Contaminated Waters Utilizing *Typha Latifolia* Plant Parts

By

Teshome Tolcha Dadi

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ABSTRACT

The objective of this thesis work is to develop various miniaturized and green sample preparation techniques for the determination of pesticide residues in matrices of different origin and the quantitative removal of pesticides from contaminated water. In this research work, an efficient and sensitive high desnsity based dispersive liquid-liquid microextraction (HD-DLLME) method coupled with gas chromatographic mass spectrometric detection (GC-MS) for the simultaneous determination of pesticide residues in water and sugarcane juice has been developed and validated. The effects of type and volume of extraction solvent, type and volume of disperser solvent, centrifuging speed and time, extraction time, sample pH and absence or presence of electrolytes was examined. The performance of the analytical technique was evaluated by extracting spiked distilled and de-ionized water at 2.5 and 5 µg/L. For 5 mL sample of water and sugarcane juice the method offers good linearity ($R^2 \geq 0.991$), repeatability (0.73–5.3%) and reproducibility (1.1–8.7%). The limit of detections (LODs) ranges from 0.005 to 0.01 µg/L. This method was successfully applied for simultaneous quantitative and qualitative determination of target analytes in water and sugarcane samples showing excellent relative recoveries (80.4–114%).

Modified salting-out-assisted liquid-liquid extraction (SALLE) followed by preconcentration using low density based dispersive liquid-liquid microextraction (LD-DLLME) for the qualitative and quantitative determination of atrazine, diazinon, ametryn, terbutryn, chlorpyrifos, dimetham-etryn, 4,4'-dichlorodiphenyldichloroethylene, 4,4'-dichlorodiphenyldichloroethane and 4,4'-dichl oro-diphenyltrichloro-ethane residues in sugar and soil samples was also developed. Various parameters affecting the extraction process such as type and volume of organic solvent, type and amount of salt, extraction time, pH of the sample solution, centrifugation speed and time were optimized. Under optimum conditions, the method offers good linear range with regression coefficients of 0.992–0.999, low limits of detection of 0.01–0.3 µg/kg. The precision (intra- and inter-day) of the method expressed as relative standard deviations (RSD) at 12.5 and 50 µg/kg were below 10% in both matrices. The recoveries obtained from spiked sugar and soil samples at 12.5 and 50 µg/kg were ranged from 79.0 to 111%. The soil sample was contaminated by atrazine and ametryn at concentrations of 0.29 and 0.23 µg/kg respectively.

A high density supercritical CO_2 (sc-CO_2) extraction method has also been developed for extraction of atrazine, 2,4´-DDD, 4,4´-DDT and endrin from onion samples. The extraction volume of sc-CO_2 and temperature were also considered as potential parameters and optimized. It was observed that increasing density of sc-CO_2 increases extraction recovery of all target analytes and the effect is significant for endrin and 2,4´-DDD. The optimum conditions were found to be 29 mL, 0.9 g /mL, 53 ^oC for volume of sc–CO_2, density sc-CO_2 and temperature, respectively. Matrix matched calibration curves showed satisfactory linearity ($R^2 \geq 0.994$). LODs ranging from 0.2 to 2 μg/kg were achieved and precision studies showed RSDs lower than 11% and recoveries in the range of 80.3 to 103%. The method was successfully applied to onion sample and none of the target analytes were found in the sample investigated. Therefore, the developed method can be used for trace analysis of the pesticide residues studied and other pollutants having related physical and chemical properties.

Similarly, multivariate optimization of combined static and dynamic mode sc-CO_2 extraction method for trace analysis of atrazine, diazinon, chlorthalonil and deltametryn residues in honey has been systematically optimized and validated. The parametrs that affect the extractability of relatively polar and non-polar pesticides including static time, temperature and pressure were optimized. The optimum extraction conditions were 11.5 min contact time in static mode, 252 bar and 70 ^oC. The proposed method has good linearity (≥ 0.998), and LODs ranging from 5.0 to 9.0 μg/kg. The precision study at two concentration levels, 250 and 1000 μg/kg was found to be in the range of 2.3–4.2% for intra-day (n = 3) and 2.1–8.0% for inter-day precisions (n = 3). The proposed method was successfully applied for selective extraction of the target compounds from complex matrices and the results obtained clearly indiacated there is no significant matrix effect on quantitative analysis of the target analytes.

Removal of atrazine, diazinon, chlorothalonil, ametryn, malathion, chlorpyrifos and dimethametryn from aqueous solutions was also investigated utilizing *Typha latifolia*. The characteristic surface chemistry of stem, leaf and flower of the *Typha latifolia* plant were analyzed by FT-IR analysis. Different experimental parameters including pH, shaking speed, contact time, adsorbent dose and initial pesticide concentration were studied. Equilibrium and kinetic models for pesticide sorption were studied by considering the effects of concentration and contact time at

the optimum conditions for each of the *Typha latifolia* plant parts. Results of the sorption equilibria were found to fit to the Langmuir isotherm model than the Freundlich adsorption model indicating monolayer homogeneous surface conditions for most analytes in all adsorbents. Atrazine, malathion and chloropyrifos for stem and atrazine, ametryn, chlorothalonil and dimethametryn for leaf powder as adsorbent fit to better Freundlich adsorption model, indicating monolayer sorption with a heterogeneous energetic distribution of active sites. On the other hand, kinetics of all pesticides, under study, sorption on the *Typa latifolia* was well defined by the pseudo-second order model. The results obtained showed that the use of this plant can be considered as one of the most promising, easily accessible and low cost adsorbent.

DEDICATION

To

My Mother Abebech Eshete and My Father Tolcha Dadi.

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ABBREVIATIONS

Abbreviation	Description
DDT	Dichlorodiphenyltrichloroethane
DLLME	Dispersive Liquid-Liquid Microextraction
DLLME-GC-MS	Dispersive Liquid-Liquid Microextraction Gas Chromatography Mass Spectrometry
DLLME-SFO	Dispersive Liquid-Liquid Microextraction Based on Solidification of Floating Organic Droplet
DMSPE-HRMS	Dispersive Micro-Solid Phase Extraction Combined with High Resolution Mass Spectrometry
EF	Enrichment Factor
EPA	Environmental Protection Authority
EU	European Union
FTIR	Fourier Transform Infrared Spectrometry
GC	Gas Chromatography
GC–ECD	Gas Chromatography Electron-Capture Detection
GC-MS	Gas Chromatography Mass Spectrometry
HD-DLLME	High Density Solvent Based Dispersive Liquid-Liquid Microextraction
HD-DLLME-GC-MS	High Density Solvent Based Dispersive Liquid-Liquid Microextraction Gas Chromatography Mass Spectrometry
HF-LPME	Hollow Fiber Based Liquid Phase Microextraction
HPLC	High Performance Liquid Chromatography
IPA-HPLC-DAD	Ion Pair Assisted High Performance Liquid Chromatography with Diode Array Detector
IPA-LLE-HPLC-DAD	Ion-Pair Assisted Liquid-Liquid Extraction Combined with High Performance Liquid Chromatography and Diode Array Detector
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection

MRL	Maximum Residue Limit
MS	Mass Spectrometry
MWCNTs	Multiwalled Carbon Nanotubes
OCP	Organochlorine Pesticides
ONP	Organonitrogen Pesticides
OPP	Organophosphorous Pesticides
MAE	Microwave Assisted Extraction
PLE	Pressurised Liquid Extraction
POPs	Persistent Organic Pollutants
4,4'-DDD	4,4'-Dichlorodiphenyldichloroethane
2,4'-DDD	2,4'-Dichlorodiphenyldichloroethane
4,4'-DDE	4,4'-Dichlorodiphenyldichloroethylene
4,4'-DDT	4,4'-Dichlorodiphenyltrichloroethane
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
R	Recovery
RR	Relative Recovery
RSD	Relative Standard Deviation
S	Solubility
SALLE	Salting-Out Liqiud-Liquid Extraction
sc-CO_2	Supercritical Carbon Dioxide
SDME	Single Drop Microextraction
SFE	Supercritical Fluid Extraction
SIM	Selective Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction

1 INTRODUCTION

Industrial and agricultural endeavors are intimately associated with the extensive use of a wide array of chemicals. Historically, chemical wastes generated through industrial processes were disposed of through the flagrant release into the environment. Gases quickly dispersed into the atmosphere; liquids were diluted into receiving waters and efficiently transported away from the site of generation. Similarly, pesticides and other agricultural chemicals which revolutionized farm and forest productivity will pollute the environment (Namieśnik and Wardencki 2002).

Pesticides are chemical substances or biological agents such as bacteria or viruses used to control, kill, or repel pests in order to increase agricultural production. The active portion of pesticide is generally formulated by the manufacturer as emulsifiable concentrates or in solid particles. Many commercial formulations have to be diluted with water before use (Pandey at al. 2011). Some of the pesticides are persistent and some degrade in the environment giving various degrees of toxicity and distribution in similar manner as their parent compounds. Thus, they can adversely affect non-target organisms and ecosystem as a whole (Battaglin and Fairchild 2002).

The application of these compounds and their degradation products has the possibility of contaminating ground and surface waters, soil (Tolcha et al. 2013) and sediments (Lee et al. 2001) posing a consequent potential danger to the environment and public health. Moreover, significant solubility of the compounds in water may allow them to be transported to the environmental water systems, such as surface water, ground, lake and river waters causing toxicity to soils, aquatic lives and human beings as they could eventually enter the food chain (Lee et al. 2001; Tolcha et al. 2013).

Most Ethiopians are engaged in agriculture and use pesticides to protect food for increased crop production by controlling pests. Others use pesticides occupationally for public health programs and in commercial applications, while many others use pesticides for lawn and garden applications and in and around the home. However, when transported outside the application area, pesticides are perceived as environmental pollutants and are therefore subject to regulation and monitoring (Anyusheva et al. 2012; Nagaraju and Huang 2007).

In developing countries lack of awareness, trained man power and disposal facility of pesticides together with improper management has resulted in adverse impacts on human health and the environment. The absence of effective controlling mechanisms for the import of pesticides and inappropriate use in Ethiopia inevitably causes devastating impacts on the health of agricultural workers and ecosystems. The negative impacts of pesticides in Ethiopia are aggravated by the limited knowledge among users on the toxicological and chemical properties of these substances (Amera and Abate 2008).

Although applications of some persistent pesticides like DDT and *s*-triazine have been banned in developed countries, developing countries like Ethiopia are still using them for agricultural and health medicinal purposes. A study carried in various agro industries in Ethiopia ensures that pollution due to pesticide residue has been identified, comprising previously used persistent organic pollutants (POPs) and currently used insecticides (Beyene et al. 2013). The investigation made by different researchers showed the presence of contaminant pesticide residues in different samples collected from various localities of Ethiopia. The results revealed the presence of 4,4´-DDT in khat, diazinon in khat and wheat (Daba et al. 2011), 4,4´-DDT and its metabolites, 4,4-DDE and 4,4-DDD in human and cow milk (Gebremichael et al. 2013) and 4,4-DDTs in fish (Beyene et al. 2013).

Due to their occurrence at trace levels and the complexity of environmental and food samples, the analysis of these pesticides requires selective and efficient sample preparation methods that can extract and preconcentrate them simultaneously prior to their instrumental determination (Cheng et al. 2011; Mnif et al. 2011, Saraji at al. 2014; Tobiszewski et al. 2009; Yang et al. 2012). Traditionally, the determination of pesticide residues at trace levels relies on the use of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) (Cheng et al. 2011; Mnif et al. 2011). However, LLE has some drawbacks, such as a difficulty of automation, high consumption of solvents and on-line connection to analytical instruments and an often tiresome formation of emulsions (Cheng et al. 2011). Conversely, SPE requires column conditioning and elution with organic solvents (Yang et al. 2012). Microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) has been also utilized for extraction of pesticide residues from complex matrices such as semi-solid and solid matrices (Chen et al. 2008).

Recently, much attention has been paid to the development of green activities such as miniaturizing and automating extraction techniques which could greatly reduce the consumption of organic solvents. Solvent microextraction techniques which are the miniaturized modes of traditional liquid-liquid extraction (LLE) technique are intensively exploited to extract pesticide residues from liquid and semi-liquid samples (Saraji at al. 2014; Tobiszewski et al. 2009).

On the other hand, there is a need to treat water which is contaminated by pesticides through agricultural, domestic and industrial activities (Bakouri et al. 2009). Adsorption technology is currently being used extensively for the removal of such pollutants from wastewaters. Removal of these contaminants requires cost effective technologies and a variety of techniques have been developed in the past decades in dealing with wastewater treatment. Currently, adsorption is believed to be a simple and effective technique for water and wastewater treatment and the success of the technique largely depends on the development of an efficient adsorbent (Bakouri et al. 2009; Gupta et al. 2011).

Biosorption is one of the effective alternative methods for the removal of pesticides in contaminated water samples. Advanced pesticide removal methods are usually needed to meet environmental quality requirements and improve the ecological system. These include combinations of biological, chemical and physical processes. Adsorption has evolved into one of the most effective physical processes for pesticide removal because the technique uses equipment that is readily available, easy to use and not energy intensive and cost effective. Various adsorbents are used for the removal of pesticides from water samples. However, the removal of multiclass pesticide residues is a challenging experimental task due to their different chemical nature.

Biosorbents are promising candidates as they can be easily adopted to low cost applications (Oboh et al. 2009). The overall process comprises the transfer of solute components in the fluid phase onto the surface or the bulk of solid adsorbent materials. The major advantages of biosorption technology include the use of locally available cheap materials, high removal efficiency, minimization of chemical and/or biological sludge regeneration (Owlad et al. 2009).

1.1 Research gap

Despite substantial technological advances in analytical instruments, sample preparation to clean up the matrices, isolate and/or concentrate the analytes of interest while rendering them in to form that is compatible with analytical systems is unavoidable in analysis (Liu et al. 2010). The development of sensitive, selective and reproducible analytical methods and techniques have always been a prerequisite for the achievement of high quality results in enforcement and monitoring programs (Nguyen et al. 2008; Merdassa et al. 2013).

Traditional sample preparation techniques such as solid-liquid extraction methods like Soxhlet extraction (Lin et al. 2011; Wang et al. 2007; Barriada-Pereira et al 2003), liquid-liquid extraction and solid-phase extraction (SPE) (Lin et al. 2011; Huo et al. 2016; Zhao et al. 2017) are still widely used for extraction of trace pesticide residues in environmental and food matrices. However, these methods are expensive, labor intensive, time consuming and requires the use of large volumes of toxic organic solvents (Merdassa et al. 2013). Recently researchers are emphasizing the development of efficient, economical, miniaturized and automated extraction techniques that could greatly reduce consumption of organic solvents. As a result, green extraction and/or miniaturized sample preparation methods are appealing in trace analysis (Hrouzková et al. 2017).

Modern sample preparation techniques including hollow-fiber liquid-phase microextraction (Gure et al. 2013), single drop microextraction (Amvrazi and Tsiropoulos 2009; Ye et al. 2007), different modes of dispersive liquid-liquid microextraction (He et al. 2010; Hrouzková et al. 2017; Matsadiq et al. 2011; Nagaraju and Huang 2007; Tolcha et al. 2013), quick, easy, cheap, effective, rugged and safe extraction (Kolberg et al. 2011), microwave-assisted extraction (Merdassa et al. 2014; Merdassa et al. 2015), salting-out assisted liquid-liquid extraction (Alemayehu et al. 2017; Gure et al. 2014; Hajkova et al. 2016; Liu et al. 2010) or solid phase microextraction (Júnior and Rè-Poppi 2007; Qiua and Cai 2010) has been developed for pesticides analysis in environmental and food samples.

Solvent-based microextraction is typically miniaturized LLE and has been extensively explored in various formats including single drop microextraction (SDME) (Amvrazi et al. 2009), hollow fiber liquid phase microextraction (Sharifi et al. 2016) and dispersive liquid-liquid microextraction

(DLLME) (Merkle et al. 2015; Wang et al. 2016). Since 2006, DLLME has been employed as a modern sample preparation procedure and thus extensively used for extraction and preconcentration of pesticides in various environmental matrices, including contaminated waters (Bedassa et al. 2015), peach juices, pulps and peels (Matsadiq et al. 2011), beverages (Çabuk and Köktürk 2013), soil (Yang et al. 2012) etc. The volume ratio of the organic solvent to the sample solution in these microextraction techniques is much less, which guarantees high enrichment factors for the extraction (Tolcha et al. 2013). Due to the small volume of extraction solvent used in DLLME, it is inconvenient as sample preparation method for solid samples.

On the other hand, the enrichment factor of SALLE method is very small compared to solvent based microextraction and thus the purpose of the extraction is mainly to clean up the matrices. It has been applied for pesticide analysis in honey, river water and human urine (Liu et al. 2010), lake water (Alemayehu et al. 2017), sea water, waste water and urine (Niu et al. 2017), banana juice (Gure et al. 2014), etc. Therefore, there is a demand to couple SALLE techniques with solvent based microextraction to enhance the enrichment factor and preconcentrate the trace level pesticide residues prior to instrumental analysis.

The use of supercritical carbon dioxide (sc-CO_2) as a green extraction solvent is also appealing in trace analysis. In general, in sc-CO_2 extraction increasing pressure has the same effect as increasing density of sc-CO_2 at constant temperature. But in multivariate optimization experiment, density of the sc-CO_2 is not only pressure dependent because temperature will not be kept constant. The effect of density of sc-CO_2 on extraction efficiency of pestide residues has not been reported so far. On the other hand, sc-CO_2 as an extraction solvent in multiresidues pesticide analysis is limited because sc-CO_2 is considered as a nonpolar solvent with a liquid solubility equal to that of hexane. However, for quantitative extraction of moderately polar and non-polar pesticides, some co-solvents such as methanol or acetonitrile have to be applied. To the best of our knowledge, the effect of co-solvent on extraction of pesticide residues from honey utilizing multivariate experimental design has not been studied. Therefore, the development of fast, selective, economical and automated extraction techniques that could greatly reduce consumption of organic co-solvents and environmentally green methods is of great demand.

Moreover, a variety of techniques have been developed in the past decades in dealing with treatment of water contaminated by pesticides including multi-walled carbon nanotubes (Dehghani et al. 2017), activated carbon (Adams et al. 1996; Hameed et al. 2009), chest nut (Memon et al. 2007), straw (Akhtar et al. 2007), tea waste (Gangadhar et al. 2016), lignocellulosic substrate from agroindustry (Boudesocque et al. 2008), baggasse fly ash (Gupta et al. 2002), coal fly ash (Singh 2009), herbal leaves powder (Chattoraj et al. 2016) and sunflower seed shells, rice husk, composted sewage sludge and agricultural soil (Rojas et al. 2015) and Neem bark dust (Yadamari et al. 2011).

Even though, a number of treatment methods have been carried out in recent years regarding the adsorption of various contaminants by using natural adsorbents, there is still a significant gap in investigation of the adsorption capabilities of *Typha latifolia* as adsorbents for the simultaneous removal of multiclass pesticides from contaminated waters. *Typha latifolia* plants, commonly known as cattails, were grown in a mixture of mature sewage sludge compost, commercial compost and perlite (Tamire et al. 2013; Manios et al. 2003). The wetland plant, *Typha latifolia*, has not been studied as a low cost adsorbent for wastewater treatment without requiring high technological expertise.

Therefore, the focus of the present study was to fill the above research gaps. In this regard, some appropriate miniaturized/green sample preparation methods for selective extraction, preconcentration and quantitative determination of trace levels of selected pesticide residues in different sample matrices were developed. In addition, the adsorption capacity of *Typha latifolia* plant parts including flower, leave and stem for the removal of multiclass pesticide residues from synthetic wastewater samples was investigated.

1.2 Objectives of the research

1.2.1 General objective

The general objective of this research is to develop different analytical sample preparation techniques which are simple, fast, miniaturized and environmentally friendly for trace analysis of pesticide residues in water, soil and food matrices and for removal techniques of pesticides residues from wastewater by locally available biosorbents.

1.2.2 Specific objectives

The specific objectives of this research are:

a) To develop a miniaturized sample preparation technique based on HD-DLLME-GC-MS for selective extraction, preconcentration and quantitative determinion of trace level pesticide residues in water and sugarcane juice samples.
b) To optimize SALLE-LD-DLLME-GC-MS for trace analysis of pesticide residues in soil and sugar samples.
c) To develop high density based sc-CO_2 extraction method coupled with GC-MS for trace enrichment of of selected pesticide residues in onion.
d) To develop combined static and dynamic mode sc-CO_2 coupled with HPLC-DAD for analysis of realatively polar and non polar pesticide residues in honey.
e) To investigate the the effect of different experimental parametrs on the removal efficiency of locally available adsorbent, *Typha latifolia*, for selected pesticides residues from contaminated waters.

2 LITERATURE REVIEW

The environment (atmosphere, hydrosphere and lithosphere) contains complex chemicals and biological systems that can be transferred from a particular environmental compartment to another by natural processes and/or human activities. For instance, different water bodies contain numerous compounds including organic and inorganic pollutants, minerals, salts, etc. originating from industrial wastes, agrochemical processes, municipal discharges and natural processes such as decay of living matters (Ahmed at al. 2010; Battaglin and Fairchild 2002; Lin et al. 2011; Pandey et al. 2010).

The use of chemical pesticides for different purposes such as forestry management, rail way, protection against infection with parasites transmitted to humans by insects, against insects and weeds in agriculture is very common all over the world. Pesticides are chemicals used to control pest (insects, weeds, mammals and microbes) infestation to the crops (Battaglin and Fairchild 2002; Lin et al. 2011; Pandey et al. 2010).

Pesticides comprise widely varying classes of compounds with very different chemical and physical properties. Some are persistent and some degrade in the environment giving various degrees of toxicity and distribution in a similar manner as their parent compounds. Thus, they can adversely affect non-target organisms and ecosystems as a whole (Battaglin and Fairchild 2002). Pesticide use in agriculture has progressively increased after World War II leading to increased world food production. The first synthetic organic pesticides were organochlorine compounds, such as dichlorodiphenyltrichloroethane (DDT), whose commercial production began in 1943 (Ahmed et al. 2010). The increasing world population demands a continually growing supply of food and food products. Because of the relative lack of new areas suitable for agriculture, the performance of the existing agricultural areas has to be substantially enhanced. Pesticides such as insecticides, molluscicides, nematicides, rodenticides, avicides, piscicides, herbicides, plant growth regulators, defoliants, fungicides, algaecides, etc. have been extensively used in agrochemical practices (Saraji and Tansazan 2009).

Large fractions of the pesticides used in agricultural areas moves with surface run-off into streams, rivers and lakes leaching into the ground water systems or volatilizing to the atmosphere (Ghosh

and Philip 2016). As a result, residues of such compounds can be main sources of environmental pollution; they may be found in the soil on which the crop was grown, may also appear in the atmosphere, in run-off water following heavy rain, irrigation, in ground water or in surface water and consequently, they can directly or indirectly pollute food and food products and biological systems. Moreover, their persistence had been a serious problem, especially in surface and ground water systems (Zhou et al. 2009).

2.1 Classification of pesticides

Active ingredients of pesticides represent a very diverse array of chemical structures including many biological agents. Many pesticide structures are very complex and cannot be categorized simply. Therefore, classification systems in use have evolved to accommodate the increasing diversity of chemical and biological agents in pest control or management (Delaplane 1996, Stoytcheva 2011). Pesticides are commonly classified based on the chemical composition, target pest species and mode of action.

2.1.1 Pesticide classification based on chemical composition

Pesticides are classified by their chemical class into organochlorines, organophosphates, organonitrogen and pyrethroids (Garrido et al. 2010; Nerin et al. 2002; Zhou et al. 2009).

2.1.1.1 Organochlorine pesticides

Organochlorine pesticides (OCPs), effective against a variety of insects, have been extensively used around the world. Their low volatility, combined with their extreme stability and probable indiscriminate use in the past, has led to their high persistence both in the environment after application and in organisms after exposure. Because of their lipophilic nature they concentrate in fatty tissues and bioaccumulate in the food chain. So, these compounds are found at higher concentrations in fatty foods and the exposure to OCPs can also occur via low level food contamination (Zhou et al. 2009). Representative compounds in this group include DDT, methoxychlor, dieldrin, chlordane, toxaphene, mirex, kepone, lindane, and benzene hexachloride.

During the last decades much attention has been given to this group of substances at the international level after it became apparent that they are transported through the environment and critical concentrations have been reached in some areas even in places where they have never been produced or used. Several countries banned the uses of OCPs during the 1970s and 1980s, although many of them continue to be used by other countries (Nerin et al. 2002).

2.1.1.2 Organophosphate and pyrethroids

Organophosphates are phosphoric acid esters or thiophosphoric acid esters. When developed in the 1930s and 1940s, their original compounds were highly toxic to mammals. Organophosphates manufactured since then are less toxic to mammals but toxic to target organisms, such as insects (Ortiz-Hernández and Sánchez-Salinas 2010).

Organophosphorus pesticides (OPPs), less persistent than OCPs, are frequently the preferred choice of treatment because they provide effective, safe and cost effective control of a wide range of pests. They can be absorbed by all routes, including inhalation, ingestion and dermal absorption. The awareness that OPPs may also concentrate along the food chain has led to the establishment of low maximum residue limits in meat, as set by the European Union (EU). Consequently, this makes necessary the control of this type of compounds in fatty matrices. OPPs are known of inducing or aggravating certain health problems in humans such as cancer, interference with immune systems and the disruption of hormonal functions (Garrido et al. 2010; Russo et al. 2002). They are also frequently used as household, garden and farm insecticides. Chlorpyrifos, parathion, diazinon, famphur, phorate, terbufos, and malathion are examples of OPPs.

Pyrethroids are synthetic derivatives obtained by modifying special chemical structures to achieve better biological performance and environmental stability compared with natural pyrethrins (Zhang et al. 2009). The pyrethroid class of insecticides was derived from natural compounds (the pyrethrins) isolated from the *Chrysanthemum* genus of plants. Although natural pyrethrins do have insecticidal activity, they also are inherently unstable when exposed to light. Therefore, the pyrethrin structure was modified to produce more stable compounds that retained the desirable insecticidal and toxicologic properties (Shafer 2005).

Over the last decades, traditional organophosphate, organonitrogen and organochlorine pesticides have been increasingly replaced by synthetic pyrethroid pesticides; this pesticide family has been used worldwide for the control of agricultural pests because of their relatively low mammalian toxicity, selective insecticide activity and low environmental persistence. They behave very similarly to natural pyrethrins, which are derived from chrysanthemum flowers and are extremely toxic to fish, aquatic arthropods and honeybees, even at low concentrations. However, repeated exposure increases the risk of anaphylaxis and allergic reaction at very low concentrations and should be monitored (Chowdhury et al. 2012; Rao et al. 2002; Zhang et al. 2009). Some examples of this class of pesticides are acetamiprid, lamda-cyhalothrin, imidacloprid and deltametrin.

2.1.1.3 Organonitrogen pesticides

Organonitrogen pesticides (ONP) is an umbrella term covering a large number of different compounds. In practice, one uses the names of the various chemical groups in a particular category of plant-protection products. In the literature, ONP is taken to mean carbamates and triazines and their derivatives (Berg et al. 2002; Chowdhury et al. 2012). Carbamate insecticides, used to kill or control insects, are made from carbamic acid. There are many forms of carbamates, each different in the way they work and in their poisonous effects. Carbamates break down in the environment within weeks or months (Chowdhury et al. 2012).

Carbamates are used as sprays or baits to kill insects by affecting their brains and nervous systems. They are used on crops and in the home to kill cockroaches, ants, fleas, crickets, aphids, scale, whitefly, lace bugs and mealy bugs. Some carbamates are used to control mosquitoes. Some carbamates have been found in groundwater at levels high enough to cause concern (Ortiz-Hernández and Sánchez-Salinas 2010).

Carbamate exposure can cause headaches, dizziness or weakness. It can make you feel like you will throw up. It can also cause shaking, stomach cramps, diarrhea and sweating. Skin exposure to carbamates causes a minor rash. Longterm exposure can result in loss of appetite, weakness, weight loss and a general feeling of sickness. There is not enough information about carbamates to know if they cause cancer in humans (Chowdhury et al. 2012). Included in this group are

aldicarb, carbofuran, carbaryl, ethienocarb, fenobucarb, oxamyl, and methomyl. These insecticides kill insects by reversibly inactivating the enzyme acetylcholinesterase (Ortiz-Hernández and Sánchez-Salinas 2010).

Triazine herbicides are six membered aromatic compounds used in weed control. They are heterocyclic ring with three nitrogen atoms replacing carbon-hydrogen units in the benzene ring structure. The word triazine is derived from two words, tri-means three and azine indicates a nitrogen-containing ring. The three isomers; 1, 2, 3-triazine, 1, 2, 4-triazine, and 1, 3, 5-triazine (also known as *s*-triazines or symmetrical triazines) are named based on which of the carbon-hydrogen units on the benzene ring positions have been replaced by nitrogen. Although 1, 3, 5-triazines are the most common and one of the oldest known classes of organic molecules, synthetic methods for the preparation of the analogs containing different substituent at each carbon are limited (Berg et al. 2002).

Triazine herbicides are somewhat persistent in water and mobile in soil. The physicochemical properties of triazines make them especially susceptible to leaching into ground water and runoff from the site of application to surface waters. The high water solubility of triazines has resulted in the contamination of surface and ground waters (Wang et al. 2010). Toxicity refers to a product's ability to cause injury or illness to living organisms. Triazine herbicides are generally of low acute toxicity for birds and mammals, although certain species show unexpected vulnerability for some of them.

A pesticide's acute toxicity is the basis for assigning its toxicity category. Acute toxicity is based on a single, short-term exposure by one of three routes-swallowing (ingestion), breathing (inhalation), or through the skin (dermal). Acute toxicity is usually expressed as LD_{50} (lethal dose 50). This is the amount of the product lethal by ingestion to 50 percent of a population of test animals (usually rats) under laboratory conditions. LD_{50} values are expressed in milligrams of pesticide per kilogram of body weight (mg/kg). The larger the LD50 value, the less toxic the chemical (Baggiani et al. 2007; Regan et al. 1996; Jackson and Finley 2011; Yu 2005).

These herbicides are inhibitors of photosynthesis and include both the asymmetrical and symmetrical triazines. Examples of symmetrical triazines are chloro-*s*-triazines (simazine,

atrazine, propazine and cyanazine); the thiomethyl-*s*-triazines (ametryn, prometryn, terbutryn) and the methoxy-*s*-triazine (prometon). Epidemiologic studies have documented that long term exposure to triazine herbicides is associated with increased risk of ovarian cancer in female farm workers in Italy and with breast cancer in the USA. Upon chronic exposure, atrazine may cause weight loss, cardiovascular damage, retinal degeneration, and mammary tumors in rats (Wang et al. 2010; Baggiani et al. 2007).

2.1.2 Pesticides classification based on target pest

2.1.2.1 Herbicides

Herbicides are the most widely used pesticides class worldwide, followed by insecticides and fungicides. They are widely used in agriculture, industry and urban areas to control weeds. They can provide cost-effective weed control with a minimum of labor. Use of herbicides has brought stable crop production; they protect crops from undue competition from weeds and enhance the nutritional quality of food. Herbicides are generally used as pre- and post-emergence for the control of weeds in agricultural crops (Gao et al. 2009; Heiligman and Krause 2007).

When use of an herbicide is desired, the selection of a specific herbicide is usually based on a number of considerations. The first consideration is the technique being utilized. Herbicides are labeled for specific uses. An herbicide may, for example, be labeled for girdling or injection but not for basal spraying. It is important that herbicides be used only for their labeled purposes. The second important considerations when selecting an herbicide include ease of use, relative availability, worker exposure, environmental safety, personal experience, and the relative effectiveness of the herbicide in controlling the target plant species. While the relative importance of these considerations may vary with the situation and the individual, it is always important to select herbicide that will effectively control the target species (Aysegul et al. 2002; Heiligman and Krause 2007).

Plants can also develop resistance to herbicides. For instance, some plants have developed resistance to atrazine and, more recently, to glyphosate herbicides. Marestail is one weed that has

developed glyphosate resistance. Glyphosate-resistant weeds are present in the vast majority of soybean, cotton and corn farms in some U.S states.

2.1.2.2 Insecticides

Insecticides are chemical or biological agents that control insects. Control may result from killing the insect or otherwise preventing it from engaging in behaviors deemed destructive (Nakao and Banba 2015). Insecticides may be natural or manmade and are applied to target pests in a myriad of formulations and delivery systems (sprays, baits, slow-release diffusion, etc.). The science of biotechnology has, in recent years, even incorporated bacterial genes coding for insecticidal proteins into various crop plants that deal death to unsuspecting pests that feed on them (Sparks and Nauen 2015). Malathion, mercarbam, DDT, aldicarb, carbofuran, pyrethrum and allethrin are classified under this catogry of pesticides.

2.1.2.3 Fungicides

Fungicides are chemical compounds or biological organisms used to kill fungi or fungal spores which cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. They can be applied directly to the soil or sprayed over crop fields (Merdassa et al. 2014). A fungicide with broad-spectrum activity is effective against a large variety of pathogenic fungi. Examples of broad-spectrum fungicides include captan, sulfur, and mancozeb. Some fungicides have a very narrow spectrum of activity; for example, mefenoxam is effective only against oomycetes like phytophthora.

2.1.3 Pesticide classification based on mode of action

2.1.3.1 Selective or nonselective pesticides

Selective pesticides are the ones that kill some organisms when applied to a mixed population, without causing serious injury to other species. For instance, selective herbicides are used in crop areas, lawns, gardens, and grasslands. 2, 4-D, atrazine, trifluralin, alachlor, butachlor, fluchloralin and pendimethalin are some of the examples of selective herbicides used on crop land (Diggle et al. 2003). On the other hand, non-selective pesticides are the ones that kill pests without regard to

species, for example, paraquat, diquat, sodium chlorate, weed oils, and acrolein. Non-selective herbicides are employed for general vegetation control on industrial sites, fallow land, and in aquatics and tennis courts (Diggle et al. 2003; Monaco et al. 2002).

2.1.3.2 Contact or systemic pesticides

A contact herbicide kills only the portion of pest that is contacted. Therefore, uniform spray coverage and particle size are essential for adequate control. Some common contact pesticides are paraquat, diquat, propanil and petroleum oils (Ashton and Crafts 1973). Systemic pesticides are extensively translocated in a plant's vascular system from point of absorption to sites of action. An example for this class of pesticide is glyphosate (Monaco et al. 2002).

2.2 Sample preparations methods for the analysis of pesticide residues in environmental and food matrices

As pesticide pollutants are generally present at low concentrations, they have to be separated from the majority of the components of the accompanying matrix and enriched before analysis (Wieteska and Drzewihska 1996; Koning et al. 2009). Major advances, in the development of analytical instruments have been made during the last two decades in the area of trace analysis in environmental samples. Since such instruments cannot handle environmental samples directly, analysis must be preceded by appropriate sample preparation or sample pre-treatment steps that enrich analytes and remove interferences (Gilar et al. 2001).

Sample preparation is a crucial step in analysis and is often a bottleneck to rapidly obtaining an accurate and sensitive result in the determination of trace pollutants. The selection of the most effective extraction and preconcentration procedure is of paramount importance for the reliable measurement of pesticides. Sample preparation is a step in which components of interest are isolated from a sample matrix into forms that are suitable for the analytical procedure and generate sub-fractions of the original samples enriched in all the substances of analytical interest, removing other components which may interfere in the analysis. The analytes separated from the matrix are preconcentrated to improve the selectivity, sensitivity, reliability, accuracy and reproducibility of

analysis (Dabiri et al. 2005; Koning et al. 2009; Somenatb 2009). In most cases, sample preparation is necessary for one of the following reasons (Shegefti et al. 2009):

a) The sample is in the wrong physical state for the analytical method (e.g., the method requires a liquid sample but the sample is solid).
b) The sample has interfering matrix components that may give false or negative readings in the measurement.
c) The sample has too low analyte concentration to be detected by the instrument.

Analytical methodologies employed must be capable of residue measurement at very low levels and must also provide unambiguous evidence to confirm both the identity and the concentration of any residue detected. Therefore, a lot of research efforts in separation science and related fields have been focused on the development of new sample preparation techniques which are less time consuming, more effective and require smaller amounts of organic solvents (Gilar et al. 2001; Shegefti et al. 2009; Xiao et al. 2009).

2.2.1 Classical sample preparation techniques

Quite a large number of conventional analytical methodologies including LLE and SPE have been utilized for selective and quantitative extraction of pesticide residues in samples of environmental, biological, food, pharmaceutical, and other origins.

2.2.1.1 Liquid-liquid extraction

Liquid-liquid extraction is a versatile classical sample preparation technique. LLE is a separation method based on the difference in solubility of a compound in two immiscible solvents at an appropriate pH (Raikos et al. 2009). LLE has been widely applied for extraction of non polar pesticides such as organochlorine and organophosphorous pesticides from water samples using organic solvents such as hexane and cyclohexane. Its application also tends to medium polarity organic compounds; carbamates, triazine and urea pesticides have been successfully extracted using dichloromethane or chloroform (Tadeo et al. 2008). LLE has been used for other matrices including analysis of multiresidues of pesticides from fruits and vegetables with acetone followed

by partitioning with dichloromethane (Gelsomino et al. 1997), grapes and wine (Miliadis 1999), honey with acetonitrile:ethyl acetate (Paulino de Pinho et al. 2010), rice and crayfish samples with methylene chloride (Zhou et al. 1996), grain, seeds, and rice with acetonitrile (Akiyama 2002).

LLE offers significant advantages in trace analysis such as preconcentration of toxic substances, simplicity, low cost and compatibility with analytical systems. LLE has disadvantages such as the use of toxic and expensive organic solvents which potentially entails problems to the health of the staff of analytical laboratories and environmental safety and the occurrence of emulsions which may lead to time-consuming extraction steps. The procedure itself is time consuming and often requires preconcentration prior to analysis (Arthur and Pawliszyn 1990; Buszewski and Ligor 2002). In the last decades, researchers have focused on developing simplified, miniaturized and improved sample pretreatment and clean-up procedures that can modify or substitute this method.

2.2.1.2 Solid phase extraction

One of the alternative sample preparation methods for LLE is SPE which was introduced in early 1970 and developed during 1980-1990. SPE process is based on distribution of analytes between solid sorbent packed in a cartridge and liquid sample which moves through the solid phase. Solid phase usually consists of small porous particles of silica with or without bonded organic phase, organic polymers and ion exchangers. Mechanisms of extractions are based on adsorption, partitioning or ion exchange according to the kind of solid phase (Ye et al. 2007). The applicability of SPE is mainly determined by the sorbent used in the extraction column. Nowadays a large number of sorbents are available, and the most frequently used group of sorbents are: chemically modified silica gel, polymer sorbents, graphitized or porous carbon (Żwir-Ferenc and Biziuk 2006).

The two basic approaches to SPE (Ye et al. 2007) are that the analyte of interest is retained and the matrix interferences are washed through or the analyte of interest is washed through and the matrix interferences are retained. SPE cartridges are available in a wide variety of chemistries, adsorbents, and sizes. Selecting the most suitable product for each application and sample can be very important. As a rough guide, the phases are categorized by the primary interaction mechanism with the analyte of interest:

a) Reversed phase: extraction of hydrophobic analytes from aqueous matrix.
b) Normal phase: extraction of polar analytes from non-polar organic solvents.
c) Ion exchange: extraction of charged analytes from aqueous or non-polar organic samples.
d) Mixed mode phases: phases with combined multiple interaction mechanisms.

SPE is used for extraction of both organic and inorganic compounds. SPE has many attractive features in comparison with classical solvent extraction methods. However, it has limitations. Some of the main limitations of SPE are listed below (Buszewski and Ligor 2002; Ye et al. 2007; Żwir-Ferenc and Biziuk 2006).

a) Clogging the pores of the solid phase by large biomolecules, oily materials and fine solids in the sample.
b) Despite decrease in solvent consumption in SPE in comparison with LLE, SPE needs at least 100 μL of solvent.
c) It is a time consuming method due to several steps of operation including; conditioning, sample loading and elution.

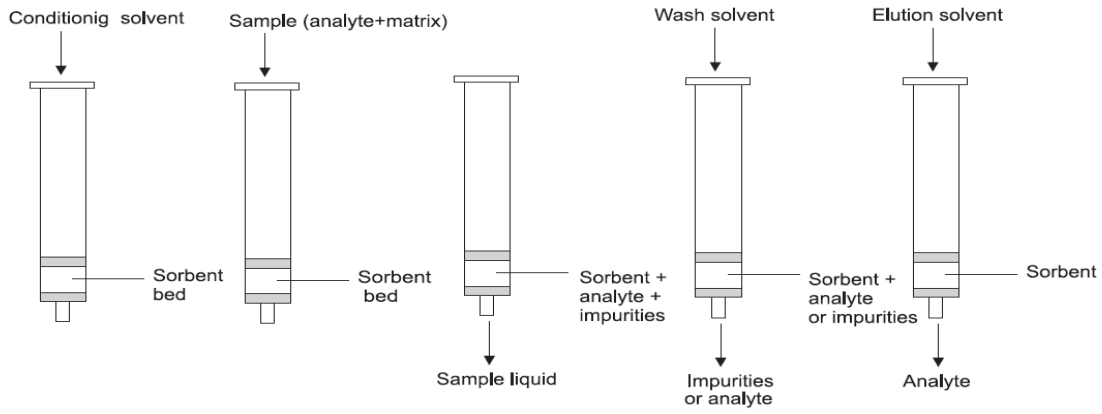


Figure 2.1.1 Solid phase extraction steps.

2.2.2 Modern sample preparation techniques for pesticides analysis

Recent research interests have received emphasis towards development of efficient, economical, miniaturized and automated extraction techniques that could greatly reduce consumption of toxic organic solvents (Saraji et al. 2014; Tobiszewski et al. 2009). To this end, DLLME (Chen et al.

2010), solid phase microextraction (SPME), SDME and hollow-fiber liquid-phase microextraction (HF-LPME) techniques have been developed and widely used as methods of choice for critically minimizing or avoiding uses of organic solvents in sample preparation procedures (Merdassa et al. 2015). However, SPME is expensive, its fiber is fragile and has limited lifetime, usually it has carry over effects and requires long-time for sorbent conditioning (Peruga et al. 2013). On the other hand, drop instability in SDME (Merdassa et al. 2015) and poor reproducibility due to manual cutting of the membranes in HF-LPME are some drawbacks in these methods (Peruga et al. 2013).

2.2.2.1 Dispersive liquid-liquid microextraction (DLLME)

Dispersive liquid-liquid microextraction technique was introduced in 2006 by Assadi and coworkers. It is a modified version solvent extraction technique in which acceptor to donor phase ratio is greatly reduced compared to other methods used for similar purposes (Chen et al. 2010; Farajzadeh et al. 2009; Zacharis et al. 2010). The principle of trace enrichment in DLLME is based on a ternary component solvent system in which extraction and disperser solvents are rapidly introduced into the aqueous sample to form a cloudy solution.

Extraction equilibrium is quickly achieved, mainly because of extensive surface contacts between the droplets of the extraction solvent and aqueous sample solution. The stability of the tiny extraction droplets in the dispersed system depends on the nature of the emulsion interface, surface electrical charge and Van der Waals forces. Factors as speed of agitation, temperature, bulk viscosity and presence of impurities, can play an important role in the effectiveness of demulsification (Chen et al. 2010; Farajzadeh et al. 2009). After centrifugation, the extraction solvent is normally sedimented at the bottom of the tube (if the density of extractant is higher than that of water) or at the top of the tube (if the density of extractant is lower than that of water) and taken with a microsyringe for chromatographic analysis. DLLME has advantages of simplicity of operation, rapidity, low cost, high recovery, use of cheap and commonly available laboratory devices and environmental benignity (Ma et al. 2012).

During sample extraction utilizing DLLME, determinations of the enrichment factor and percentage recovery, based on the measured results, are performed utilizing equations 2.1 and 2.2,

respectively. The preconcentration or enrichment factor (EF) is the ratio of the analyte concentration in organic phase to analyte concentration in the sample. EF is a measure of the rate of mass transfer from aqueous phase to organic phase at specified condition (Guo et al. 2009).

$$EF = C_{org}/C_{aq} \quad (2.1)$$

Where, C_{org} and C_{aq} are the concentration of analyte in the organic phase (with volume as V_{org}) and the initial aqueous samples (with volume as V_{aq}), respectively. Extraction recovery of the analyte (R %) is the percentage of the extracted analyte in the organic phase as described in equation 2.3, where n_{aq} is the amount of analyte in the sample prior to extraction and n_{org} is the amount of analyte in organic phase (Guo et al. 2009; Juybari et al. 2017).

$$R\ \% = \frac{n_{org}}{n_{aq}} x100 \quad (2.2)$$

$$R\% = \frac{C_{org}V_{org}}{C_{aq}V_{aq}} x100 \quad (2.3)$$

$$R\% = \frac{EF\ x\ V_{org}}{V_{aq}} x100 \quad (2.4)$$

The relative recovery (RR%), which is defined as the ratios of the peak areas of spiked real water extracts to the peak areas of spiked ultra-pure water extract is given by:

$$RR\% = \frac{\text{Peak areas of spiked real water extracts}}{\text{Peak areas of spiked ultra}-\text{pure water extracts}} x\ 100 \quad (2.5)$$

In DLLME, the factors that can affect the extraction efficiency include types and volumes of extraction and disperser solvent, pH, ionic strength, extraction time and temperature.

2.2.2.1.1 Factors affecting extraction efficiency of DLLME

2.2.2.1.1.1 Type of extraction solvent and volume

It is clear that the extraction solvent is the most important parameter in a DLLME method. Solubility of the extraction solvent in water has an inverse relation with stability of cloudy suspension (Martins et al. 2014). A proper extraction solvent must meet some primary requirements such as low water solubility, good chromatographic behavior and high extraction capacity for the target analytes (Guo et al. 2009; Martins et al. 2014).

Matsadiq et al. (2011) examined the effect of the the nature of the extraction solvent on extraction efficiency of DLLME for extraction of polychlorinated biphenyls (PCB), organochlorine pesticides (OCP) and pyrethroid pesticides in peach juices, pulps and peels. Different kinds of extractants including dodecan-1-ol, undecan-1-ol, n-tetradecane and n-hexadecane were evaluated. For n-tetradecane (melting point: 5.8 ^{o}C) and n-hexadecane (melting point: 18 ^{o}C), their hydrophobicity was so strong that it could not be solved in the common dispersive solvent. Moreover, the organic droplet formed a thin flat shape on the surface of the solution, so it melted quickly and was difficult to handle. The best result was achieved when using dodecan-1-ol (melting point: 24 ^{o}C) and undecan-1-ol (melting point: 11 ^{o}C) due to their suitable melting point and good affinity with target analytes.

Chen et al. (2010) also studied the effect of type of extraction solvent in DLLME for determination of carbamate pesticides in water samples. Toluene, cyclohexane, n-hexane and 1-octanol having different polarity and water solubility were tested. Toluene, followed by cyclohexane, n-hexane and octanol, have the highest extraction efficiency. It seems that ring structure and aromatic group of organic solvents are beneficial for the extraction of pesticides, which have aromatic group in the molecular structure. In this respect, toluene was selected.

The volume of extraction solvent is also an important experimental determinant in DLLME. Cheng et al. (2011) observed that the extraction efficiency of DLLME is not the same over the range of 20-50 μL of 1-dodecanol as extraction solvent for the determination of diethofencarb and pyrimethanil by DLLME from aqueous samples. Volumes of smaller than 20 μL could not form droplets. When the volume of extraction solvent was increased from 20 to 50 μL the enrichment factor (EF) decreased. They concluded that the volume of dodecanol leading to the highest EF was optimized at 20 μL. Cheng et al. (2010) also observed the same effect of extraction solvent volume on the extraction efficiency, different volumes of CCl_4 (40.0-90.0 μL) for extraction of fungicides in environmental water by DLLME. Increasing the volume of the extraction solvent results in dilution. They found that 50 μL CCl_4 was the optimum the extraction solvent volume.

2.2.2.1.1.2 Type and volume of disperser solvent

The role of an ideal disperser solvent is making more fine droplets of extraction solvent and dispersion of extraction phase in the bulk sample. Disperser solvent acts as a bridge between two immiscible phases, the sample and the extractant. Miscibility in both organic phase (extraction solvent and aqueous phase) is the main point for selection of dispersive solvent for the emulsification of extraction solvent. Methanol, ethanol, acetone and acetonitrile are usually used as disperser solvents due to their low toxicity as well as low cost (Tolcha et al. 2013; Zhou et al. 2009).

Xi et al. (2016) evaluated the effect of nature of disperser solvent for determination of benzoylurea insecticide in fruit juice by DLLME. For this purpose, various experiments were performed using 120 µL of each disperser solvent containing 20 µL of 1-dodecanol as the extraction solvent. Ethanol was found to result in the best extraction recovery among acetone and acetonitrile. Ethanol is less toxic and cheaper than acetone and acetonitrile. Yang et al. (2012) observed high extraction efficiency of DLLME for organophosphorus pesticides in soil with acetonitrile than acetone.

A series of sample solutions were investigated by using 1 mL each of the disperser solvents containing 20 mL chlorobenzene. Wu et al. (2009) also reported acetone as the best disperser solvent with $CHCl_3$ as extraction solvent for extraction of carbamate pesticides in water samples. Acetonitrile, acetone, or 1,4-dioxane as dispersive solvent could produce a two-phase system. They observed that cetone gives the best extraction efficiency for pirimicarb and diethofencarb but a little bit lower extraction efficiency for carbofuran and carbaryl than 1,4-dioxane. Overall consideration, acetone was selected as the dispersive solvent.

The disperser solvent volume was another important factor that affects extraction efficiency in DLLME. At low disperser volume, the organic extractant droplets cannot form properly. At high disperser volume, the solubility of organic analytes in aqueous phase will increase due to the increasing partitioning of disperser solvent in water (Lin et al. 2011; Tolcha et al.2013).

Zacharis et al. (2010) investigated the effect of the volume of disperser/terminating solvent on the extraction capacity of DLLME for determination of trace organochlorine pesticides in

environmental water samples. The total ACN volume was varied between 500 and 2000 µL at equal disperser/terminating volumes (250 + 250, 500 + 500, 750 + 750 and 1000 + 1000, µL+µL). Higher extraction efficiency was achieved for total ACN volumes of 1500 µL (750 + 750) due to more efficient dispersing/terminating actions at higher ACN volumes. A volume of ACN of 1500 µL (750 µL + 750 µL) was finally chosen. Lin et al. (2011) observed the same results on varying disperser solvent from 0-1.2 mL of acetonitrile containing a fixed volume of $CHCl_3$. They realized that extraction recoveries increased by increasing of the volume of acetonitrile up to 1 mL, and then decreased at higher volumes. A 1 mL of acetonitrile was chosen as the optimum disperser solvent volume for quantitative extraction of N-methyl carbamate pesticides in vegetables.

Chang et al. (2011) also examined the dispersive solvent volume, at various volumes of methanol (200, 300, 400 and 500 mL) containing 11 mL of 1-nonanol were tested. They observed that the peak area increased with increasing dispersive solvent volume (200–400 mL) due to the fact that a small volume of the dispersive solvent cannot disperse the extraction solvent effectively. A dispersive solvent volume of 400 mL resulted in larger peak areas and a further increase in the volume of the dispersive solvent may result in decreasing water phase polarity and increasing the solubility of the analytes in water. A dispersive solvent volume of 400 mL was selected as optimum for organochlorine pesticides from water.

2.2.2.1.1.3 Extraction time

In DLLME, extraction time means the time interval from the beginning of the dispersion and its end just before addition of the terminating solvent. Mass transfer is a time dependent process and one of the most important factors in most of the extraction procedures, especially in microextraction methods such as DLLME, SPME and LPME (Matsadiq et al. 2011, Saraji and Tansazan 2009). Equilibrium state is reached in a very short time and the target analytes diffuse into extraction solvent quickly due to the formation of infinitely large surface area of extraction solvent droplets (Saraji and Tansazan 2009; Zhou et al. 2009).

Nagaraju et al. (2007) observed extraction time has no influence of on extraction efficiency of DLLME for determination of triazine herbicides in aqueous samples. The reason out that in DLLME the surface area between the extraction solvent and the aqueous phase is extremely large.

The effect of extraction time was studied over the range of 0–160 min. extraction efficiency. Pusvaškienė et al. (2009) obtained the same result for the determination of volatile aromatic hydrocarbons in water by DLLME. They investigated extraction time up to 30 min and observed the peak area variations at different extraction times were not significant.

2.2.2.1.1.4 pH of sample solution

The pH of sample solution is another important parameter that may have an influence on the extraction performance. This may be due to the stability of the target analytes in the weakly acidic condition and/or weakly alkaline environment, while they were easily degraded in strong acidic and/or alkali conditions (Saraji and Tansazan 2009). For instance, for basic triazines to prevent the protonation of the weak bases in the acidic solution, the sample solution should not be rather acidic (Zhou et al. 2009).

Zhou et al. (2009) investigated effect of sample pH over the pH range of 3–11 on the extraction of atrazine and simazine in environmental water samples. Their results indicated that the best performance obtained at pH 5. They concluded atrazine and simazine were stable in the weakly acidic condition and weakly alkaline environment, while they were easily degraded in strong acidic and alkali conditions. A pH 5 was selected as optimum experimental condition.

Bedassa et al. (2015) carried out a series of experiments to investigate the effect of pH on the extraction efficiency of the DLLME for multiclass pesticides residue in environmental water over the pH range of 1.5–4. The experimental response of all the target analytes increased with rise in pH of the sample solution up to pH 2 and then decreases on further increase in pH of the sample solution. In a more acidic solution, lower peak areas were observed probably due to hydrolysis of the pesticide and higher pH, the target analytes might not be completely transformed to their neutral forms. A sample solution of pH 2 was chosen as the optimum.

2.2.2.1.1.5 Ionic strength

The addition of salt decreases the solubility of analytes in the aqueous sample and enhances their partitioning into the adsorbent (for SPME) or organic phase (LLE) (Tsai and Huang 2009; Viñas

et al. 2014). The salting-out effect has been commonly used in microextraction techniques such as LPME and SPME. In DLLME experiments, extraction of analytes can be enhanced or retarded by addition of salts depending on the nature of the analytes. This is possible because electrostatic interaction increases by adding salt and viscosity of the sample solution also increases, which reduces the analytes to move into the extraction phase (Chen et al. 2017).

Sanagi et al. (2012) studied the effect of adding NaCl on the DLLME-SFO efficiency for extracting triazine herbicides in water and sugarcane samples. They evaluated in the range of (0-8%, w/v). The experimental results showed that the peak area increased with an increase in NaCl from 0% to 5% (w/v) and remained constant or no significant effect beyond 5%. Thus, 5% of NaCl was selected as the optimum concentration of NaCl. Wu et al. (2009) reported the negative effect of addition of NaCl on the extraction efficiency of DLLME for extraction of carbamate pesticides in water samples. The effect of NaCl was evaluated in the range from 0% to 15% (w/v). They observed that the best extraction efficiencies for each target analyte were obtained without the addition of NaCl.

2.2.2.1.1.6 Temperature

Temperature is an important parameter in all of the equilibrium systems. But in DLLME temperature is a critical parameter because, boiling point of the extraction and disperser solvents are limited to the process. The extraction efficiency decreases due to the increase in solubility of target analytes in aqueous solution with increasing temperature (Tsai and Huang 2009; Xiao-Huan et al. 2009).

The effect of temperature on extraction efficiency of DLLME for analysis of *s*-triazine herbicides in water was investigated by Tolcha et al. (2013) by varying it from 20 to 40 ^{o}C. The experimental results indicated that the peak area increases with increasing temperature from 20 to 25 ^{o}C. However, increasing temperature beyond 25 ^{o}C showed a slight decrease in the extraction efficiency. They explain the results as it could be due to the gradual losses of the extraction solvents, beyond 30 ^{o}C. An extraction temperature of 25 ^{o}C was selected.

2.2.2.1.2 Types of DLLME

2.2.2.1.2.1 High density organic solvent based DLLME

The extraction steps of DLLME are illustrated in Figure 2.2.1. Acetone, methanol and acetonitrile can be used as dispersers, whereas high density solvents such as chlorobenzene, carbon tetrachloride and tetrachloroethylene are used as extraction solvent (Gure et al. 2013). In this techniques certain volume of sample solution is placed in a screw cap glass test tube with conical bottom (A), followed by the rapid injection of disperser solvent containing extraction solvent into the sample solution with a syringe or pipette.

Then, the mixture is gently shaken, thus, a cloudy solution (water/disperser solvent/extraction solvent) is formed in the test tube (B). After that, the surface area between extraction solvent and aqueous phase (sample) is infinitely large, thereby, transition of analyte from aqueous phase (sample) to extraction phase is fast. Subsequently, equilibrium state is achieved quickly, resulting in a very short extraction time, which is the remarkable advantage of DLLME compared with those of other techniques. Finally, the dispersed fine particles of extraction phase are sedimented in the bottom of conical test tube through centrifugation (C) and certain volume of the sedimented phase is injected into chromatographic system using a microsyringe for further analysis (D) (Chen et al. 2010).

Due to the immiscibility of the extraction solvent with water, the most commonly preferred techniques for the final analysis of organic analytes is GC instrument. If HPLC is chosen, the solvent should be first evaporated and then reconstituted with a HPLC compatible solvent before the final analysis (Herrera-Herrera et al. 2010). High density solvent based DLLME has been employed for extraction of various types of organic and inorganic compounds from environmental waters as well as other matrices such as foods.

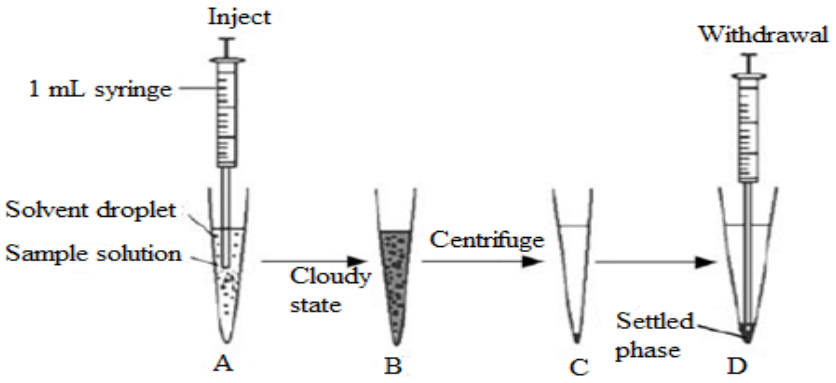


Figure 2.2. 1 Schematic diagram of high density solvent based DLLME procedure.

3.2.2.1.2.2 Low density organic solvent based solvent demulsification DLLME

Acetone, methanol and acetonitrile can be used as dispersers, whereas lower density solvents such as 1-octanol, toluene, n-hexane and cyclohexane are used as extraction solvent (Tolcha et al. 2013). The principle of this techniques is the same as high density organic solvent based DLLME except the type of organic solvent used and the organic phase is floated at the top of the aqueous sample. The organic layer is taken with a microsyringe for its later chromatographic analysis (Farajzadeh et al 2009).

Recently, many researchers have attempted to use low toxicity solvents with density lower than that of water in DLLME (Mei-I and Huang 2009). One possible way of enabling the application of such solvents in DLLME is the use of special extraction devices, such as specially designed centrifugation tubes and pipette collection tubes as shown in fig. 2: a) special extraction vessel DLLME, b) glass tube with narrow neck DLLME, c) centrifuge glass vial designed in-house DLLME, d) special flask equipped with two narrow open ports DLLME, e) 5 mL polyethylene Pasteur pipette DLLME, f) 5 mL syringe as sample vial DLLME and g) disposable polyethylene pipette DLLME.

2.2.2.1.2.3 Low density solvent based DLLME solidification of floating organic drop (DLLME-SFO)

DLLME-SFO has been presented by Leong and Shang for organic compounds determination. In this technique, the extractant with lower density than water, low toxicity and proper melting point near room temperature (in the range of 10-30^{o}C) was used. The advantages of DLLME-SFO method are simplicity of operation, rapidity, low cost, high recovery, compatibility of the extraction solvent with the instruments for analysis. DLLME-SFO promises to have a wide application prospect in trace analysis area (Chang et al. 2010; Xua et al 2009). Large contact surface between the sample solution and the extraction solvent speeded up mass transfer, as far as DLLME (Chang et al. 2010).

Preconcentration factor (PF) was calculated based on the following equations,

$$PF = \frac{C_{floating}}{C_O} \quad (2.6)$$

where, PF, $C_{floating}$ and C_0 are the preconcentration factor, concentration of the analyte in the floating organic drop and initial concentration of the analyte in the aqueous sample (Xua et al 2009).

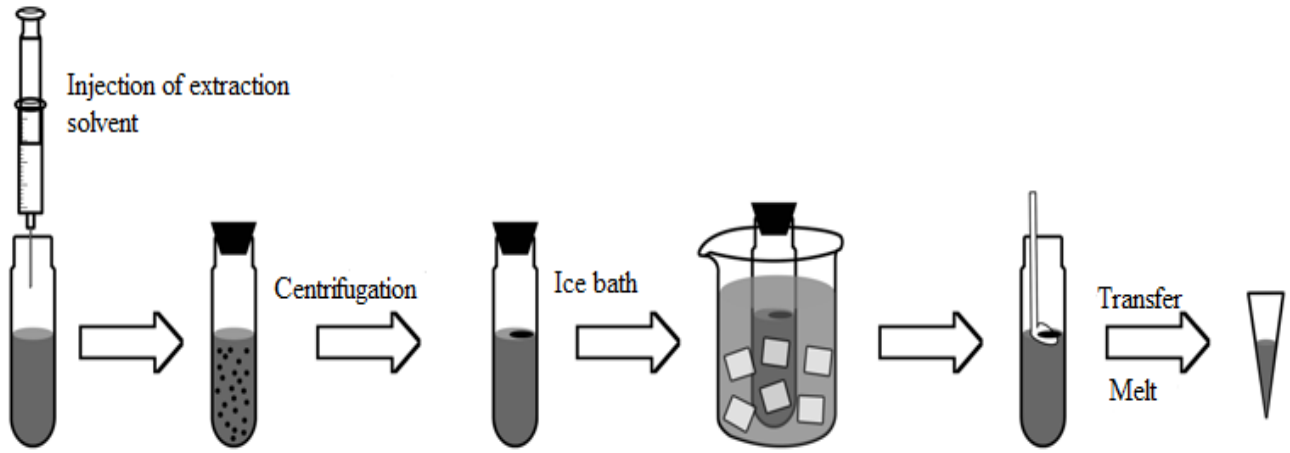


Figure 2.2.2 Schematic diagram of the proposed DLLME-SFO procedure.

2.2.2.1.2.4 Ionic liquid based DLLME (IL-DLLME)

Ionic liquids (ILs), which are composed of organic cations and organic or inorganic anions, are liquids near room temperature (by convention below 100 ^{0}C). The widely used cations in ionic liquids are based on ammonium, sulfonium, phosphonium, imidazolium, pyridinium and picolinium with different substitution. The anion of ionic liquid maybe organic or inorganic. Anion includes $[BF_4]^-$, $[SbF_6]^-$, $[PF_6]^-$, $[SF_3SO_3]^-$ and alkyl sulfate (Singh and Kumar 2008). Since the first report in 1982, many ILs containing a great variety of cations and anions of different sizes have been synthesized to provide desirable characteristics. They are relatively thermally stable, low toxicity, non-coordination and provide no detectable vapor pressure, thus, avoiding environmental and safety problems due to volatilization (Feng et al. 2010; Zhang and Lee 2012). ILs had also been widely used in different fields (e.g., chromatography, electrochemistry or extraction) because of their unique properties, such as high thermal and chemical stability, negligible vapor pressure, tunable viscosity and miscibility with water and organic solvents (Dupont et al. 2002; Olivier-Bourbigou and Magna 2002). ILs have been proposed as extractants in DLLME (IL-DLLME) which applied widely as preconcentration technique for the determination of organic compounds in environmental water samples. Several kinds of IL-DLLME have been developed recently, such as conventional IL-DLLME, temperature controlled IL-DLLME (TC-IL-DLLME), IL cold induced aggregation DLLME (IL-CIA-DLLME) and ultrasound assisted IL-DLLME (Dupont et al. 2002).

Conventional IL-DLLME used IL as the extraction solvent of DLLME and it was a biphasic process, since the aqueous samples and the extractant were immiscible during extraction. The selection of IL depended on the good performance of the disperser solvent (Ma et al. 2012). TC procedure is only applicable when the solubility of the extraction solvent in the aqueous sample has a high dependence on temperature. One function of temperature is to promote the dispersing of IL into the solution and forming the fine drops and increasing the chance of the analytes extracted into ionic liquid phase and the other is to perform the phase separation (Zhang et al. 2010).

Room temperature ionic liquids (RTILs) are regarded as green solvents. There has been significant attention being focused on the application of ILs in analytical chemistry. ILs have been investigated as extraction solvents for SDME, HF-LPME and DLLME. With respected to ionic liquid-based DLLME (IL-DLLME), RTILs could replace the commonly employed highly toxic chlorinated extraction solvents, with direct extract injection into HPLC systems for analysis (Zhang and Lee 2012).

2.2.2.1.3 DLLME combined with other other techniques

2.2.2.1.3.1 DLLME combined with SPE

SPE is commonly used sample preparation technique for the extraction and selective isolation of selected analytes, usually from a gas, fluid or liquid phases. Since DLLME is not suitable for complex matrices such as highly saline solution, a combination of SPE and DLLME was introduced by Rezaee et al. (2006) for determine carbamazepine in biological fluids and water samples. SPE-DLLME is an efficient hyphenated technique that offers the advantages of both methods such as simplicity, low solvent usage and exposure, low disposal cost and extraction time, with high recovery and enrichment factor, and it can be also used in complex matrices.

After concentration and purification of the sample using SPE C-18 sorbent, 1.5 mL of acetonitrile containing 60 µL of chloroform was injected into 5 mL pure water. After extraction and centrifuging, the sedimentated phase was evaporated and the residue was dissolved in 30 µL methanol and injected into the HPLC system. This new method provides detection limits of 0.8 µg/L and 1.7 µg/L in urine and plasma samples, respectively. The calibration graphs are linear in the range of 2.5–500 and 5–500 µg/L in urine and plasma, respectively.

In another study, SPE-DLLME was applied to the preconcentration and analysis of short-chained dodecyl alcohol ethoxylates and dodecyl alcohol. The results showed that the analytes were preconcentrated 700 times with the use of small sample volume. The developed method was used for the analysis of short-chained dodecyl alcohol ethoxylates and dodecyl alcohol in both sewage effluents from sewage treatment plants and river water samples (Zgola-Grzeskowiak and Grzeskowiak, 2012).

2.2.2.1.3.2 Single-drop microextraction (SDME) combined with DLLME

The single-drop microextraction (SDME) is performed by suspending a microliter drop of water-immiscible solvent in the stirred aqueous solution or placing the drop in the headspace of sample bottle. However, this technique suffers much from the instability of the suspending drop and approaching high signal intensity of analyte usually takes quite some time for extraction. The integration of these two techniques, i.e. DLLME and SDME is presented as a new extraction method for environmental analysis. This proposed process allows the individual shortcomings of each technology to be overcome and leads to an optimum process configuration (Zgola-Grzeskowiak and Grzeskowiak, 2012). Furthermore, DLLME is used mainly for the pre-concentration of the analytes into an extraction organic solvent which afterwards using GC as detection system and SDME is used for the back extraction of the analytes into the water samples which can provide situation for using HPLC method.

The combination of DLLME and SDME as a new pre-concentration technique is developed for the separation and determination of acidic non-steroidal anti-inflammatory pharmaceutical compounds, namely, naproxen, diclofenac, and ibuprofen, in water samples using HPLC-UV. Good linearity range of 0.11000 μg/L, acceptable reproducibilities (RSDs, 4.5–8.8%), low limits of detection (0.03–0.2 μg/L), and satisfactory relative recoveries were obtained.

Moreover, the low-density solvent-based DLLME combined with SDME was developed in a new format of fast three-phase microextraction for the first time (Li et al., 2013c). In a study, a volume of low-density solvent (toluene) was used as organic phase and injected into the aqueous sample (donor phase) with disperser (methanol). The analytes were pre-extracted into the organic sample within 2 min. Afterwards the layer of the organic phase was formed on the top of the aqueous phase by a 2 min centrifugation. Then a drop of acceptor solution was introduced into the upper layer of toluene and the SDME was carried out for back extraction. After extraction, the acceptor drop was withdrawn and directly injected into a HPLC-UV for analysis. In this procedure, the high speed and efficiency of DLLME make the typical stirring step in SDME unnecessary and the total extraction time noticeably short.

2.2.2.1.3.3 Supercritical fluid extraction (SFE) combined with DLLME

Supercritical fluid extraction (SFE) has been adopted to extract different substances from solid matrices since three decades ago. As is well known, in spite of substantial advantages of DLLME, it is not suitable for extraction of compounds from solid samples and sometimes extra steps such as drying and filtering processes in sample preparation before DLLME are time-consuming (Zgola-Grzeskowiak and Grzeskowiak, 2012).

Rezaee et al. (2010b) reported for the first time, a combination of SFE and DLLME, as a sample preparation method was developed for determination of ten PAHs in marine sediment samples. In SFE-DLLME, the collecting solvents such as methanol and acetonitrile in SFE can be used as disperser solvent in DLLME. After performing SFE and collecting the extracted analytes in the disperser solvent, a suitable volume of the extracting solvent was added into the collecting solvent. Finally, the mixture was injected to the aqueous sample. The other steps were similar to DLLME method. SFE-DLLME leads to high preconcentration factor for determining organic compounds in solid samples, easy use of DLLME in solid samples and can eliminate the need to evaporate the collecting solvent at the end of SFE. The performance of SFE-DLLME in the extraction of polycyclic aromatic hydrocarbons (PAHs) from different marine sediment samples with various matrices was excellent.

PAHs were employed as model compounds to assess the extraction procedure and were determined by gas chromatography-flame ionization detection (GC-FID). SFE of PAHs was performed at 313 K and 253.2 bar, at static and dynamic time 10 and 30 min, respectively. The extracted PAHs were collected in 1 mL of acetonitrile. Subsequently, 16 µL of chlorobenzene (as extraction solvent) was added to collecting solvent (1 mL of acetonitrile). Then, the mixture was injected rapidly into 5 mL of aqueous solution. After centrifugation, the PAHs in the sedimentated phase were analyzed by GC-FID. Under the optimum conditions, the calibration plots were linear in the range of 0.4-41.6 mg/kg and the limits of detection were 0.2 mg/kg for all of the analytes. Analysis of PAHs in different solid samples showed that the improved technique has great potential for PAHs analysis in marine sediments.

2.2.2.2 Salting out liquid-liquid extraction (SALLE)

Recently researchers emphasized on development of efficient, economical, miniaturized and automated extraction techniques that could greatly reduce consumption of toxic organic solvents. As a result, green extraction and/or miniaturized methods are appealing in trace analysis (Hrouzková et al. 2017). A SALLE was found to be simple, fast, cheap and environmentally friendly extraction techniques (Alemayehu et al. 2017).

A SALLE method is based on LLE in which addition of an appropriate amount of salt to a mixture of aqueous sample and water-miscible organic solvent which results in separation of the solvent from the mixture and thus the formation of two-layer system (Gure et al. 2015). The target analytes are extracted into the organic phase. Compared with conventional LLE, SALLE is more efficient, extractability range for the analytes is wider and greener. However, the enrichment factor of SALLE method is very small compared to solvent based microextraction and thus the purpose of the extraction is mainly to clean up the matrices and has been applied for pesticides analysis in honey, river water and human urine (Liu et al. 2010), lake water (Alemayehu et al. 2017), sea water, waste water and urine (Niu et al. 2017), banana juice (Gure at al. 2014), etc. Therefore, there is a demand to couple SALLE techniques with solvent based microextraction to enhance the enrichment factor and preconcentrate the trace level pesticide residues prior to instrumental analysis.

2.2.2.2.1 Factors affecting SALLE

2.2.2.2.1.1 Selection of the type and volume of organic solvent

Selecting an appropriate extracting solvent is based on two important parameters, the solubility of the target compound and the penetrability into the matrix must be considered. Due to miscibility of extracting solvent in water, SALLE was applied for extraction, preconcentration and clean-up of polar compounds from water or liquid samples. Therefore, it is important to choice an appropriate extraction solvent with suitable polarity for maximum analyte or analytes extraction during SALLE steps (Rezaeepour et al. 2015).

Gure et al. (2014) observed the significant effect in their investigation of SALLE for sulfonylurea pesticides analysis in water. Under the following extraction condition; using 3 mLwater sample containing 0.1mol/L citrate buffer (pH 2) and 25%NaCl (w/v) and 2 mL of each organic solvent and with exception of methanol, in which the two phase system was not observed. Their result depicted acetonitrile is the best extraction solvent for the pesticides investigated. Thus, they selected acetonitrile as extraction solvent. Liu et al. (2010) also observed that the nature of organic solvent for extraction of sulfonamides by SALLE from honey, water and human urine has significant effect on extraction efficiency. They observed that minimum volume for phase separation was approximately 500 µL, 300 µL and 800 µL for isopropyl alcohol, acetonitrile and acetone, respectively. It indicated that the consumption of acetonitrile might be the least among the three. The less volume of organic solvent could lead to better extraction performance and they selected acetonitrile.

The volume of the extraction solvent also plays a great role on the extraction performance of the SALLE procedure. At low volumes, phase separation between organic solvent and aqueous phases may not be clear and thus, collection of the organic phase will difficult (Gure et al. 2014). On the other hand, at higher volumes, the volume of organic phase obtained after phase separation is higher than its initial volume, indicating the existence of dissolved water in the organic phases (Liu et al. 2010).

Alemayehu et al. (2017) have studied the effect of volume of extraction solvent (acetonitrile) on the performance of SALLE for the extraction of arbaryl, atrazine, propazine, chlorothalonil, dimethametryn and terbutryn from environmental water samples. The volume of acetonitrile, as extraction solvent, was varied over the range of 0.5-2.5 mL. With lower volumes of acetonitrile, i.e., 0.5 and 1 mL, phase separation between the aqueous and organic phases was found to be insufficient. The extraction efficiency of SALLE decreased when volume of acetonitrile is greater than 1.5 mL which is attributed to dilution of the organic phase. They concluded that the highest instrumental responses were obtained with 1.5 mL of acetonitrile and thus used as optimum extraction solvent volume.

2.2.2.2.1.2 Effect of salt type and amount

In SALLE, water molecules preferentially form hydration sphere around the salt ions leading to separation of organic phase rich in analytes. Thus the nature of salt is responsible for strong hydration to enhance the extraction efficiency (Jain et al. 2015). Razmara et al. (2011) evaluated the effect of ionic strength on the extraction efficiency of SALLE. In order to obtain phase separation and the optimum extraction efficiency, several salts, i.e. $(NH_4)_2SO_4$, NaCl, K_2HPO_4, and Na_2CO_3 were tested. Their experimental results demonstrated that $(NH_4)_2SO_4$ provided higher extraction efficiency than other salts. This may be due to stronger ability of salting-out and more solubility of $(NH_4)_2SO_4$ in water.

Concentration of the salt is also important factor affecting the extraction efficiency of the method (Sereshti et al. 2014). The concentration of the salt must be large enough to induce the required phase separation. The quantity of salt added must be enough to clearly separate the two phases, but should not be added in excess beyond saturation to avoid adsorption of analytes on solid phase (Jain et al. 2015). Razmara et al. (2011) also reported the effect of the ionic strength of $(NH_4)_2SO_4$ over the range of 1 to 3 g and observed 2.25 g was the optimum experimental value.

2.2.2.2.1.3 Effect of extraction time

Extraction time is also another vital parameter in DLLME since mass transfer is time dependent. The extraction time was studied by Alshishania et al. (2018) from 30-75 second for extraction of biguanides in biological and environmental samples by SALLE. There was no significant increase in extraction recovery over the investigated period which was expected as the two phases were completely miscible before the addition of the salt. Therefore, 30 second was chosen for further studies. Gure et al. (2014) also reported the significance of extraction time for the determination of sulfonylurea herbicides in environmental water and banana juice samples by SALLE. The extraction time was evaluated in the range of 1-7 min and the maximum extraction recoveries were obtained at the 3 min. Thus, 3 min was chosen as the optimum extraction time.

2.2.2.2.1.4 Effect of pH

As to liquid microextraction of analytes with weak acid or base properties, the environmental pH determines their ionization status and thus is influential to transfer of the analytes from aqueous phase to organic phase (Fan et al. 2014). This parameter should consistent with the isoelectric point of the analyte. In the isoelectric point net charge of analyte is zero and thus mass transfer to organic phase increases (Rezaeepour et al. 2015. Low and high pHs may decreases extraction efficiency due to decrease in the stability of non polar or medium polar organic pollutant and increasing its surface charge (Teju et al. 2017).

Bedassa et al. (2017) reported the effect of pH for efficient extraction of ionizable and relatively polar compounds pesticides from alcoholic beverages. They have studied its effect by adjusting the sample solution over the range of 4–8, keeping other experimental parameters constant and observed that the peak areas of all the target analytes increased with the rise in pH of the sample solution up to pH 7 and then started to decrease on further increase in pH of the sample solution. They concluded that lower peak areas observed at higher pH might be most likely due to the hydrolysis of the pesticides and the lower peak areas of the target analytes at lower pH might be attributed to their incomplete conversion to their neutral form and thus, complete transfer of the analytes from the sample solution to the organic phase could not be achieved. They chose a sample solution of pH 7 as the optimum.

2.2.2.2.1.5 Effect of centrifugation speed and time

Centrifugation another important parameter in SALLE and it accelerate the phase separation (Farajzadeh and Khoshmaram 2015). Centrifugation of the sample solution influences kinetics of the extraction and enhances contact between the organic solvent and aqueous solution and thus facilitates formation of the two-phase system in the SALLE technique (Razmara et al. 2011).

Alemayehu et al. (2017) studied the effect of centrifugation speed in the range of 2000 to 4000 rpm, with interval of 500 rpm. The peak area of all target analyte increases with increasing centrifugation speed. However, centrifugations speed of higher than 4000 rpm were not conducted due to the instrumental limitations and 4000 rpm was utilized as optimum centrifugation speed.

Optimizing the time required for phase separation is also important analytical step, in order to obtain a clear extract. Centrifugation time investigated was used after salt addition to separate the two phases. Alshishania et al. (2018) evaluated the effect of centrifugation time on SALLE performance from 1–12 min and selected 3 min.

2.2.2.3 Supercritical fluid extraction (SFE)

SFE, substance whose temperature and pressure exceed the critical point, has gained increased attention as a potential replacement for conventional liquid solvent extraction due to its properties of supercritical fluids such as higher diffusivity and low viscosity which allow selective extractions of different chemicals without additional clean-up as well as the use of little sample amounts (Rissato et al. 2004). The solvents in supercritical state show intermediate physical-chemical properties similar to that of liquid and gas, which increases the extracting power of the solvent. The high density of these fluids gives them a high solvation power, whereas its high diffusion and low viscosity values provide a desired penetration power in the solid matrix. The high solvation power of supercritical solvents is related to their density. This characteristic promotes higher solubility of the compounds in supercritical fluids when compared with organic solvents (Machado et al. 2013).

SFE is interesting because this technique considerably reduces sample preparation time due to the excellent mass transfer properties and ease of control *via* temperature, pressure or modifier (Rissato et al. 2004; Aguilera et al. 2003). Generally, the solubility of the solutes in supercritical fluids increases with temperature at constant pressure. However, the effect of temperature on pressurized systems is complex due to two factors: an increase in temperature increases the vapor pressure of the solute, promoting an increase of its solubility in supercritical fluids; on the other hand, an increase in temperature also decreases the solvent density, reducing the solubility of the solute in the solvent (Machado et al. 2013).

The key parts of an SFE system are the high-pressure pump which delivers the fluid and the restrictor which maintains the pressure inside the system. Extraction is performed inside a high-pressure cell (containing the sample), maintained at the correct temperature. The fluid may simply fill the cell (static mode), or continuously flow through the vessel (dynamic mode). The extracted

solutes are entrained by the supercritical fluid flow out of the cell; their collection is usually achieved as the fluid is depressurized by passing through the restrictor (Pourmortazavi et al. 2014).

SFE results are strongly dependent on the physical nature of the matrix and the polarity of the pesticides. Consequently, SFE must be regarded as a four-stage process: desorption of the compound from the matrix with subsequent diffusion into the matrix; solubilization of the analyte by the supercritical fluid; sweeping out of the extraction cell by the fluid; trapping of the extracted solutes upon depressurization of the fluid. Each part of the process has to be carefully optimized in order to obtain quantitative and reproducible recoveries. Most of the time, the first step remains the most difficult to control, as solute-matrix interactions are very difficult to hinder and to predict. This problem is crucial when dealing with samples that contain native pesticides (Camel 1998).

Supercritical carbon dioxide extraction (sc-CO_2) is considered as an appropriate and particularly interesting, since the method does not degrade the extracted agrochemicals, selectivity and does not use toxic organic solvents (Sartori et al. 2017). Carbon dioxide can be easily converted to the supercritical state, and its critical temperature and pressure are 31.1 ^{o}C and 7.38 MPa, respectively. A sc-CO_2 is commonly used as the mobile phase in chromatography and as an extraction solvent because it is nontoxic, nonreactive, inexpensive, and easy to handle (Ishibashi et al. 2012).

2.2.2.3.1 Factors affecting SFE

2.2.2.3.1.1 Modifier effects

The use of pure CO_2 in multiresidue pesticide analysis is limited because CO_2 is considered a nonpolar solvent with a liquid solubility equal to that of hexane. However, for quantitative extraction of moderately polar and polar pesticides, a modifier such as methanol or acetonitrile has been applied in order to obtain satisfactory results (Lanças et al. 2000). Based on the nature of sample matrix and the analyte's retaining nature on the matrix, the modifier may influence the extraction in three different ways:

a) Increasing the analyte's solubility in the supercritical fluid, as a result of analyte-modifier interactions in the fluid phase.

b) Facilitating the analyte desorption-the molecules of polar modifiers are able to interact with the matrix and compete efficiently with the analytes for the active sites in the matrix.
c) Distorting the matrix-analyte diffusion and penetration of the supercritical fluid inside the matrix are favored when the modifier swells the matrix.

There are two main procedures to study with co-solvents or modifiers in sc- CO_2 are; the first one, and the most common, accounts for a mixing of the modifier with the CO_2 flow while the second mixes the modifier with raw material in the extraction cell. This procedure is always associated to a static extraction step in which the modifier, in intimate contact with the sample matrix, is able to substitute the analyte molecules bound in active centers of the matrix and release them into the supercritical fluid phase (Abbas et al. 2008).

Nemeto et al. (1997) was studied the effect of modifier on the extraction efficiency of supercritical CO_2 for extraction of different pesticides from spiked Celite. For the effect of modifiers, nine solvents (water, methanol, ethanol, 2-propanol, acetonitrile, acetone, ethyl acetate, dichloromethane, and n-hexane) were evaluated in duplicate extractions. Non polar pesticides, the modifiers had little influence on recoveries, whereas the recoveries of group polar pesticides improved with the addition of modifiers. For some of pesticides, water and the alcohols (methanol, ethanol, and 2-propanol) gave higher average recoveries of pesticides than the other modifiers. Water and the alcohols were the effective modifiers for improvement in recovery of pesticides. Therefore, the increased solubility of the pesticides in CO_2 by modifiers (hypothesis a) is probably the main mechanism of the effect of modifiers.

Studies on the influence of different static modifier conditions on pesticide recoveries were conducted on gazpacho samples with 6% olive oil (Aguilera et al. 2003). Ethyl acetate and methanol as modifier were performed and compared with recoveries from extraction with no modifier. Recoveries obtained by using ethyl acetate as modifier were slightly higher than those obtained with methanol, except for the most polar pesticides methamidophos and acephate, for which better recoveries were obtained by using methanol as modifier.

2.2.2.3.1.2 Effect of extraction time

The length of extraction time influenced the extraction efficiency and selectivity of the fluid. It is important to maximize the contact of the supercritical fluid solvent with the sample material in order to enhance the efficiency of SFE. Shorter extraction time could cause incomplete extraction and longer extraction time could be time and solvent wasting (Pourmortazavi et al. 2007). The SFE system used in this study can accommodate two extraction modes: static extraction mode (in which the sample is allowed to steep in CO_2 fluid) and dynamic extraction mode (in which CO_2 fluid continuously flows through the sample). The static mode, however, is often used when modifiers and derivatizing reagents are employed, especially when a modifier or derivatizing reagent is directly added to the extraction vessel prior to pressurization. Moreover, a static extraction is often done before dynamic extraction for the supercritical fluid extraction of pesticides from other matrices such as soil or agricultural products (Nemoto et al. 1997).

Rissato et al. (2004) evaluated the effect of extraction time on the extractability of different pesticide residues in honey sample. The extraction time was studied at 10, 20 and 30 min and. The results showed that by increasing the period from 10 to 20 min, improved the extraction efficiency of the studied pesticides in more than 25%. However, the increase in the extraction time from 20 to 30 min and insignificant effects on the extraction efficiencies of pesticides.

The effect of extraction time on extaraction recovery of pesticides from soil using supercritical carbon dioxide has also studied by Forero-Mendieta (2012) at different stages. They observed that when the extraction was performed using 3 stages of 10 min, the % R was low for most pesticides, except for iprodione. It can also be seen that in general the results for almost all compounds in the extraction time, 3 steps of 15 min, 4 steps of 10 min and 4 steps of 15 min each, were statistically equal, with some exceptions such as beta-endosulfan, pp-DDT, chlorpyrifos and fenamiphos. They suggested that a higher rate of mass transfer between soil and sc- sc-CO_2 /MeOH is reached in the three (of 15 min) or four (of 10 min) initial extraction steps, so that the additional steps (4th step of 15 min and 5th step of 10 min each) did not significantly affect the % R. A 4 stages of 10 min were selected as extraction time.

Patil et al. (2014) also evaluated the effect of dynamic extraction time for extraction of wedelolactone. In order to obtain high yield of wedelolactone, an important extraction step of static extraction (10 min) was performed to make a better penetration of the fluid into the matrix compared with the only dynamic extraction mode. This step was followed by a dynamic extraction to enhance solubility of wedelolactone in the supercritical fluid. To evaluate the effect of dynamic extraction time on sc-CO_2 of wedelolactone, extraction was performed for 30, 60, and 90 min separately. The experimental results indicate the extraction yield of wedelolactone increases significantly in the extension of extraction time.

2.2.2.3.1.3 Effects of extraction pressure

The pressure of the fluid is the main parameter that influences the recovery of organic compounds. The maximum fluid density could be obtained at high pressures at a given temperature, which can enhance the strength of the solvent (Reindl and Höfler 1994). For a given temperature, the fluid density is proportional to the pressure, so that increasing the pressure is beneficial to the solubility of analytes into the fluid. The density of a supercritical fluid is extremely sensitive to minor changes in temperature and pressure near the critical point. The densities of the fluids are closer to that of organic liquids but the solubility of solids can be 3-10 orders of magnitude higher (Karale et al. 2011).

A class of compounds may be characterized by its "threshold pressure" (i.e. the pressure above which they begin to be soluble in the fluid). Consequently, a correct choice of the pressure may lead to selective extractions; thus, it should allow the successive extraction of classes of pesticides, and/or the extraction of pesticides without simultaneous extraction of matrix interferents (Camel 1998). The study conducted by Rissato et al. (2005) show pressure has an effect on the extraction efficiency of SFE. To study the effect of pressure, a set of experiments was carried out at three different pressures (19971, 44935 and 69898 kPa) using 10% of methanol as a modifier for simultaneous determination of organophosphorus, organohalogen, organonitrogen and pyretroids pesticides in fruit and vegetables. The results demonstrate that pressure up to 44935 kPa resulted in increasing recovery in relation to 19971 kPa. The recoveries obtained were higher than 81% for all matrices studied and for 69898 kPa no significant increase in recovery was observed. According

to Rissato et al. if an analyte is very soluble in supercritical fluid at a low pressure, this solubility will increase or remain the same at higher pressures.

2.2.2.3.1.4 Effects of extraction temperature

Temperature is another important experimental variable for sc-CO_2 as it affects three extraction steps: desorption, diffusion and dissolution. While the CO_2 density may decrease with the increase of temperature at constant pressure, the solubility of many organic compounds can dramatically increase because of an increase in the solute's vapor pressure (Hawthorne 1995). However, very little solubility data are available in the literature to assess the effects of elevated temperature. Consequently, isolating the effects of temperature on analyte-matrix interactions is extremely difficult (Hauthal 2001).

Nemato et al. (1997) have confirmed the effect of temperature on the extraction recovery of multiresidue analysis of pesticides. The extraction was performed at extraction temperatures of 40, 50, 60, and 70 °C and a constant CO_2 density of 0.70 g/mL. For thiometon and quinalphos, varying extraction temperature had little influence on recoveries. The recoveries of malathion and chlorfenvinphos increased slightly from 50 to 70 °C. For azinphos-methyl, which yielded poor recovery (23.1%) at an extraction temperature of 40°C, the recovery increased slightly from 50 to 60 °C, and then a greater than twofold increase in recovery was observed at 70 °C. Similar trends were observed for azinphos-ethyl, pirimicarb, and benalaxyl. Dimethoate was not recovered at 40-60 °C but showed a significant increase in recovery at 70 °C. In particular, the recoveries of fensulfothion, dimethoate, mefenacet, propiconazole, and dimethipin were dramatically increased at 70 °C. Thus, extraction temperature was an important parameter in improving the recovery of some pesticides. The recoveries of almost all of the pesticides investigated were at least 80% at 70 °C. However, even at 70 °C, myclobutanil, triadimenol, lenacil, bitertanol, and methamidophos showed poor recoveries (17-48%), and acephate and propamocarb were not recovered at all without the addition of modifier.

2.2.2.3.1.5 Effect of CO_2 flow rate

In general, a lower flow rate results in a lower linear velocity and usually increases the extraction efficiency as a result of an extended contact between the supercritical fluid and the analytes (Hawthorne 1995). The slower the fluid velocity, the deeper it penetrates the matrix. The quantitative collection of the extracted analytes is simpler at lower extraction flow rates, particularly when the analytes are volatile. Moreover, a lower flow rate increases the trapping efficiency of analytes at the analyte trap (Pourmortazavi et al. 2007).

Nemato et al. (1997) studied the effect of CO_2 over a range of 1 to 4 mL/min. Pesticides were extracted from fortified Celite added with 0.40 mL of water as a modifier at a CO_2 density of 0.70 g/mL and an extraction temperature of 50 °C. In these experiments, no static extraction was used. The overall average recovery of the 88 pesticides at CO_2 flow rates of 1, 2, 3, and 4 mL/min were 92.3-93.1% indicating there were no significant differences in the overall average recovery and precision of the 88 pesticides at each CO_2 flow rate evaluated. In general, a lower flow rate results in a lower linear velocity and usually increases the extraction efficiency as a result of an extended contact between the supercritical fluid and the analytes. They selected 2 mL/min as a CO_2 flow rate.

2.2.2.3.1.6 Effect of fluid volume

Fluid volume is another important parameter to be considered in to enhance the efficiency of SFE. Berglöf et al. (1999) studied the influence of the volume of supercritical carbon dioxide used in the recovery was studied during the extraction of silica. Extractions were performed at a density of 0.85 g/mL, a temperature of 55°C, and a modifier addition of 200 μL methanol. It was found that the main fraction of the pesticides elutes during the first two mL, and no detectable increase in recovery was found after 5 mL. Thus, the fluid volume of 20 mL used in this investigation could be reduced with preserved recovery.

2.3 Removal of pesticides using biosorbents

Adsorption technology is currently being used extensively for the removal of pollutants from wastewaters. Removal of these contaminants requires cost effective technologies and a variety of techniques have been developed in the past decades in dealing with wastewater treatment. Currently, adsorption is believed to be a simple and effective technique for water and wastewater treatment and the success of the technique largely depends on the development of an efficient adsorbent (Gupta et al. 2011; Memon et al. 2007).

2.3.1 Factor affecting removal efficiency of sorbent

The different operating parameters such as pH, shaking speed, contact time, adsorbent dose and initial concentration of adsorbent have significant effect on the removal efficiency of sorbet by sorbent.

2.3.1.1 Effect of pH

The pH of the medium in which biosorption occurs has a pronounced effect on biosorption efficiency. It was reported that biosorption of dichlorodiphenyldrichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE) has not the same trend in the range of pH 2.0-9.0 by bagasse fly ash (BFA). This means that increasing the pH to a given value increases biosorption capacity, after which the reverse trend starts. Herein, by increasing pH from 2 to 7, the biosorption efficiencies increased from 0.21 to 3600 mg/kg for DDD and from 0.11 to 3420 mg/kg for DDE. In more basic solutions, removal efficiency decreased. They concluded that pH 7 is optimum for both of the pesticides (Gupta and Ali 2001). Gupta et al. (2002) studied the behavior of biosorption of lindane and malathion onto BFA in the pH range from 2 to 9. Increasing pH from 2 to 6 had positive effect, after which removal efficiency remained constant. Therefore, pH 6 was selected as optimum.

2.3.1.2 Effect of shaking speed

Shaking speed is used to homogenize the solution and it assists a high contact between the adsorbent and sorbet. Memon et al (2007) studied the sorption of carbofuran and methyl parathion

as a function of shaking speed in the range of 25-150 rpm. It was found that percent sorption increases with increasing shaking speed and attains a maximum sorption at 100 rpm and then declined with increasing shaking speed. They selected 100 rpm as an optimum shaking speed for both carbofuran and methyl parathion.

2.3.1.3 Effect of contact time

Contact time is a fundamental parameter in all mass transfer phenomena including bio-sorption. Gangadhar et al. (2016) have studied the effect of contact time for the removal of malathion and phorate pesticides by Tea (Camellia sinensis) waste over the range of 25-225 ^{o}C. They observed that, 3 h is the equilibrium time resulting 95 and 94 % removal respectively at 2.0 g/L of initial adsorbate concentration. Gupta et al. (2011) also studied the uptake of dye on mesoporous activated carbon prepared from waste rubber tire (RTAC) and activated carbon (CAC) and depicted that the sorption is quite rapid initially, gradually slows down and then reaches the equilibrium. In both systems nearly 50-60% of the ultimate adsorption occurred within 15 min of contact for RTAC and 28 min of contact for AC.

Equilibrium was attained at 40 min for RTAC-Acid Blue 113 and 60 min for CAC-Acid Blue 113 systems. The uptake of dye molecules by the adsorbents, and the time required for establishment of equilibrium suggest the effectiveness of these materials for wastewater treatment. They concluded that, decrease in amount of dye adsorbed with time may be due to aggregation of dye molecules around the adsorbent particles. This aggregation may hinder the migration of the adsorbate, as the adsorption sites become saturated, and resistance to diffusion of dye molecules in the adsorbents increases. The difference in the equilibrium time between the two adsorbents might be due to differences in the surface properties of the adsorbents.

2.3.1.4 Effect of adsorbent dose

Sorbent dose determines the capacity of a sorbent for a given initial concentration of sorbate. Some researchers wanted to know if it is possible to use lower values of this ratio while keeping the removal efficiencies satisfactory. Increasing the sorbent mass in a solution having fixed concentration of solute will increase the sorbent efficiency usually defined as mg sorbate/g sorbent.

The higher sorption capacity means that the sorbent has been utilized more efficiently since most of the sorbent removal capacity is exhausted and that less of the waste is produced (Memon et al. 2007; Memon et al. 2007). In most cases removal efficiency rises with increasing sorbate dose, reported rapidly increasing of percent sorption up to 90% by increasing the amount of sorbent from 0.1 to 0.4 g, and then remain almost constant up to 1 g of sorbent dose (Memon et al. 2007). However, in some cases, after some sorbent concentration, removal efficiency decreases (Aktar et al. 2007). This was explained as predominance of sorbent-sorbent interaction over sorbent-sorbate interaction.

2.3.1.5 Effect of initial concentration of pesticides

In many cases suppression of the all mass transfer resistances of the sorbate between the aqueous and solid phases can be provided as an important driving force by initial sorbate concentration parameter. The study done by Akhtar et al. (2007). observed that during the consideration of initial concentration of methyl prathion removal by BFA of sugarcane and rice bran, and RH under the optimized conditions. The results obtained by Ju et al. (1997) indicated that the increasing the biosorption capacity of lindane by dried bacteria of Z. ramigera, E. coli, B. subtilis, and B. megaterium from 370 to 2800, from 98 to 500, from 100 to 600, and 100 to 700 mg/g, respectively, was relatively involved by increasing of initial lindane concentration from 1 to 4 mg/L at a constant cell concentration of 8 g/L.

2.3.1.6 Temperature

In general, solubility and stability of pesticides depends on temperature and it is very important parameter in biosorption process. The effect of temperatures was examined by Gupta and Ali (2001) for the biosorption of DDD and DDE by bagasse fly ash from 30 to 50 ^{o}C. It was found that as long as the initial sorbate concentrations were kept low changing the temperature had no effect on removal capacities. On the other hand, at higher initial concentrations, increasing the temperature decreased removal efficiencies. For both of the sorbents, maximum removal capacity occurred at 308 ^{o}C, indicating that the biosorption process is exothermic.

El Bakouri et al. (2009) studied the effect of varying temperature in the range from 15 to 358 ^{o}C on the biosorption of endosulfan sulfate by 10 different natural organic substances. They observed that as the temperature raises removal efficiency decreases, which is explained by greater solubility of pesticides at higher temperature reducing the affinity of endosulfan sulfate for biosorption on the surface of the sorbents.

2.3.2 Adsorption isotherms and kinetics

2.3.2.1 Adsorption isotherms

Adsorption isotherm expresses the relation between the amounts of adsorbate (mg) removed from the liquid-phase by unit mass of biosorbent (g) at fixed temperature. To find the relationship between aqueous concentration (C_e) and sorbed quantity (q_e) at equilibrium, mostly isotherms models are used for fitting the data (Gupta and Babu 2006; Gupta et al. 2011; Memon et al. 2007). The Langmuir and Freundlich isotherm models are used to describe the biosorption equilibrium. The Langmuir model represents monolayer sorption on a set of distinct localized sorption sites with uniform energies and with no transmigration of sorbate in the plane of the surfaces. The following equations have been used to evaluate the efficiency of the sorbent materials (Abdullah and Prasad 2009; Abdullah and Prasad 2009 2014; Gupta and Babu 2006):

$$q_e = (C_0 - C_e) \times \frac{V}{M} \tag{2.7}$$

Where q_e is the equilibrium pesticide concentration on the adsorbent (mg/g), C_0 and C_e are the initial and equilibrium pesticide concentration in the solution (mgL^{-1}), respectively; V is the solution volume (L); and M *is* the mass of biosorbent (g). Langmuir parameters can be determined from a linear equation given below (Abdullah and Prasad 2009).

$$\frac{1}{q_e} = \frac{1}{q_{\max}} + \frac{1}{K_L q_{\max}} . \frac{1}{C_e} \tag{2.8}$$

The plot of $\frac{1}{q_e}$ versus $\frac{1}{C_e}$ gives a straight line of slope $\frac{1}{q_{\max} K_L}$ and intercept $\frac{1}{q_{\max}}$, so the values of Langmuir constants (K_L, q_{max}) are calculated from the slope and intercept of the linear plot respectively. The essential feature of Langmuir isotherm model can be expressed by means of a separation factor or equilibrium parameter (R_L), which is calculated according to the following equation (Abdullah and Prasad 2014):

$$R_L = \frac{1}{1 + K_L C_i} \tag{2.9}$$

Where C_i initial concentration of any value. The value of R_L indicates the type of biosorption isotherm is linear (R_L =1), favorable (0 < R_L <1), unfavorable (R_L >1) and irreversible (R_L = 0). The Freundlich isotherm is a nonlinear sorption model. This model proposes a monolayer sorption with a heterogeneous energetic distribution of active sites, accompanied by interactions between adsorbed molecules, in which it is characterized by the heterogeneity factor $\frac{1}{n}$ and the general form of this model is (Gupta and Babu 2006):

$$q_e = K_f C_e^{\frac{1}{n}} \tag{2.10}$$

The logarithmic form of Equation 2.10 is:

$$\log q_e = \log K_f + \frac{1}{n} \log C_e \tag{2.11}$$

Where K_f a constant related to the biosorption capacity and $\frac{1}{n}$ is an empirical parameter related to the biosorption intensity of the adsorbent. Typically, 1/n values range from 1 downwards. A value of $1/n \leq 1$ signifies that the biosorption is favorable. When the value of $1/n < 1$, K_f decreases with concentration which means unfavorable biosorption indicative of saturation of adsorption sites available to the chemical, resulting in relatively less adsorption. The Freundlich isotherm constants (n, K_f) are calculated from the slopes and intercepts of the linear plot of $\log q_e$ versus $\log C_e$ (Adams and Watson 1996; Memon et al. 2007, Gupta and Babu 2006).

2.3.2.2 Sorption kinetics

The rate of sorption onto a sorbent surface depends upon a number of parameters such as structural properties of the sorbent, initial concentration of the solute and the interaction between the solute and the active sites of the sorbent. Rate of adsorption is usually measured by determining the change in concentration of the adsorbate in contact with the adsorbent as a function of time. The controlling mechanism of the biosorption process was investigated by fitting first and second-order kinetic models to the experimental data (Abdullah and Prasad 2009). If the kinetic data obtained were fitted to linear form of pseudo-first-order, the rate of occupation of biosorption sites is proportional to the number of unoccupied sites. The pseudo-first-order kinetic model known as the Lagergen equation is expressed as (Oliveira et al. 2008; Vinodhini and Das 2010):

$$\log(q_e - q_t) = \log q_e - 0.4342 k_1 t \qquad (2.12)$$

Where q_e and q_t are the amounts of pesticide adsorbed per unit mass of biosorbent (mg/g) at equilibrium and at time *t*, respectively, and k_1 is the rate constant for first-order adsorption (min^{-1}). The slope and intercept of the plot, $\log(q_e - q_t)$ versus t were used to obtain the first-order rate constant k_1 and equilibrium adsorption capacity q_e.

For pseudo second-order model, the sorption data were analyzed according to the pseudo second-order kinetic model. This kinetic model is based on the assumption that the biosorption process follows a second order mechanism, with chemisorption as the rate limiting step. Therefore, the occupation rate of adsorption sites is proportional to the square of the number of unoccupied sites and can be expressed as follows (Vinodhini and Das 2010; Rozaini et al. 2010):

$$\frac{t}{q_t} = \frac{1}{k_2 (q_e)^2} + \frac{t}{q_e} \qquad (2.13)$$

Where k_2 is rate constant for the second-order adsorption kinetics (g mg^{-1} min^{-1}). The straight-line plot of $\frac{t}{q_t}$ against t was used to determine the rate constants and correlation coefficients for second order kinetic models; q_e and k_2 can be calculated from the slope and intercept of the line.

Percent removal of pesticide by adsorbent was also evaluated using the formula (Bakouri et al. 2009; Chowdhury et al. 2012):

$$\text{Removal } (\%) = \frac{C_o - C_e}{C_o} \text{ x } 100 \qquad (2.14)$$

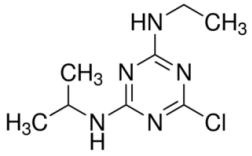
where C_0 and C_e are the initial and final concentrations of pesticides in the water sample, respectively.

3 EXPERIMENTAL

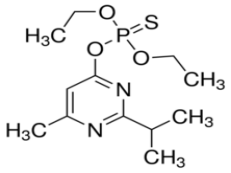
3.1 High density based dispersive liquid-liquid microextraction combined with gas chromatography-mass spectrometry (HD-DLLME-GC-MS) for simultaneous determination of multiclass pesticide residues in water and sugarcane juice samples

3.1.1 Chemicals and reagents

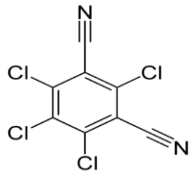
All the standards of pesticide compounds; *viz*., atrazine, diazinon, chlorothalonil, ametryn, malathion, chlorpyrifos and dimethametryn (Figure 3.1.1) are of analytical reagent grade and were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The disperser and extraction solvents were obtained from different sources: methanol was purchased from Acros organics (New Jersey, USA); acetone from Scharlau (Barcelona, Spain) and acetonitrile from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), carbon tetrachloride from BDH Chemicals Ltd. (Poole, England); tetrachloroethylene from May and Baker Ltd. (Dagenham, England) and chloroform from Sigma Aldrich (Seelze, Germany). Extra pure sodium chloride was purchased from Oxford laboratory (Mumbai, India) and used to study the effect of ionic strength. Sodium hydroxide pellets were from BDH Laboratory Supplies (Poole, England) and hydrochloric acid from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used to adjust the sample pH. Ultrapure water used as reagent water during method development and application was obtained by purifying with double distiller, A8000 Aquatron water Still (Bibby Scientific, Staffordshire, UK) and deionizer (EASYPure LF, Dubuque).



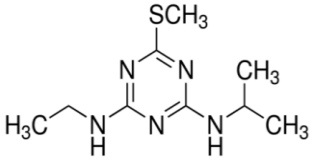
Atrazine
(Log P: -0.97, pK_a: 1.7)



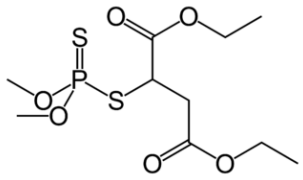
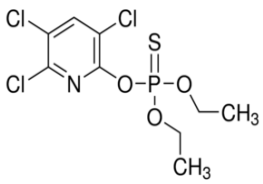
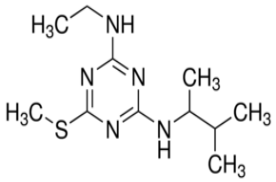
Diazinon
(Log P: 3.69, pK_a: 2.6)



Chlorothalonil
(Log P: 2.94, pK_a: na)



Ametryn
(Log P: na, pK_a: 4.1)

Malathion (Log P:3.74, pK_a:4.3)

Chlorpyrifos (Log P: 4.0, pK_a: na)

Dimethametryn (Log P: 3.9, pK_a: 4.0)

K_a: acid dissociation constant, na: not available, P: octanol water partition coefficient

Figure 3.1.1 Chemical structures, common names, log P and pK_a of the analyte considered in this study (Mnif et al. 2011; Megersa and Jönsson 1998).

3.1.2 Preparation of standard solutions

Stock standard solution of 100 mg/L of the target analytes was prepared in methanol. Working solutions of 1 mg/L were also prepared by diluting the standard stock solution in methanol. A series of solutions for calibration were prepared in reagent water, at five concentration levels, from the working solution, to study the method linearity. Reagent water was spiked by diluted standard solution, 5 µg/L, containing the mixture of the target analytes and used for parameter optimization. Similarly, 2.5 and 5 µg/L mixture of the pesticide compounds were also prepared, in the same manner as above, for determination of the percent recovery (%RR). All solutions were stored in the dark at 4 ^oC until the time of analysis.

3.1.3 Sampling sites and sample collection

Two different water samples were collected from Hawassa Lake; located at 7^{o}01'52.7"N latitude and 38^{o}25'18.8"E longitude with elevation of 1685 m above the sea level and Wonji Shoa sugar factory irrigation; at latitude of 8^{o}27'15.4"N and longitude of 39^{o}13'49.3"E with elevation of 1552 m above the sea level. Similarly, sugarcane juice sample was also collected from Wonji Shoa sugar factory, all in Ethiopia. All the samples collected for this study were stored in brown glass bottles and transported to the analytical laboratory of the Addis Ababa University. They were then kept in a refrigerator, at 4 ^oC in the dark, for a maximum of 24 h. The samples were then filtered through 0.45 cellulose acetate filter papers (0.45 µm, MicroScience and 110 mm Smith F1/KA4, Germany) for further analysis. A 5 mL sugarcane juice sample was diluted to 15 mL with reagent water. The

sugarcane juice sample was centrifuged for 15 min at 3800 rpm (Scientific Ltd, K240, UK). After centrifugation, 5 mL of the supernatant was subjected to the DLLME procedure.

3.1.4 GC-MS analysis

Agilent Technologies, 7820A gas chromatography (GC) equipped with Agilent Technologies, 5977E inert mass spectrometry (MS) detector was used to analyze the pesticide compounds. GC separations were carried out on Hp-5MS ultra inert capillary column (30 m x250 µm 0.25 µm). Helium gas (99.999%) was used as carrier gas at a flow rate of 1 mL min^{-1} and the analytical results obtained were interpreted using mass hunter Chem-Station. The oven temperature programme employed for separation were as follows: 130 ^{o}C for 0 min; increased at 25 ^{o}C/min to 185 ^{o}C held for 1 min; then increased at 9 ^{o}C/min to 200 ^{o}C for 1 min and 10 ^{o}C/min to 290 ^{o}C held for 1 min. The GC oven and injection port temperature were maintained at 290 ^{o}C and 250 ^{o}C, respectively.

All injections were made in splitless mode. The mass detector was used in the full scan mode and scanned over the range m/z 50-550 to confirm the retention times of the analytes. Selective ion monitoring (SIM) mode was used for determination of all the analytes. For identity, confirmation of pesticides was made by selecting the most abundant characteristic ions of each pesticide and two characteristic fragment ions. The m/z selected for SIM mode detection was as follows: atrazine (215.1, 200.1 and 173.1), diazinon (152.1, 137.1 and 124.1), chlorothalonil (267.9, 265.9 and 263.9), ametryn (228.1, 227.1, 226.1), malathion (178.1, 174.1 and 158.1), chlorpyrifos (198.9, 196.9 and 179.1) and dimethametryn (213.1, 212.1 and 196.1).

3.1.5 HD-DLLME-GC-MS procedure

A modified 5 mL micropipette tip was used as extraction vessel. The micropipette tip was burned using match flame to close the tip. Initially, 5 mL of the pretreated water and sugarcane samples were adjusted to pH 7, separately in a beaker. Each of the resulting solution was spiked into appropriate quantity of the standard solution containing the mixture of the analytes under study. Afterwards, a mixture containing 40 µL chloroform and 0.4 mL methanol was injected rapidly into the sample solution, in the modified micropipette tip. Then, the contents (sample solution, extraction solvent and disperser solvent) in the tip ended up with emulsion formation. The cloudy solution was left to stand

for 3 min and then the content was centrifuged at 4000 rpm (Centrifuge model 800, China) for 3 min, for allowing phase separation. This was followed by collection of the sedimented phase, using 100 µL microsyringe, (microliter®#710), as indicated in Figure 3.1.2. Finally, 1 µL of each extract was injected into a GC-MS system for analysis.

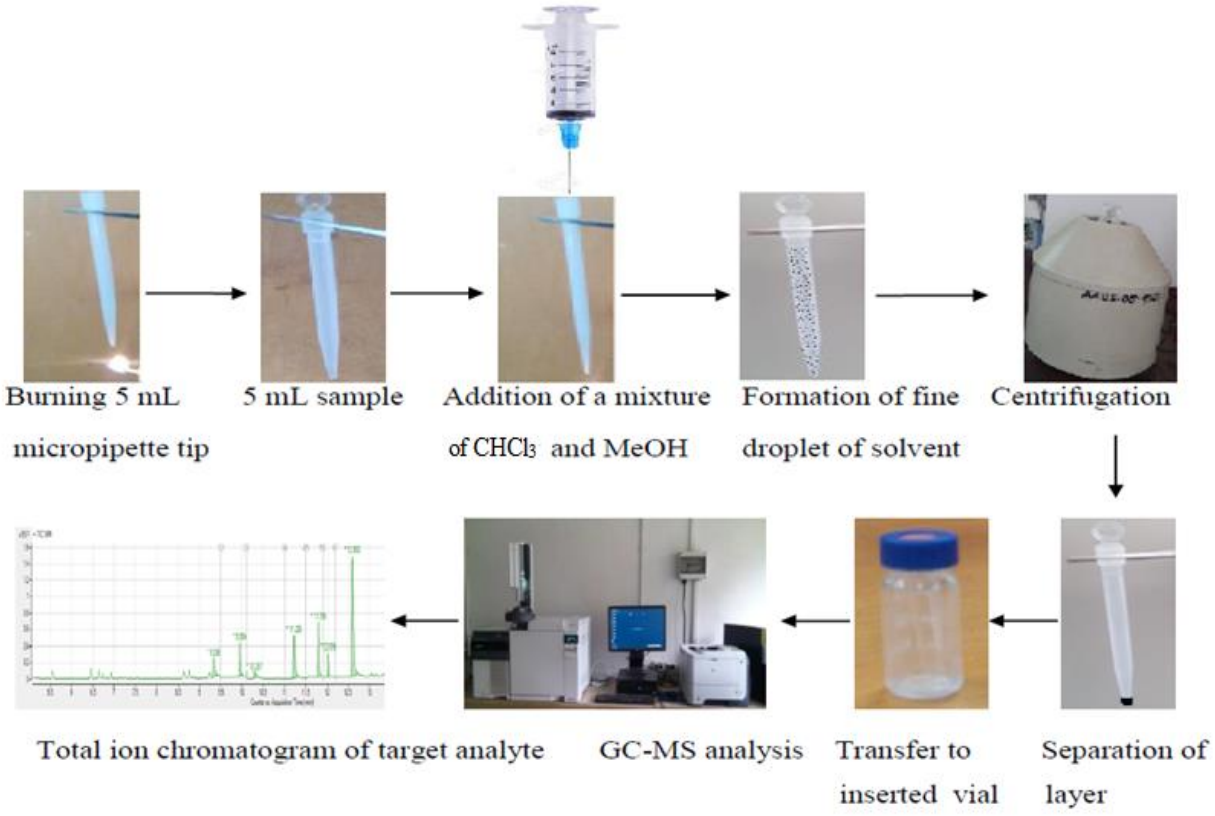
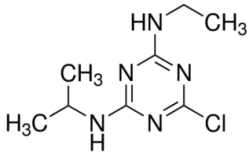


Figure 3.1.2 Experimental set-up of the proposed HD-DLLME extraction procedure.

3.2 Modified SALLE combined with LD-DLLME for multiclass pesticide residues analysis in sugar and soil using gas chromatography-mass spectrometry

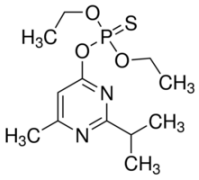
3.2.1 Chemicals and reagents

All of the target pesticides given in Figure 3.2.1 are of analytical grade and purchased from Dr. Ehrenstorfer (Augsburg, Germany).



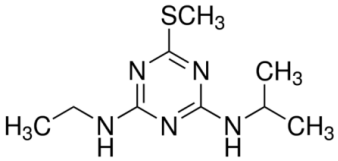
Atrazine

(Log P: -0.97, pK_a: 1.7)



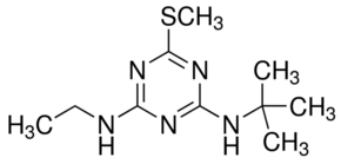
Diazinon

(Log P: 3.69, pK_a: 2.6)



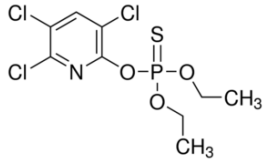
Ametryn

(Log P: na, pK_a: 4.1)



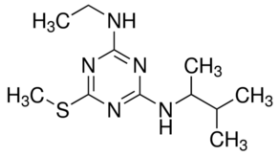
Terbutryn

(Log P:3.74, pK_a:4.3)



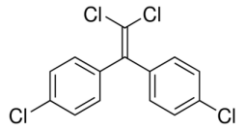
Chlorpyrifos

(Log P: 4.0, pK_a: na)



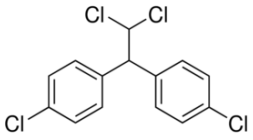
Dimethametryn

(Log P: 3.9, pK_a: 4.0)



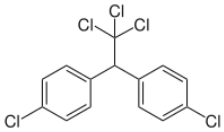
4,4'-DDE

(Log P: 6.51, pK_a: na)



4,4'-DDD

(Log P: 6.02, pK_a: na)



4,4'- DDT

(Log P: 6.51, pK_a: na)

Figure 3.2.1 Chemical structures, physical contstants and common names of the pesticides used in this study.

A 100 mg/L stock standard solutions of atrazine, diazinon, ametryn, terbutryn and dimethametryn were prepared by dissolving an appropriate amount of each standard in methanol. In the same way

100 mg/L stock standard solutions of chlorpyrifos, 4,4'-DDE, 4,4'-DDD and 4,4'-DDT were prepared by dissolving in small amount of ethyl acetate until completely dissolved and diluting to the required volume with methanol. An intermediate working solution containing 5 mg/L of each analyte was also prepared in the mixture of methanol for use during optimization of the extraction parameters and stored in fridge at 4 °C. All chemicals and reagents used in this study were of analytical grade, while the solvents were of HPLC grade. Acetone, methanol and acetonitrile were purchased from Scharlau (Barcelona, Spain), Techno Pharmchem (New Jersey, USA) and Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), respectively. Ethyl acetate was supplied by VWR BDH Prolabo (West Chester, PA, USA). The extraction solvent for LD-DLLME, toluene (99+ %) was obtained from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) and magnesium sulphate ($MgSO_4$) were purchased from BDH Chemicals Ltd (Poole, England). Sodium hydroxide pellet and hydrochloric acid were obtained from BDH Laboratory Supplies (Poole, England) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany), respectively to adjust the sample solution pH.

3.2.2 Instruments

Chromatographic analyses were performed using Agilent Technologies, 7820A gas chromatography (GC) equipped with Agilent Technologies 5977E inert mass spectrometry (MS) detector and separations were achieved on DB-5MS (USA) ultra inert capillary column (30 m x 250 µm and 0.25 µm i.d.). Data acquisition and processing were accomplished with Agilent mass hunter Chem-Station software. An electronic balance (Adam Equipment Company, UK) was used for weighing during various experiments. To measure pH values Adwa pH meter, model 1020 (Romania) were used. A centrifuge, Model 800 (China, Beijing), vacuum oven (LABLINE INSTRUMENTS, England), ultrasonic heater (Decon F5100b, England), filtrating apparatus with vacuum pump (Quick FIT, England), cellulose acetate filter papers (0.45 µm, MicroScience and 110-mm Smith F1/KA4, Germany) and deionizer (EASY Pure LF, Dubuque) were used in the process of sample preparation and analysis.

3.2.3 Chromatographic conditions

Chromatographic separation was performed on DB-5MS. The mobile phase used was helium gas (99.999 %) and delivered at a flow rate of 1 mL/min. The oven temperature program was set up as follows: 70 ^{o}C for 0 min; increased at 30 ^{o}C/min to 150 ^{o}C held for 1 min; then increased at 45 ^{o}C/min to 290 ^{o}C for 3 min. The GC oven and injection port temperature were maintained at 290 ^{o}C and 250 ^{o}C, respectively. In order to confirm the retention times of the analytes, the mass detector was scanned in full mode over the range *m/z* 50-550. Selective ion monitoring (SIM) mode was used by selecting the most abundant characteristic ions of each pesticide and two characteristic fragment ions for quantitative determination of all the analytes. The *m/z* selected for SIM mode detection were as follows: atrazine (215.1, 200.1, 173.1), diazinon (152.1, 137.1, 124.1), ametryn (228.1, 227.1, 226.1), terbutryn (170.0, 227.1, 226.1), chlorpyrifos (198.9, 196.9, 179.1), dimethametryn (213.1, 212.1, 196.1), 4,4'-DDE (176.1, 246, 317.9), 4,4'-DDD (165.1, 235, 237), 4,4'-DDT (199, 235, 246). All injections were made in splitless mode.

3.2.4 Sampling and sample preparation

Both sugar and soil samples were collected from Wonji Shoa sugar factory and farm lands, Oromia Regional States of Ethiopia, respectively. The geographical location of sampling place is 8^{o}27'14.9"N latitude and 39^{o}13'49.4"E longitude with elevation of 1552 m above sea level. A composite of sugar sample (5 portion) was taken from the factory at a time interval of 30 min randomly. A composite of soil sample (10 portion) was also taken from sugarcane farm lands according to the procedure described by Merdassa et al. (Merdassa et al. 2013). Ten holes of 25 cm depth were made using a spade. Then, a 5 cm thickness slices along the vertical wall of the holes were taken. All sugar and soil collected were pooled separately on methanol rinsed aluminum sheet each having an area of 3 m^2 and mixed manually. Each samples, sugar and soil, samples were divided into six places. A small amount was taken from each portion to make a sub sample of 1 kg and transported to the laboratory in a chilled insulating box. The soil samples were air dried, grounded with electric mill, sieved through a 0.25 mm pore size, wrapped in a methanol rinsed aluminum foil and kept in a polyethylene plastic bag.

3.2.5 SALLE-LD-DLLME procedure

A 1 g of soil or sugar sample was accurately weighed on an aluminum sheet and transferred into centrifuge tube and then subsequently spiked with appropriate concentrations of the target analyte using a mixture of standard solution. A 5 mL of distilled and deionized; ultra pure water; water adjusted to pH 7 was added to dissolve the solid samples, to make the sample matrices more accessible to the extraction solvent and to remove water soluble components. Then, 1.5 mL of acetonitrile (ACN) was added to the resulting solution and the mixture was shaken to homogenize. After keeping for 3 min to establish equilibration, 25% (*w/v*) $(NH_4)_2SO_4$ was added to the mixture solution and the mixture solution was shaken until the salt was dissolved. The solution was separated into two clear phases after centrifugation at 3000 rpm for 3 min. The upper solution of sugar sample was eluted through packed florisil SPE cartridge (500 mg), conditioned by 5 mL ACN, and further eluted with 2 mL of ACN. The soil sample extract was also diluted by 2 mL ACN and transferred to the d-SPE tube containing 12 mg Supel^{TM} QuE PSA/C18 sorbent containing a mixture of 150 mg Supelclean PSA, 150 mg Discovery DSC-18 and 900 mg of $MgSO_4$, preconditioned with 5 mL of ACN, for cleanup and shaked manually. Then, it was centrifuged for 3 minutes at 4000 rpm. The collected organic phase extract was dried in vacuum oven and the residue was dissolved in 0.6 mL of acetone and the resulted solution was subjected to LD-DLLME procedure.

A LD-DLLME was adopted from our previous work (Tolcha et al. 2013). For the DLLME, 5 mL aqueous solution of NaCl, 10% *w/v*, adjusted to pH 7 was placed in home designed modified Pasteur pipettes (Figure 3.2.2). The mixture of 50 µL of toluene and 0.6 mL of the acetone containing the pesticide residue (as a disperser solvent) was injected rapidly at room temperature by using 1 mL syringe and shaked vigorously. After 10 min, another 0.5 mL of acetone, as a demulsifier, was injected slowly to the resulted solution to break up the emulsion. This was followed by collection of the organic phase using a microsyringe and transferred into inserted GC injection vial. Finally, 1 µL of the extract was injected directly into GC-MS system for further instrumental analysis and peak area was used as an instrumental response.

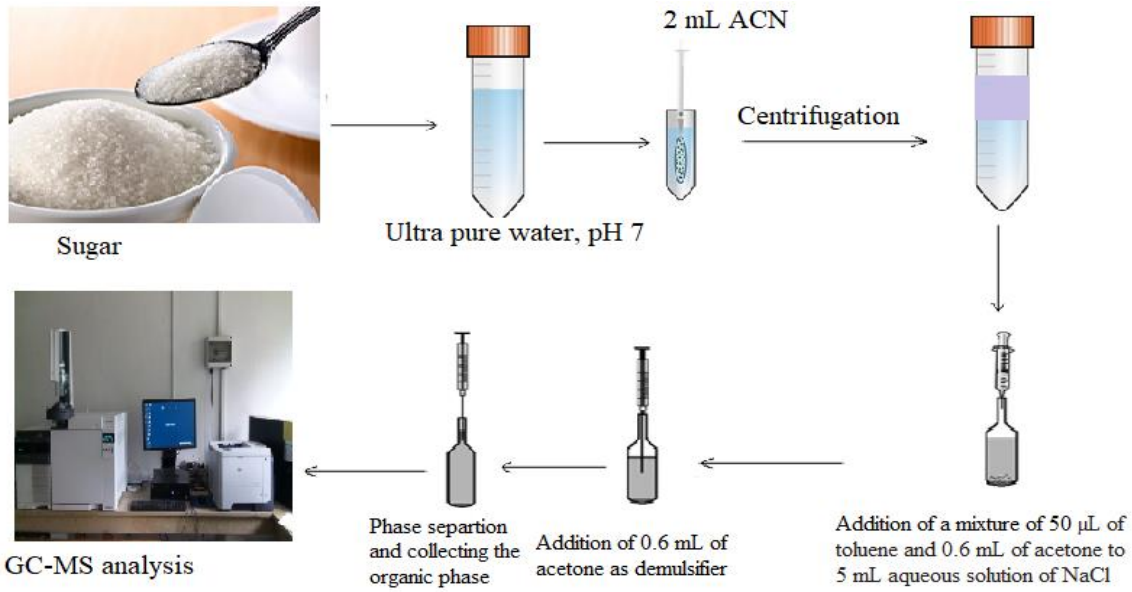
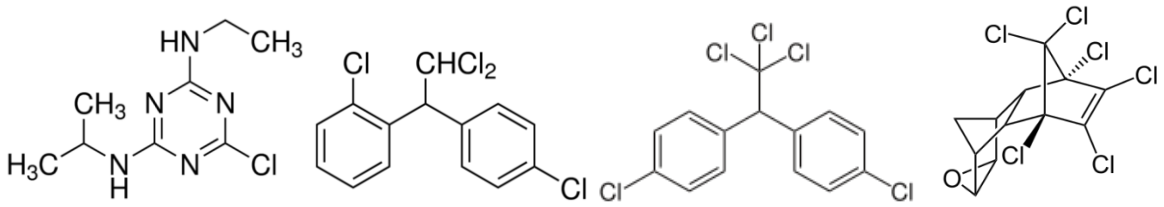


Figure 3.2.2 A systematic diagram for SALLE-LD-DLLME procedure used in this particular work.

3.3 High density sc-CO_2 extraction for quantitative analysis of *s*-triazine and organochlorine pesticides in onion

3.3.1 Chemical reagents and standards

Methanol and n-heptane were the organic solvents of chromatographic grade and purchased from VWR BDH Chemiclas (Poland) and Fisher Chemicals (UK), respectively. Analytical grade standards including atrazine, 2,4'-DDD, 4,4'-DDT and endrin (Figure 3.3.1) were purchased from the sigma Aldrich (Buchis, Siwizerland). The stock solution was prepared by weighing 2.5 mg of each standard and dissolving in small amount of ethyl acetate and the resulting solution was diluted with methanol in a 25 mL volumetric flask and stored at −19.5 ^{0}C. Fresh working solutions were prepared by proper dilution of the stock solution with methanol.



Atrazine (Log P: -0.97, pK_a: 1.7) 2,4'-DDD (Log P: 6.02, pK_a: na) 4,4'-DDT (Log P: 6.51, pK_a: na) Endrin (Log P: 4.8, pK_a: na)

na: not available

Figure 3.3.1 Chemical structures, physical constants and common names of the pesticides for this work.

3.3.2 Equipments

Chromatographic analyses were performed using G2614A gas chromatography (GC) equipped with G1099A mass spectrometry (MS) supplied by (HEWLETT PACKARD, USA**)**. A HP-5 MS (5% Phenylpolysilixam) (USA) ultra inert capillary column (30 m x 250 µm and 0.25 µm i.d.) and helium gas (99.999%) was used for GC separations and carrier gas respectively. Data acquisition and processing were accomplished with enhanced data analysis software (HEWLETT PACKARD, USA**)**. Nitrogen drier, electronic balance, 5 mL extraction cell, 100 µL micropipette and test tube were used during extraction process.

3.3.3 GC-MS analysis

A HEWLETT G2614A gas chromatography (GC) equipped with G1099A mass spectrometry, MS (HEWLETT PACKARD, USA**)** with an inert ion source was used to analyze the pesticide residues level. GC separation was accomplished by HP-5MS. Helium gas of purity 99.999% was used as carrier gas at a flow rate at 1 mL/min with constant pressure of 252 Kpa. The oven temperature program used during analysis was set as follows: 82 ^oC for 1 min; increased to 185 ^oC at 25 ^oC/min ramp rate and held at this temperature for 1 min; then increased to 250 ^oC at 9 ^oC/min ramp rate and maintained at this temperature for 1 min. The GC oven and injection port temperatures were kept at 260 ^oC and 250 ^oC, respectively. Splitless injection mode was used during the whole

analyses. Peaks were identified by their retention time and mass spectra after acquisition of the total ion chromatogram. In order to confirm the retention times of all analytes, scan mode was carried over the range m/z 50-550. Selective ion monitoring (SIM), of each pesticide and two characteristic fragment qualifier ions were selected to identity of pesticides. The *m/z* selected for SIM mode detection was: atrazine (173, 200, 2015), 2,4'-DDD (199, 235, 237), 4,4'-DDT (212, 235, 239) and endrin (245, 263, 281). Peak area was utilized as an instrumental response. Quantitative determination of the instrumental responses was based on the peak areas.

3.3.4 Sampling and sample pretreatment

Onion samples were collected randomly from local market in Addis Ababa, Ethiopia. The geographical location of sampling place is 8º58'50.2"N and longitude 38º48'27.9"E with elevation of 2296.1 m above sea level. The onion was cut into small pieces using iron knife and air dried. It was further dried in oven at 92 ºC until constant mass was obtained. Then, the sample was grounded with electric mill, sieved through a 0.25 mm pore size, wrapped in a methanol rinsed aluminum foil and kept in a polyethylene plastic bag.

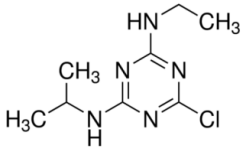
3.3.5 A sc-CO_2 extraction procedure

A sc-CO_2 extraction of pesticides were carried out by using the Waters ASFE MV10 extraction system, Waters SFC 5 mL vessel (USA), an SFX-200 controller, 100 DX syringe pump (ISCO, Sweden), restrictor heater (PEWEE BOXER ®, Thialand) and a liquid carbon dioxide (CO_2) cylinder that was pressurized up to work pressure. A 1 g of onion sample was fortified by adding an appropriate volume of standard working solution and mixed with 1g of boiling beads to reduce volume in Waters SFC 5 mL extraction cell in a sandwich mode, using a silanized glass wool at both the bottom and the top of the cell to protect cell sealing. Then, it was heated in GC oven for 5 min at 53 ºC and extraction was continued in dynamic mode by 29 mL of CO_2 at 3 mL/min. The density of CO_2 was kept at 0.9 g/mL. Then the extraction was flushed with 2 mL of n-heptane and dried under gentle N_2 stream. Finally, it was reconstituted by 100 µL of n-heptane and 2 µL was injected to GC-MS for analysis.

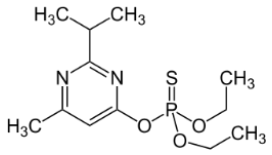
3.4 Multivariate optimization of combined static and dynamic mode sc-CO_2 for trace analysis of pesticide residues in honey

3.4.1 Chemicals and reagents

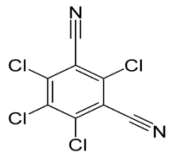
The organic solvents used were methanol which was purchased from VWR BDH Chemicals (Poland), acetone from Fisher Chemicals (UK, England) and acetonitrile from Schrlau Chemie S.A. (Spain) and all are of chromatographic grade. Analytical standards including atrazine, diazinon, chlorothalonil and deltametryn (98% >) were purchased from the sigma Aldrich (Buchis, Siwizerland). A solution in methanol were also prepared and used as a high performance liquid chromatography photo diode array detector mobile phase. The stock solution of 100 mg/L in methanol were prepared and stored in a deep freezer at −19.5 ^{0}C. Fresh working solution of 40 mg/L were prepared by proper dilution of the stock solution with methanol.



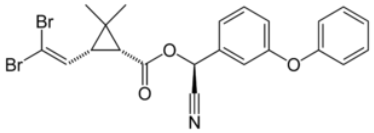
Atrazine (S = 30 mg/L, 25 ^{o}C)



Diazinon (S = 40 mg/L, 25 ^{o}C)



Chlorothalonil (S = 0.81 mg/L, 25 ^{o}C)



Deltametryn (S = 0.002 mg/L, 25 ^{o}C)

Figure 3.4.1 Chemical structures, solubility (S) and common names of the pesticides studied in this work.

3.4.2 Equipments

Chromatographic analyses were performed using DIONEX Ultimate 3000 ultra high pressure liquid chromatography 3000 photodiode array detector (HPLC-PDAD). Separation was performed

using Zorbax SB-C18 (USA) capillary column (2.1 x 150 mm 3.5 µm i.d.). A binary solvent system; milli-Q water and methanol was used as a mobile phase. Data acquisition and processing were accomplished with Chromeleon Ultimate-300 software. Waters ASFE MV10 extraction system, Waters SFC 5 mL vessel (USA), an SFX-200 controller, 100 DX syringe pump (ISCO, Sweden), restrictor heater (PEWEE BOXER ®, Thialand) and a syphonated carbon dioxide (CO_2) cylinder that was pressurized up to work pressure, nitrogen drier, 5 mL extraction cell, electronic balance, test tube, inserted vials and 100 µL micropipette were also used during extraction process.

3.4.3 HPLC-UV-VIS analysis

A DIONEX Ultimate 3000 ultra high pressure liquid chromatography was used to analyze the pesticide residues level. A separation was accomplished by Zorbax SB-C18 (USA) capillary column (2.1 x 150 mm and 3.5 µm i.d.). A binary mobile phase comprising of solvent A (Milli-Q water) and solvent C (methanol) was used in a gradient elution mode Table 3.4.1. The detection wavelength was adjusted at 221 nm with bandwidth of 4 nm in reference to wavelength 360 nm having bandwidth 100 nm. The autosampler and column temperature were kept at 20 ^{o}C and 40 ^{o}C respectively. Peak identification was based on the retension time and peak area was used as instrumental response.

Table 3.4.1 HPLC-DAD condtions.

No.	RT (min)	Flow (µL/min)	%A	%C
1	0	700	53	47
2	3.5	700	0	100
3	5	700	0	100
4	6	700	53	47
5	8	700	53	47

3.4.4 Sampling site and sample collection

Agrochemical and industrial practices are mainly practiced in the Central Oromia Region, Ethiopia because of the availability of the infrastructure and water resources. One of the booming agro-

industry in Ethiopia is a floriculture. Bishoftu town which is found Oromia regional state is known for wide cultivation of floriculture. The geographical location of Bishoftu town is 8°44'4''N latitude and 38°59'9''E longitude at an altitude of 1925 m. There are more than 15 floriculture industries established around the Wedecha River which is found 10 km away from the town. The effluents of floriculture industries are directly discharged to the river and impose impacts on the surrounding environment. A composite of honey samples was directly collected using bulk honey sampling techniques from around floriculture areas of Bishoftu town. A composite sample consisted of portions collected from 6 different hives. All honey samples were stored in glass container, maintained at room temperature until extraction and analysis.

3.4.5 A sc-CO_2 extraction procedure

The extraction was carried out by using the SFX-220 extraction system (ISCO, Lincoln, NE, USA) that consists of an SFX-220 extractor, an SFX-200 controller, 100 DX syringe pump connected to a liquid CO_2 cylinder that was pressurized up to work pressure. A 2 g of honey sample was spiked by adding an appropriate volume of standard working solution and mixed with 2 g of glass beads in a stainless steel extraction cell (5.6 cm × 1.6mm i.d.) in a sandwich mode, using a silanized glass wool at both the bottom and the top of the cell. Then, 2 mL of acetonitrile was added to the contetns as a modifier solvents and heated in GC oven for 11.5 min at 70 °C. The dynamic mode extraction was performed with 30 mL of liquid CO_2 at 3 mL/min. The pressure was kept at 252 bar. Then the extraction was flushed with 2 mL of acetonitrile and dilute the extract with 1 mL of CAN. After centrifuging at 9000 rpm for 10 min, it was dried under gentle N_2 stream. Finally, it was reconstituted by 100 µL of methanol and 10 µL was injected to HPLC-DAD for analysis.

3.5 *Typha latifolia* plant parts as a low cost adsorbent for removal of selected multi pesticide residues in contaminated aqueous samples

3.5.1 Chemicals and reagents

All the pesticide standards given in Table 3.5.1 were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Chloroform was purchased from Sigma Aldrich (Seelze, Germany) and methanol from Acros Organics (New Jersey, USA), and were used as extraction and disperser

solvent, respectively. To adjust the sample pH hydrochloric acid, from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and sodium hydroxide pellets, from BDH Laboratory Supplies (Poole, England), were used. Stock standard solution of 100 mg/L of the target compounds was prepared in methanol. Working solutions, containing a mixture of each analyte, of 10 mg/L were prepared by diluting the standard stock solution with methanol. All solutions were stored in the fridge at 4 ^{o}C when not in use.

Table 3.5.1 Physico-chemical and physical constants of pesticides used in this study (Dehghani et al. 2017; Merdassa et al. 2013; Mnif et al. 2011; Moussavi et al. 2011).

Analyte	**Molecular formula**	**Molecular weight (g/mol)**	**Solubility (mg/L)**	**Density (g/cm)**	**pK_a (25 °C)**	**Log P (25 °C)**
Atrazine	$C_8H_{14}ClN_5$	215.68	30 (20 °C)	1.19	1.7	-0.97
Diazinon	$C_{12}H_{21}N_2O_3PS$	304.35	40 (25 °C)	1.12	2.6	3.69
Chlorothalonil	$C_8Cl_4N_2$	265.91	0.81 (25 °C)	1.80	-	2.94
Ametryn	$C_9H_{17}N_5S$	227.33	185 (20 °C)	1.19	4.1	2.63
Malathion	$C_{10}H_{19}O_6PS_2$	330.35	145 (20 °C)	1.23	2.6	2.75
Chloropyrifos	$C_9H_{11}Cl_3NO_3PS$	350.59	2 (25 °C)	1.40	-	4.0
Dimethametryn	$C_{11}H_{21}N_5S$	255.38	19.6 (25 °C)	1.11	4.0	3.90

3.5.2 GC-MS analysis

GC-MS analysis and extraction procedure was adopted from section 3.1.4 and 3.1.5 respectively.

3.5.3 Preparation of adsorbents and characterization

The preparation method is the key factor for any type of biosorbent, because morphological properties such as particle size and shape, binding surface area and overall removal capacity depend on it. A simple to prepare and use, hazard free and environment friendly treatments are the major requirements for sustainable preparation of biosorbent. Considering the above, this research used a simple and non-treated preparation methods rather than the expensive and high technology and non-environment friendly acid/base pre-treatment methods (Oboh eta al. 2009; Singha et al. 2011). This is the novelty of this study. *Typha latifolia* plant samples, Figure 3.5.1 were collected from the vicinity of Muger river, North Shoa, Oromia region, Ethiopia, using pre-cleaned polyethylene bags. The geographical location of sampling site is 9º54'00.4"N latitude and 37º57'11.9"E longitude with elevation of 1548.4 m above the sea level. The collected stem, leaf and flower were washed with tap water for several minutes. Then, washed again with distilled and de-ionized water. After cutting into small pieces the biosorbents were sunlight dried and grounded using an electric mill. The powder was sieved using 250 µm sieve particle size analyzer. The powdered samples were further dried at 105 °C in an oven until constant mass of the powdered sample was obtained and stored in a polyethylene bag until it is used in the adsorption process. The fresh and pesticide loaded adsorbents were mixed separately with KBr of spectroscopic grade and made in the form of pellets. The pellet formed were scanned in the spectral range of 4000−400 cm^{-1} using a Fourier transform infrared spectrophotometer (Shimadzu, IR Prestige-21).

Figure 3.5.1 *Typha latifolia* plant at sampling site (A) and after processed; stem (B), leaf (C) and flower (D).

3.5.4 Batch adsorption experiments

Batch experiments were carried out using a series of 100.0 mL Erlenmeyer flasks to investigate the effects of pH, contact time, shaking speed, adsorbent dose and initial pesticide concentrations on the adsorption of the pesticide from aqueous solutions. A weighed amount of biosorbent was added to the Erlenmeyer flasks with 50 mL of pesticide solution, adjusted to pH 7, 8 and 6 for leaf, stem and flower respectively, of known initial concentration. Then it was agitated at a constant speed of 200 rpm for 1h for stem, 150 rpm for 1h for leaf, and 150 rpm for 2 h for flower on an orbital shaker (Model CHINCAN, WSZ-100, China). Samples were collected from the Erlenmeyer flasks at regular time intervals and the residual pesticides concentration in the solution were preconcentrated by HD-DLLME and analyzed by GC-MS.

4 RESULTS AND DISCUSSION

4.1 High density based dispersive liquid-liquid microextraction combined with gas chromatography-mass spectrometry (HD-DLLME-GC-MS) for simultaneous determination of multiclass pesticide in water and sugarcane juice samples

4.1.1 Optimization of HD-DLLME procedure

In order to realize a reliable or applicable extraction procedure utilizing HD-DLLME, investigation of the effects of various experimental parameters and determining the optimum conditions must always be considered. In this analytical method, all experimental variables affecting the performances of the technique including the effect of the type and volume of extraction solvent as well as disperser solvent, the shaking speed and time, extraction time, pH of the sample solution and variation in quantities of the salt added have been studied and optimized. For analyses of the analytes, peak areas were used to evaluate the extraction efficiency and establish the optimum extraction conditions.

4.1.1.1 Selection of extraction solvent

Selection of appropriate extraction solvent is the primary step in the optimization procedure. Generally, in the HD-DLLME techniques extraction solvent should meet the following requirements. It should (a) have high density than water, (b) be insoluble in water, (c) have high extraction capability for the target analytes and (d) have good chromatographic behavior (Matsadiq et al. 2011; Wang 2016; Yang et al. 2012). In this study, analytical performances of chloroform, tetrachloroethane and carbon tetrachloride, as extraction solvent were evaluated. Based on the experimental results obtained, Figure 4.1.1 the highest responses, as peak area, were observed when chloroform was used as extraction solvent. Thus, chloroform was chosen as extraction solvent for further studies.

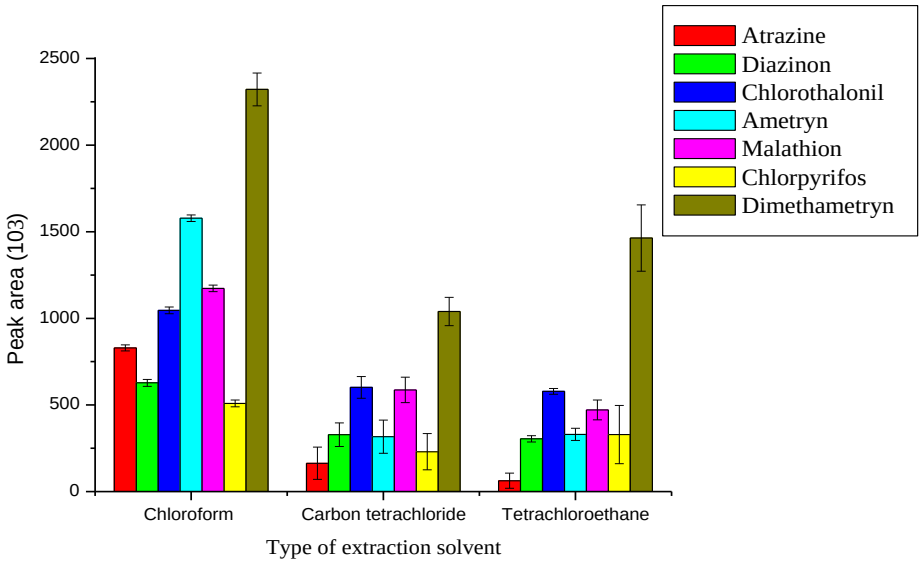


Figure 4.1.1 Effect of the type of extraction solvent on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent volume, 50 µL; disperser solvent, 0.5 mL methanol; extraction time, 1 min; centrifugation speed, 3500 rpm for 3 min; n = 3.

4.1.1.2 Selection of disperser solvent

An ideal disperser solvent, used in DLLME, must have the capacity to make finer droplets of the extraction solvent and disperse extraction solvent in the sample bulk (Nagaraju and Huang 2007; Rezaee et al. 2006). Furthermore, the disperser solvent should be miscible both in the organic and aqueous phases in order to form a distinct cloudy solution (Liang et al. 2013; Lin et al. 2011). The type of disperser solvent can also influence viscosity of the organic phase and therefore affect the stability of the cloudy solution (Hashemi et al. 2010; Tabrizi and Rezazadeh 2012).

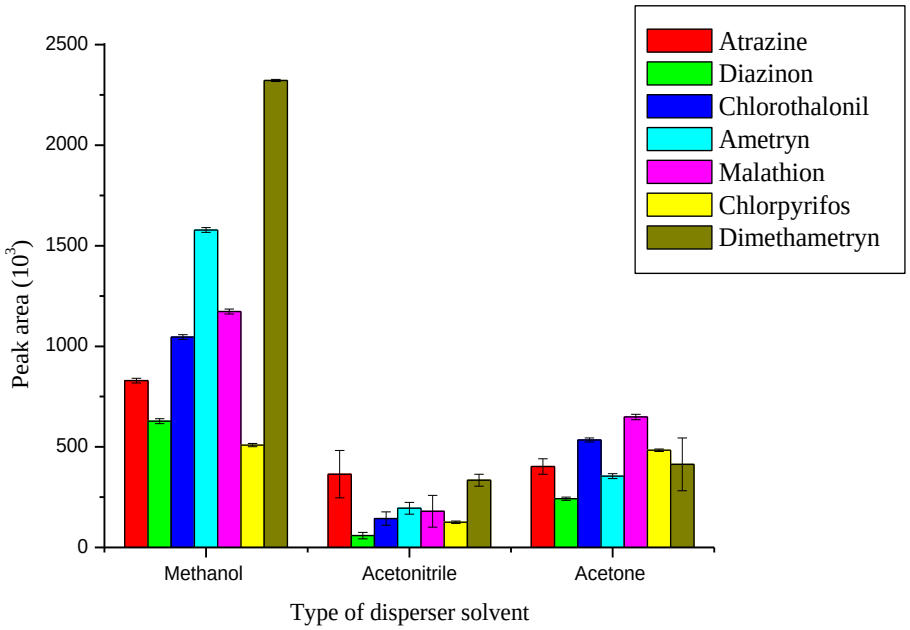


Figure 4.1.2 Effect of the disperser solvent on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent volume 0.5 mL; extraction time, 1 min; centrifugation speed 3500 rpm for 3 min; n = 3.

In the HD-DLLME technique investigated, performances of three extraction solvents including acetone, acetonitrile and methanol were evaluated. The analytical results obtained, as peak areas, were found to be the highest, when methanol was used as disperser solvent (Figure 4.1.3). It was also observed that methanol fulfills the major characteristics that the disperser solvent possesses than the other two solvents (Tabrizi and Rezazadeh 2012). As a result, methanol was selected as disperser solvent and utilized for further experiments.

4.1.1.3 Effect of volume of extraction solvent

In order to investigate the effect of extraction solvent volume on the extraction efficiency, volume of chloroform was varied from 40 to 80 µL, in a constant volume of methanol (0.5 mL) for quantitative extraction of multiclass pesticides by HD-DLLME. It was noticed that, below 40 µL

of the solvent volume, formation of the sedimented phase was not satisfactory and thus collection of the sedimented phase was found to be difficult.

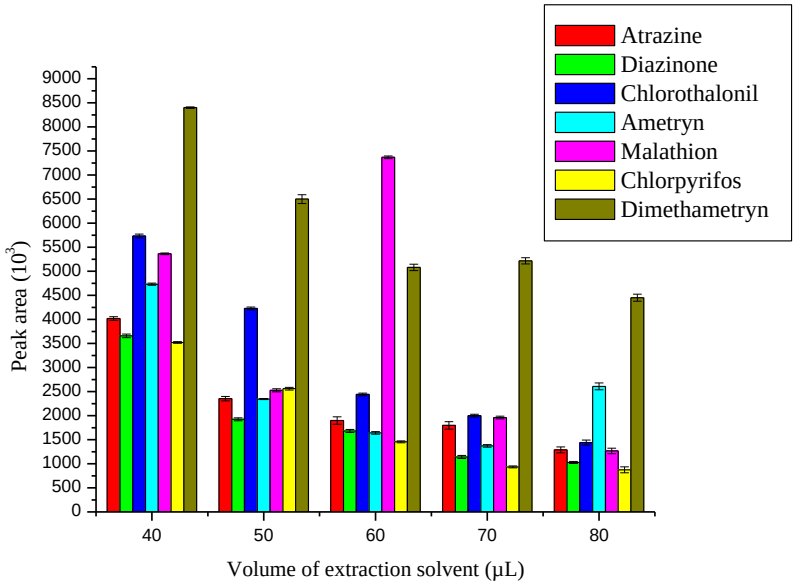


Figure 4.1.3 Effect of volume of extraction solvent on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, chloroform; disperser solvent volume, 0.5 mL methanol; extraction time, 1 min; centrifugation speed, 3500 rpm for 3 min; n = 3.

On the other hand, at higher volumes of the extraction solvent the ratio between the disperser solvent and that of extraction solvent exhibited decreasing tendencies which could probably resulted in reduced quantity of the droplets formed and thereby decreased extraction efficiency (Saraji and Boroujeni 2014). Therefore, 40 µL chloroform, which resulted in the highest signals, was chosen as the optimum volume of the extraction solvent (Figure 4.1.3).

4.1.1.4 Effect of volume of disperser solvent

The influence of the disperser solvent volume on extraction efficiency of the HD-DLLME technique was studied over the range of 0.3–0.6 mL of methanol. It was noted that below 0.3 mL of methanol, layer formation was not observed. However, the peak areas showed increasing tendency when the

volume of methanol was increased, from 0.3 to 0.4 mL (Figure 4.1.4). This may be attributed to the fact that smaller volumes, less than 0.4 mL, can't sufficiently disperse the extraction solvent. On the other hand, further increase in the disperser solvent volume, beyond 0.4 mL, decreasing the aqueous phase polarity, and thereby causing increased solubility of the target analytes in the aqueous phase (Chang et al. 2011). Thus, disperser solvent volume of 0.4 mL was chosen as the optimum volume and used in the subsequent extractions.

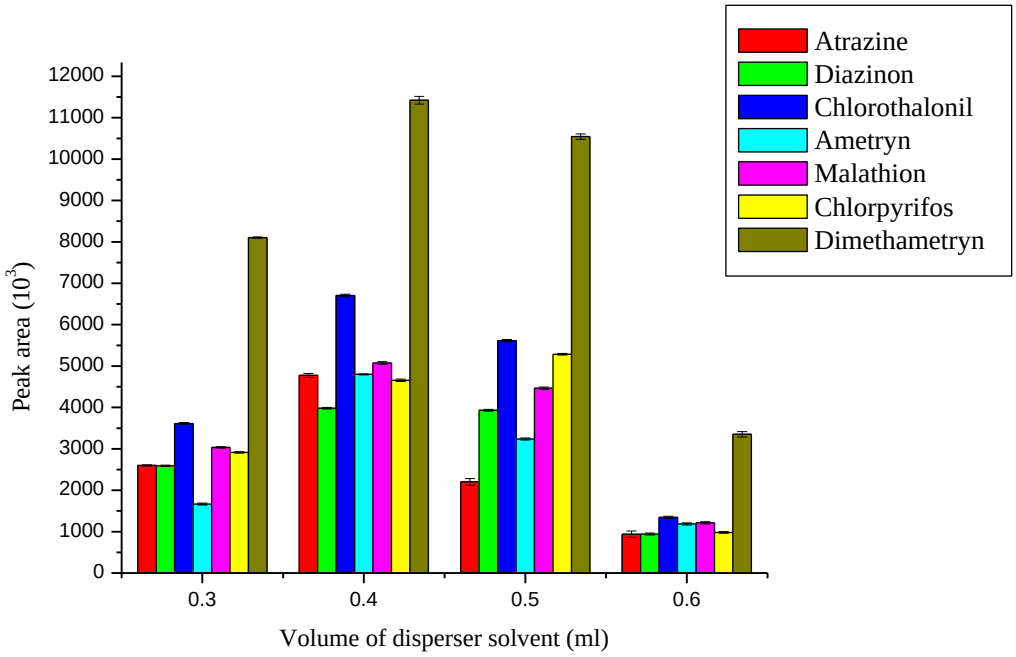


Figure 4.1.4 Effect of volume of disperser solvent on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent, methanol; extraction time, 1 min; centrifugation speed, 3500 rpm for 1 min; n = 3.

4.1.1.5 Effect of extraction time

Mass transfer is a time dependent process and is also one of the salient factors in most of the extraction procedures, particularly in miniaturized extractions such as SPME and LPME (Li et al. 2015; Nagaraju and Huang 2007). In the DLLME, equilibrium is achieved so quickly, which may mainly be attributed to the instant transition of the analytes, from aqueous phase to the extraction

solvent. This is most probably facilitated by the presence of large surface areas of contact between the extraction solvent and the aqueous phase, during formation of cloudy solution (Yang et al. 2012; Zacharis et al. 2010).

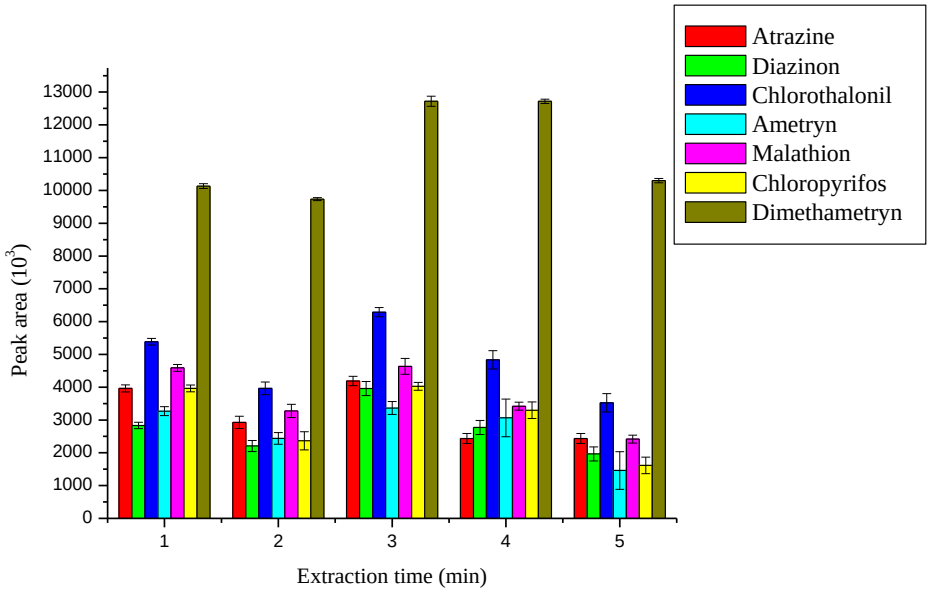


Figure 4.1.5 Effect of the extraction time on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent, 0.4 mL methanol; centrifugation speed, 3500 rpm for 3 min; n = 3.

In DLLME, extraction time is specifically defined as the time interval between injecting the mixture of disperser solvent and extraction solvent into the sample solution and starting centrifugation (Xi et al. 2016). Accordingly, in the current study the effect of extraction time on the extraction technique was investigated over the range of 1–5 min. The experimental results revealed that, for most of the target analytes, 3 min extraction time was found to be optimum. This may be attributed to the very fast mass transfer taking place initially but before establishment of the equilibrium state, which was achieved later, around 3 min (Figure 4.1.5). Therefore, extraction time of 3 min was found to be the optimum time and used throughout this study.

4.1.1.6 Effect of centrifugation speed

Centrifugation speed is one of the most important parameters in the sample preparation steps and also plays a key role in separation of the phases and thus resulting a clear solution in HD-DLLME techniques (Li et al. 2015). In order to obtain the highest signal, the speed was varied from 2500 to 4000 rpm. The corresponding experimental results revealed that the peak areas were increasing with the centrifuge speed, up to 4000 rpm (Figure 4.1.6). Extractions at higher speeds than 4000 rpm were not performed because of the instrumental limitation, i.e., 4000 rpm is the maximum speed, which was used as the optimum centrifugation speed throughout the study.

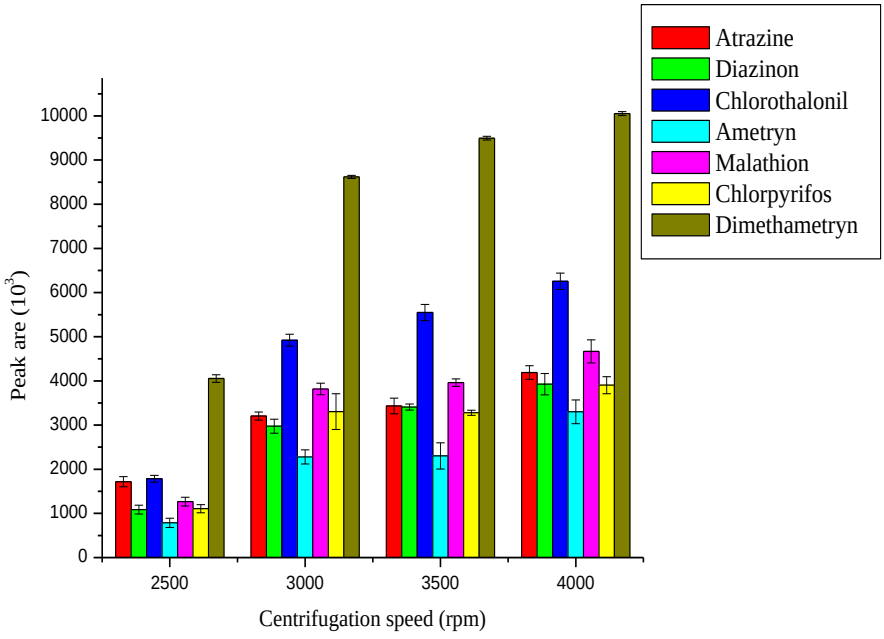


Figure 4.1.6 Effect of centrifugation speed on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent, 0.4 mL methanol; extraction time, 1 min; centrifugation time, 1 min; n = 3.

4.1.1.7 Effect of centrifugation time

In DLLME procedures, optimizing the time required for phase separation is also important analytical step, in order to obtain a clear extract (Xi et al. 2016). In order to establish the optimum conditions, time was varied from 1–5 min, at constant speed of 4000 rpm. Based on the peak areas representing the target analytes, the highest results were obtained at the centrifugation time of 3 min (Figure 4.1.7). Similar observations to that of the extraction time was also noted here for initial time of centrifugation for establishing equilibrium. Beyond 3 min, the peak areas were found to decrease gradually and thus centrifugation time of 3 min was chosen as optimum and used in the subsequent analysis.

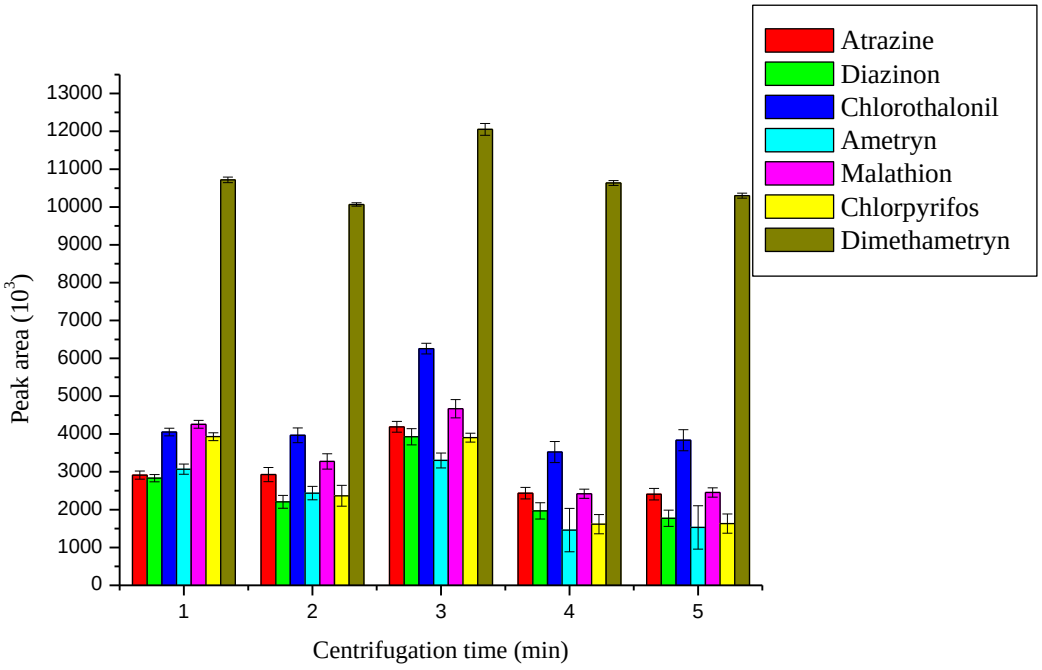


Figure 4.1.7 Effect of centrifugation time on the HD-DLLME efficiency. Extraction conditions: sample size 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent, 0.4 mL methanol; extraction time, 1 min; centrifugation speed, 4000 rpm for 3 min; n = 3.

4.1.1.8 Effect of sample solution pH

The The sample solution pH has also significant role on the extraction efficiency of the multiclass pesticides using the HD-DLLME procedure (He et al. 2010; Zhou et al. 2009). In order to evaluate the effect of this parameter, series of experiments were carried out by varying pH of the original aqueous solution from 5 to 9. The experimental results obtained revealed that pH 7 was the optimum pH, Figure 4.1.8, which may be associated with enhanced stability of the target analytes in the weakly acidic and weakly alkaline environments, while they were easily degraded in strongly acidic and alkaline conditions (Bedassa et al. 2015; Peruga et al. 2013; Tadesse et al. 2015).

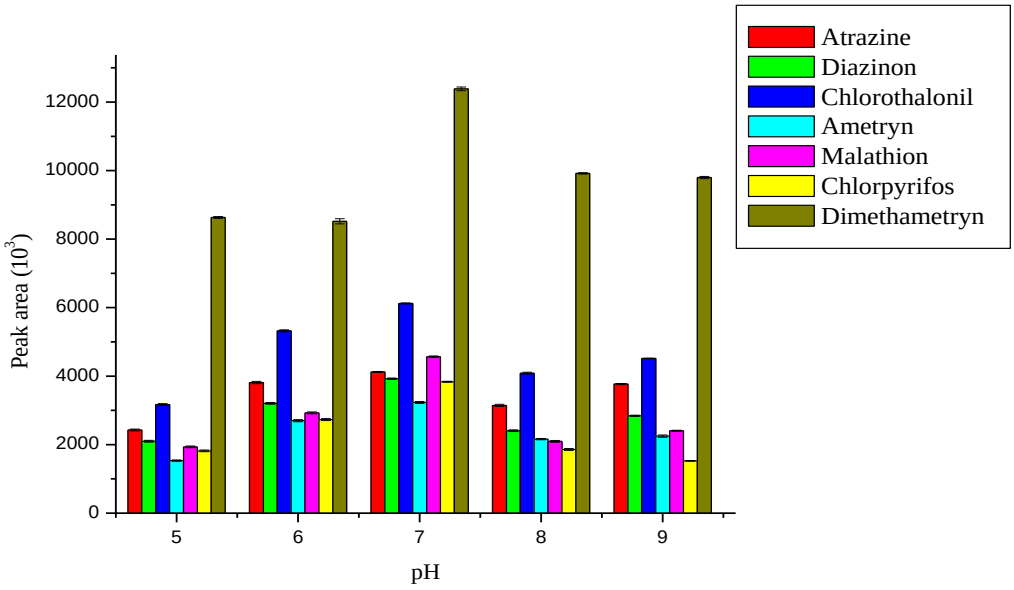


Figure 4.1.8 Effect of pH on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent, 0.4 mL methanol; extraction time, 3 min; centrifugation speed, 4000 rpm for 3 min; n = 3.

4.1.1.9 Study of salt addition

Generally, in LPME, addition of the salt to solution reduces solubility of the analytes in the aqueous sample solution and as a result enhances their partitioning into the organic phase (Gure et

al. 2015). In this study, effect of salt amount was studied by adding varied quantities of NaCl (0, 2.5, 5 and 7.5% w/v) to the aqueous sample solutions. It was observed that (Figure 4.1.9) addition of NaCl resulted in a reverse effect on extraction efficiency. This may be because salt addition decreased the peak areas of the analytes, since dissolution of sodium chloride in water may increase electrostatic interaction, which could most likely cause the extent of analytes transfer to the extraction phase to be reduced. In other words, presence of salt may also have the effect of decreasing solubility of chloroform in water, and this in turn may cause the volume of the sedimented phase to increase. The ultimate effect could thus result in a decreased extraction efficiency (Khalilian and Rezaee 2017; Lin et al. 2011; Yang et al. 2012). Based on these observations, all the subsequent experiments were carried out without addition of salt solution.

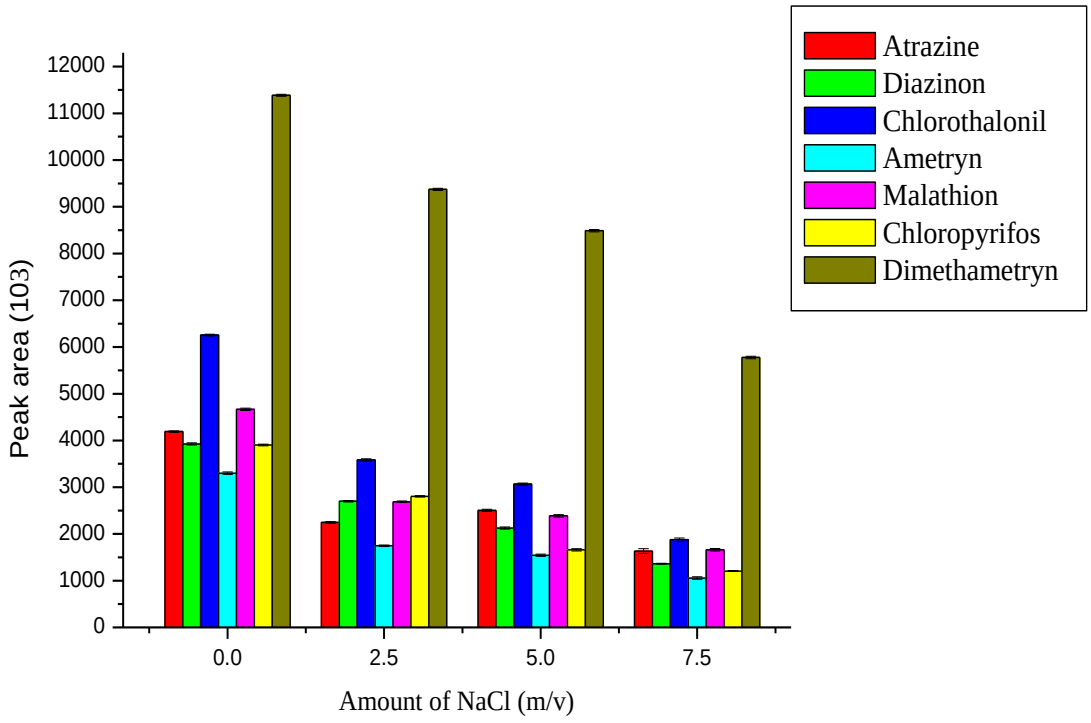


Figure 4.1.9 Effect of addition of salt on the HD-DLLME efficiency. Extraction conditions: sample size, 5 mL; spiked concentration, 5 µg/L; extraction solvent, 40 µL chloroform; disperser solvent, 0.4 mL methanol; extraction time, 3 min; centrifugation speed, 4000 rpm for 3 min; n = 3.

4.1.2 HD-DLLME method validation

The proposed analytical method was validated by evaluating linearity, precisions and limits of detection (LOD). The linearity was established for five different concentration levels. Each concentration level was extracted in triplicate, following the optimized procedure and each extract was also injected in triplicate. Good lineary, ranging from 0.991 to 0.999, were obtained for all the target analytes considered in this study. Repeatability (intra-day precision) was investigated by extracting the spiked reagent water at 5 µg/L, prepared and injected in triplicate on the same day, under the same experimental conditions. Similarly, reproducibility (interday precision) of the method was evaluated using reagent water spiked at the same concentration levels, used for repeatability, during three consecutive days. As provided in Table 4.1.1, satisfactory precisions (RSD less than 10%) were obtained in all cases (SANTE 2015). The limit of detection (LODs) was determined as the lowest concentration yielding a signal to noise (S/N) ratio of 3. The LODs of the analytes ranged from 0.005 to 0.02 µg/L.

Table 4.1.1 Performance characteristics of the proposed analytical method.

Analyte	Linear range (µg/L)	Regression equation	R^2	LOD (µg/L)	Repeatabilty (%RSD, n = 3)	Reproducibilty (%RSD, n = 3)
Atrazine	0.10–100	y = 20141x + 31927	0.995	0.01	1.4	1.1
Diazinon	0.02–100	y = 15334x + 37096	0.999	0.007	3.9	3.9
Chlorothalonil	0.10–100	y = 28260x + 86439	0.997	0.02	0.7	4.9
Ametryn	0.02–100	y = 12677x + 16514	0.991	0.008	0.8	8.7
Malathion	0.02–100	y = 26235x + 14121	0.991	0.006	2.3	3.7
Chlorpyrifos	0.02–100	y = 14695x + 94087	0.993	0.009	5.3	4.2
Dimethametryn	0.02–100	y = 59031x + 11517	0.996	0.005	4.0	4.8

4.1.3 Application of the method to real samples

The proposed HD-DLLME-GC-MS technique was applied for selective and quantitative extraction and determination of seven pesticides in the water samples collected from Hawassa Lake and Wonji Shoa sugarcane irrigation water, and sugarcane juice samples from Wonji Shoa sugar factory, following the optimized analytical method. The matrix effect on the selective isolation and quantitative determination of the trace levels of the target analytes by the developed method was evaluated by percent relative recovery (%RR). It is defined as the ratio of the peak area of the spiked real water extract to that of spiked reagent water extract spiked at the same level (Saraji and Tansazan 2009). To study the matrix effect, two spiking levels (2.5 and 5 µg/L) were considered and the resulting %RR were found to vary from 80.4 to 114% with RSD varying from 0.27-11% (Table 4.1.2); which were found to vary within the acceptable range (SANTE 2015). These results demonstrate that the matrices had insignificant effect on the novel HD-DLLME technique developed in this study.

The presence of the seven pesticide residues in the three samples were investigated and only ametryn was detected in the water sample from Lake Hawassa at 1.5 µg/L level. Similarly, atrazine and ametryn were detected in the water sample collected from Wonji Shoa sugarcane irrigation at concentration levels 4.1 and 4.8 µg/L, respectively. Ametryn was also detected in the sample of sugarcane juice at the concentration level of 7.1 µg/L. The United States Environmental Protection Agency (US EPA) set the maximum allowable level of atrazine at 3 µg/L in water for human consumption. Similarly, the European Union (EU) set the maximum residue levels for individual pesticide at 0.1 µg/L and 0.5 µg/L for mixtures of pesticides (Chen et al. 2015). Furthermore, EU has set the maximum residue levels (MRL) of 14 µg/L in the surface water and 250 µg/L in sugarcane juice for ametryn (US EPA archive document 2015).

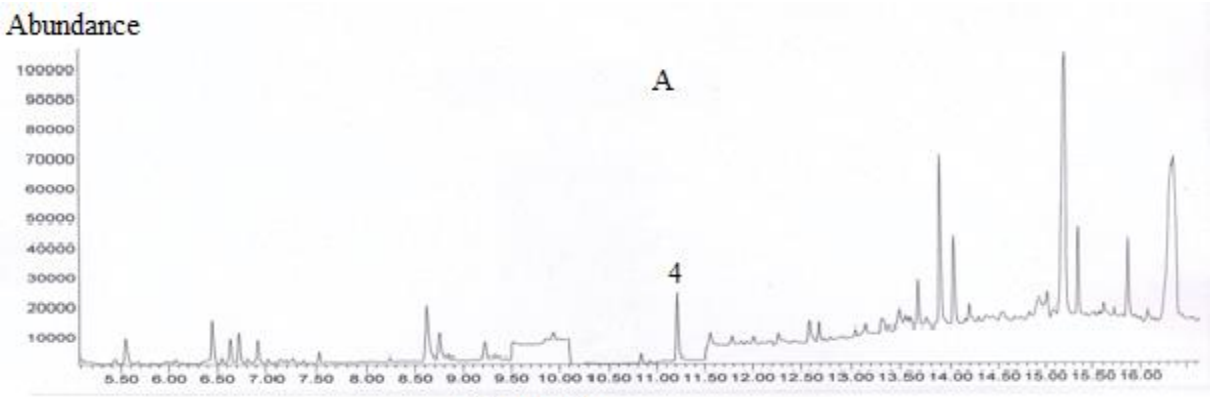
Based on the findings of this study, atrazine was detected only in the water sample of Wonji Shoa sugarcane irrigation, and the residue level determined, 4.1 µg/L, which is higher than the limit set by EPA (Chen et al. 2015), even if the water from this source is not used for drinking purposes. On the other hand, the amounts of ametryn found in both water samples

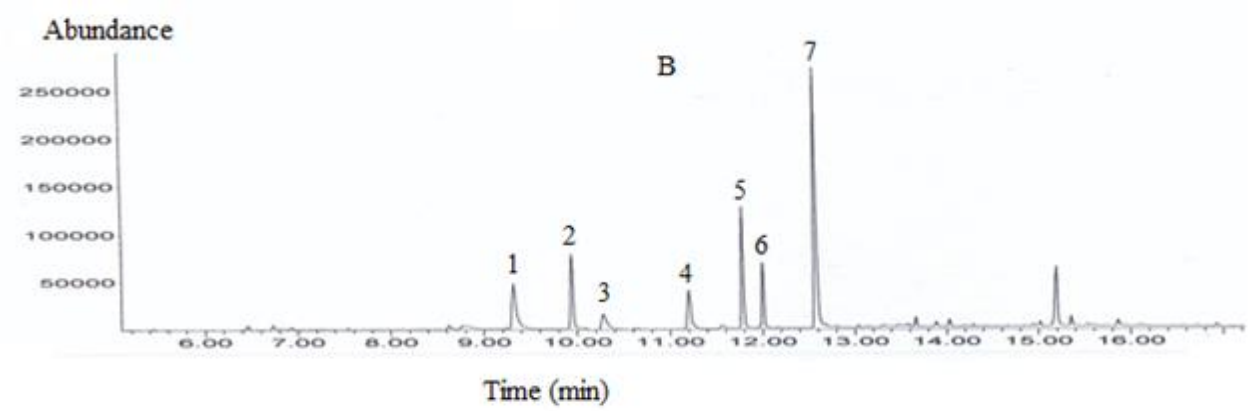
were below the MRL set by the EU for surface water. Similarly, the quantity of ametryn detected in the sugarcane juice was also below the MRL recommended by the EU. However, the findings of this study could be used as a warning alarm for the need of continuous monitoring program in order to protect the environmental deterioration, and minimizing human and animal health risks possibly caused by future accumulation of the pesticide residues in the study areas. Typical GC-MS chromatograms for the spiked and non-spiked Wonji Shoa sugar factory irrigation water Hawassa Lake water and sugarcane juice samples are shown in Figure 4.1.10.

Table 4.1.2 Recovery values of the proposed method in environmental water and sugarcane juice samples.

Analyte	**Spiking level (µg/L)**	**Lake Hawassa water samples**	**Wonji Shoa sugar factory irrigation water samples**	**Sugarcane juice samples**
		%RR (%RSD, n = 3)	**%RR (%RSD, n = 3)**	**%RR (%RSD, n = 3)**
Atrazine	0	nd	d (4.1 µg/L)	nd
	0.25	90.3 (7.5)	114 (5.2)a	93.8 (5.9)
	5	109 (3.5)	104 (2.5)	110 (4.8)
Diazinon	0	nd	nd	nd
	0.25	110 (1.0)	96.4 (0.27)	81.4 (5.2)
	5	109 (5.7)	81.5 (5.3)	91.5 (7.7)
Chlorothalonil	0	nd	nd	nd
	0.25	96.0 (8.8)	83.0 (3.4)	83.9 (9.7)
	5	110 (5.6)	95.7 (10)	98.7 (11)
Ametryn	0	d (1.5 µg/L)	d (4.8 µg/L)	d (7.1 µg/L)
	0.25	80.6 (8.0)	86.5 (4.9)	82.0 (6.7)
	5	86 (5.6)	98.4 (3.8)	80.4 (4.6)
Malathion	0	nd	nd	nd
	0.25	97.4 (0.96)	84.5 (7.7)	81.7 (2.0)
	5	108.3 (2.5)	90.1 (7.2)	112 (3.3)
Chlorpyrifos	0	nd	nd	nd
	0.25	100 (1.6)	103 (6.1)	90.6 (5.3)
	5	110 (2.2)	82.6 (1.5)	81.7 (0.7)
Dimethametryn	0	nd	nd	nd
	0.25	95.0 (9.5)	80.5 (2.4)	84.2 (11)
	5	112 (4.8)	86.0 (5.4)	101 (7.5)

nd: not detected, d: detected


Abundance
A
4
Time (min)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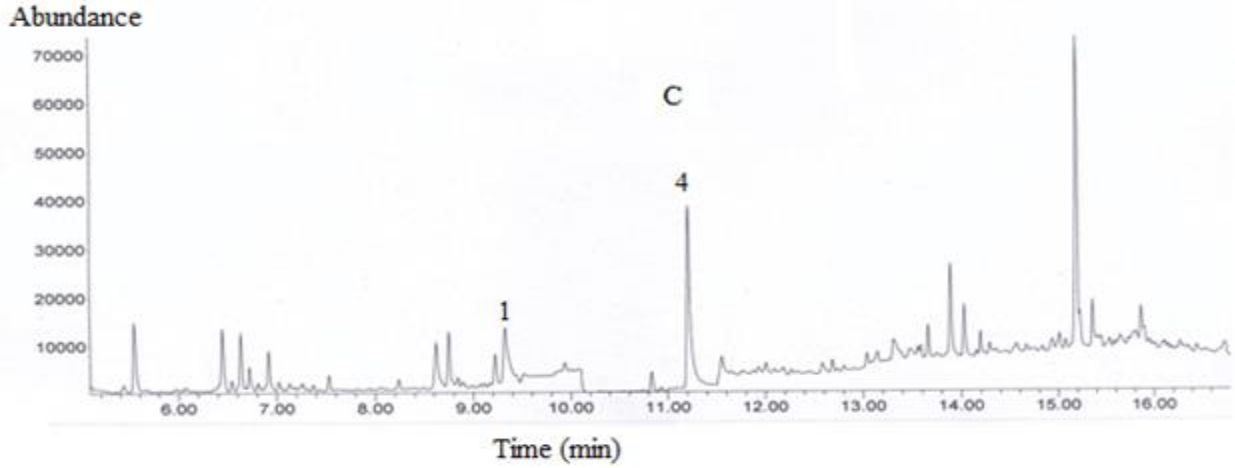
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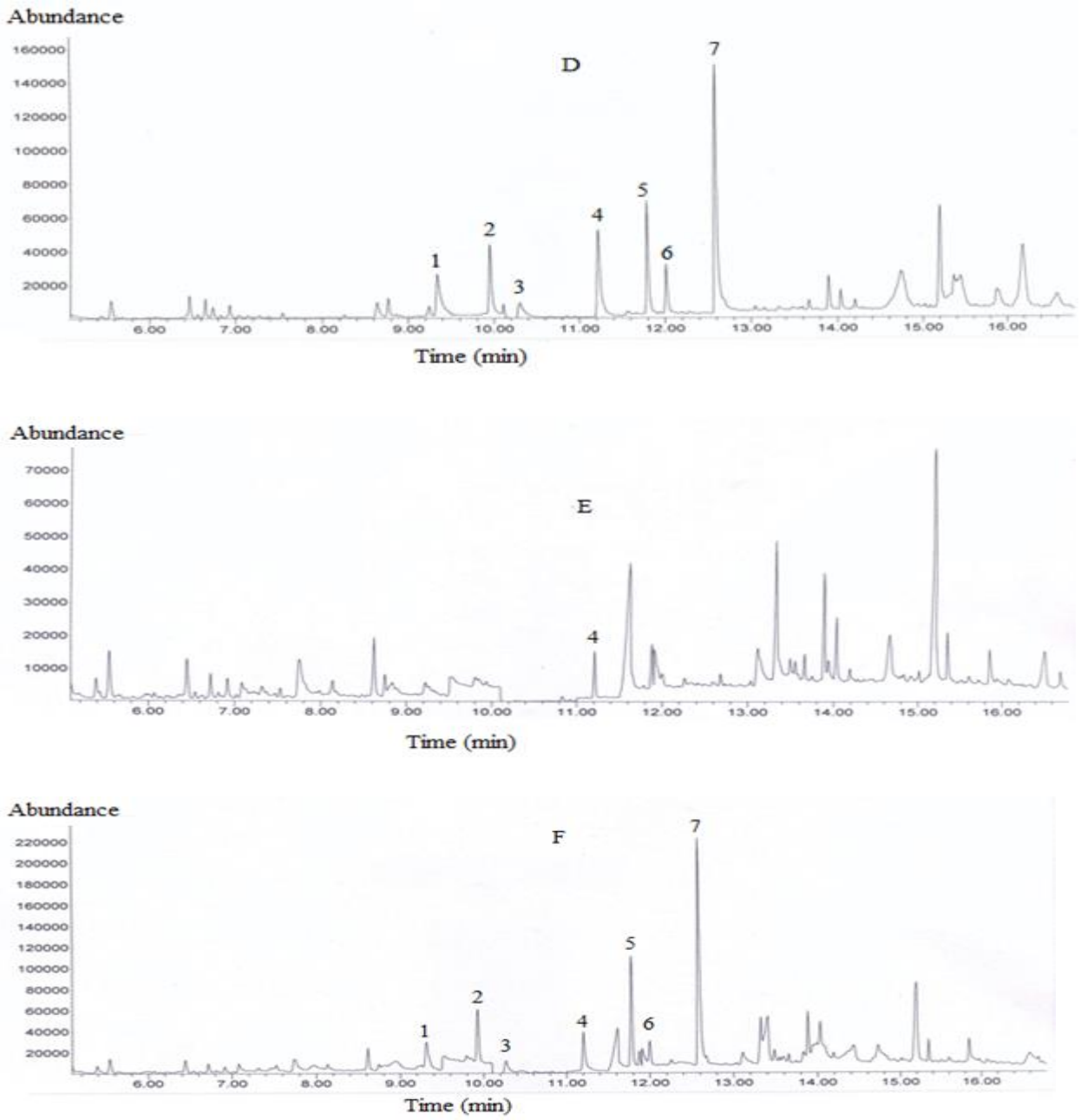


Figure 4.1.10 Typical chromatograms for blank (A) and spiked (B) Lake Hawassa water; blank (C) and spiked (D) Wonji Shoa irrigation water; blank (E) and spiked (F) sugarcane juice samples. Peaks: 1, atrazine; 2, diazinon; 3, chlorothalonil; 4, ametryn; 5, malathion; 6, chlorpyrifos; 7, dimethametryn.

4.1.4 Comparison of the proposed method with similar literature reports

The figures of merit of the HD-DLLME method developed in the presented study for determination of seven pesticides in water and sugarcane juice samples have been compared with other recently reported techniques including DLLME-GC-MS (Nagaraju and Huang 2007; Rocha et al. 2008), ion-pair assisted liquid-liquid extraction combined with high performance liquid chromatography diode array detection (IPA-LLE-HPLC-DAD) (Gure et al 2014), dispersive micro-solid phase extraction combined with high resolution mass spectrometry (DMSPE-HRMS) (Chen et al. 2015) and dispersive liquid-liquid microextraction based on solidification of floating organic droplet (DLLME-SFO) (Cheng et al. 2011).

Details of the relevant results of the methods and that of this study are provided in Table 4.1.3. Based on these findings, comparison was made and it was observed that the proposed method involves minimum labor and requires short extraction time. In addition, performances of the developed technique were compared with that of the previously reported techniques in terms of relative recovery, LOD and regression coefficient (R^2) and the findings confirmed that the developed technique are found to be comparable or better. Furthermore, it could also be noted that the developed method utilizes simpler and classical laboratory equipment and uses microliter amount of organic solvents, which could be accessible in most common research laboratories.

Table 4.1.3 Comparison of the proposed HD-DLLME method with various modes of extraction methods.

Methods	**Analyte**	**Extraction time (min)**	**Recovery**	**LOD (µg/L)**	**R^2**	**References**
DLLME-GC-MS	Chlorothalonil and chlorpyrifos	2	75.0–113	0.002–0.5	0.998–0.999	Rocha et al. 2008
IPA-LLE-HPLC-DAD	Chlorpyrifos, diazinon, fenitrothion and others	20	73.0–105	0.5–3.0	0.993–0.997	Gure et al 2014
DLLME-GC-MS	Ametryn, desmetryn, dimethametryn, diprometryn, metoprotryn, prometryn and terbumetryn	-	85.2–115	0.021–0.12	0.978–0.999	Nagaraju and Huang 2007
DMSPE-HRMS	Ametryn and atrazine dimethametryn	1.5	71.1–91.5	0.0003–0.006	0.999–0.999	Chen et al. 2015
DLLME-SFO	Diethofencarb and pyrimethanil	5	86.2–105	0.24 and 0.09	0.999–-0.999	Chen et al. 2011
HD-DLLME-GC-MS	Atrazine, diazinon, chlorothalonil, ametryn, malathion, chlorpyrifos and dimethametryn	3	80.4–114	0.005–0.02	0.991–0.999	This study

4.2 Modified SALLE combined with LD-DLLME for multiclass pesticide residues analysis in sugar and soil using gas chromatography-mass spectrometry

4.2.1 Optimization of SALLE parameters

The SALLE techniques were optimized by using sugar samples as a representative matrix. A sugar samples was spiked at 50 µg/kg by weighing 1 g of each samples into centrifuge tube and spiking with an appropriate volume of working solution containing each of target pesticides with a microsyringe, ensuring that the solution is homogenized. All experiments were further enriched by LD-DLLME and repeated in triplicate.

4.2.1.1 Selection of type and volume of organic solvent

The selection of an appropriate extraction solvent is an important step in the optimization for successful application of the SALLE method. The extraction solvent of choice has to meet certain requirements such as highly polar, miscibility with the aqueous phase (Gure et al. 2014), extraction capability for the analytes of interest, having a density lower than water, ability to form phase separation up on addition of the appropriate salt and being environmentally friendly (Teju et al. 2017; Xu et al. 2015). In this study, acetonitrile (density 0.790 g/mL), acetone (density 0.788 g/mL) and methanol (0.792 g/mL) were investigated for extraction efficiency for target analytes based on these considerations.

Accordingly, series of experiments were performed using 1 g of sugar, placed in centrifuging tube, spiked at 50 µg/kg containing all the target analytes and adding 5 mL of deionized water of pH 7. A 2 mL of extraction solvent was added and allowed to equilibrate for 2 min and then 25% (w/v) of NaCl was added. The resulting solution was centrifuged at 4000 rpm for 3 min. Under this experimental condition, it was observed that only acetonitrile formed phase separation. Despite acetonitrile miscibility with water, there is the potential to partition a solution into two layers when salts or/ and the other organic solvent is added (Hajkova eta al. 2016). Therefore, ACN was selected for further experiment.

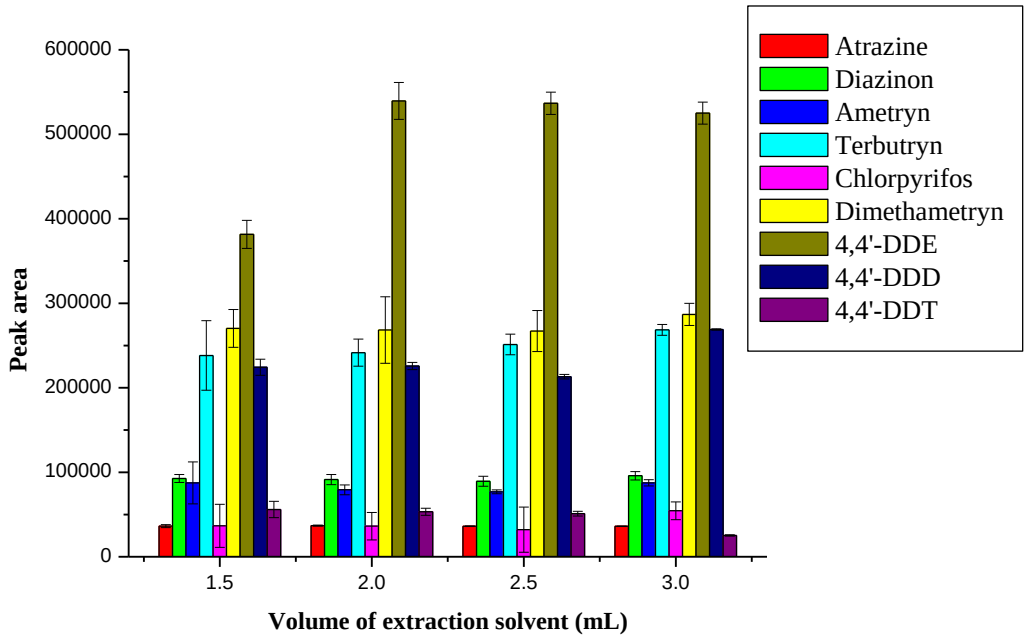


Figure 4.2.1 Effect of volume of extraction solvent. Extraction conditions: 1 g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, acetonitrile; amount of salt, 25% (w/v) NaCl; centrifugation speed, 4000 rpm; centrifugation time, 2 min; extraction time, 2 min; n = 3.

To evaluate the effect of ACN volume, 1.5, 2, 2.5 and 3 mL were studied. Below 1.5 mL the layer formed was not sufficient and it was very difficult to collect the upper organic phase separately. The curve of variation of extraction peak area versus the volume of ACN as extraction solvent is shown in Figure 4.2.1. As the volume of ACN increased, the peak area of most target analytes was slightly increased. From the obtained results, 2 mL of ACN was chosen as the optimal volume for the extraction solvent.

4.2.1.2 Effect of salt type and amount

Increasing ionic strength of the sample solution decreases the solublity of organic pollutants in water (Wen et al. 2013). In this study, the effect of three different salts; NaCl, $MgSO_4$ and $(NH_4)_2SO_4$ was investigated at 25% (w/v) of each salt, as a potential salting-out reagent. As shown in Figure 4.2.2, the highest peak area was obtained when $MgSO_4$ used as potential salting-out reagent. This may be due to its high ionic strength per unit concentration in aqueous phase.

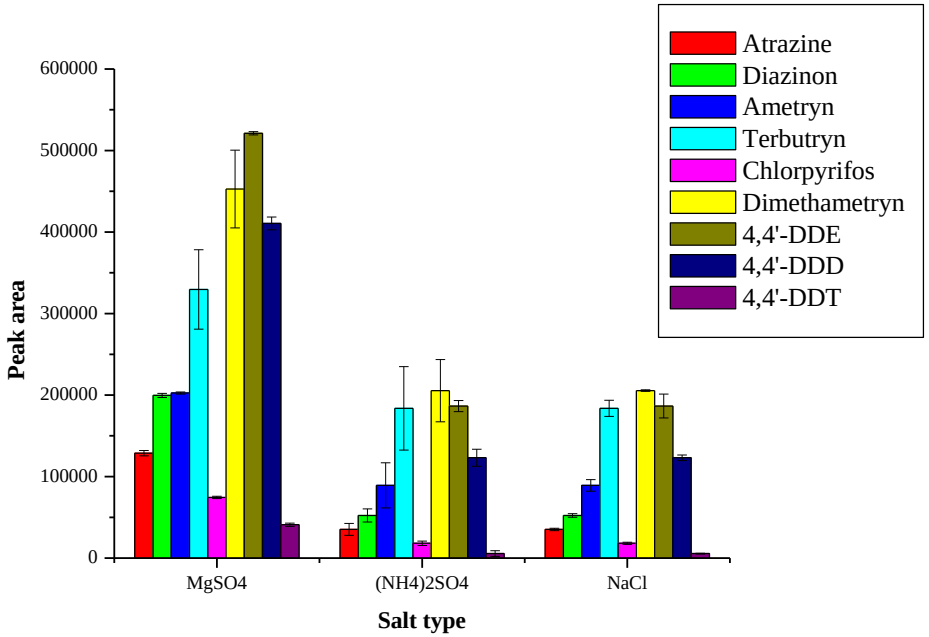


Figure 4.2.2 Effect of salt type. Extraction conditions: 1g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, 2 mL acetonitrile; amount salt, 25% (w/v); centrifugation speed, 4000 rpm; centrifugation time, 2 min; extraction time, 2 min; n = 3.

It should be pointed that any strong Lewis base could have interaction with magnesium and impact on the extraction efficiency because magnesium is a strong Lewis acid (Wen et al. 2013; Zhang et al. 2009). Thus, $MgSO_4$ will be used for further experiments. Although acetonitrile is miscible with water in any proportion at room temperature addition of salt significantly reduced the mutual miscibility, even resulting in phase separation of acetonitrile from aqueous phase. Additionally, the presence of salt mainly leads to an increase of partition coefficients of pesticides to a hydrophobic material (Teju et al. 2017). The effect of salt was assessed by adding 24–27% (*w/v*) of $MgSO_4$. It was observed that 24% of $MgSO_4$ fails to form a phase separation. The highest peak area of all target analytes was obtained when 25% of $MgSO_4$ used and slightly decreased beyond 25% of $MgSO_4$ (Figugure 4.2.3). Therefore, 25% of $MgSO_4$ was selected as an optimum amount of the salt for further experiments.

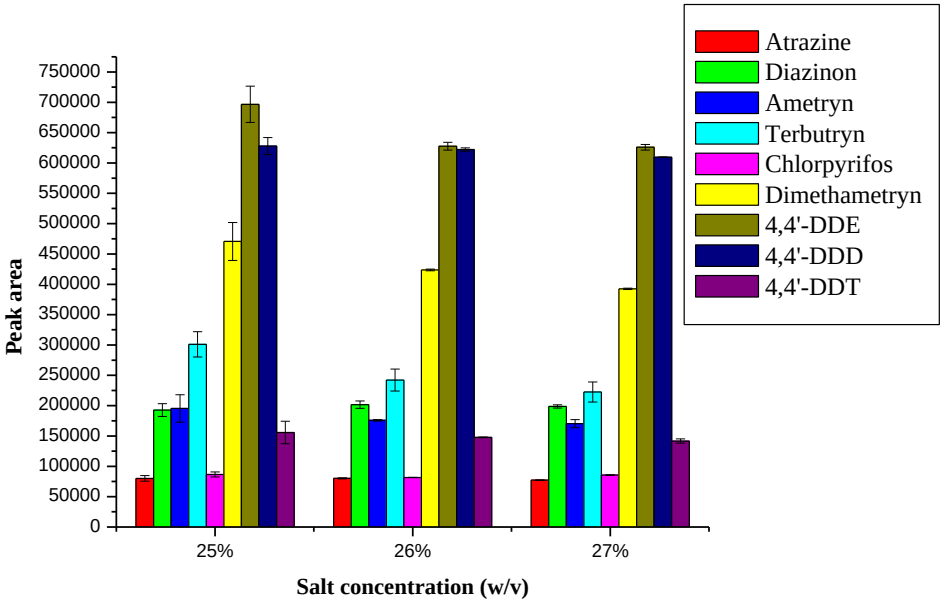


Figure 4.2.3 Effect of amount of salt. Extraction conditions: 1 g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, 2 mL acetonitrile; salt, $MgSO_4$; centrifugation speed, 4000 rpm; centrifugation time, 2 min; extraction time, 2 min; n = 3.

4.2.1.3 Effect of extraction time

The extraction time, the time interval from adding the extraction solvent and before starting centrifugation (Chang et al. 2011), is studied over a range of 1–5 min. Initially extraction efficiency increased as extraction time was increased and peak area of all of the target analytes was enhanced at 3 min (Figure 4.2.4). The peak area decreased when extraction time was further extended. This may be due to the fact that long extraction time would result in a decrease of peak areas which might be due to slow dissolution of ACN in water after phase separation which may in turn increase the solubility of target analytes (Tolcha et al. 2013). Hence, an extraction time of 3 min was chosen as the optimum extraction time.

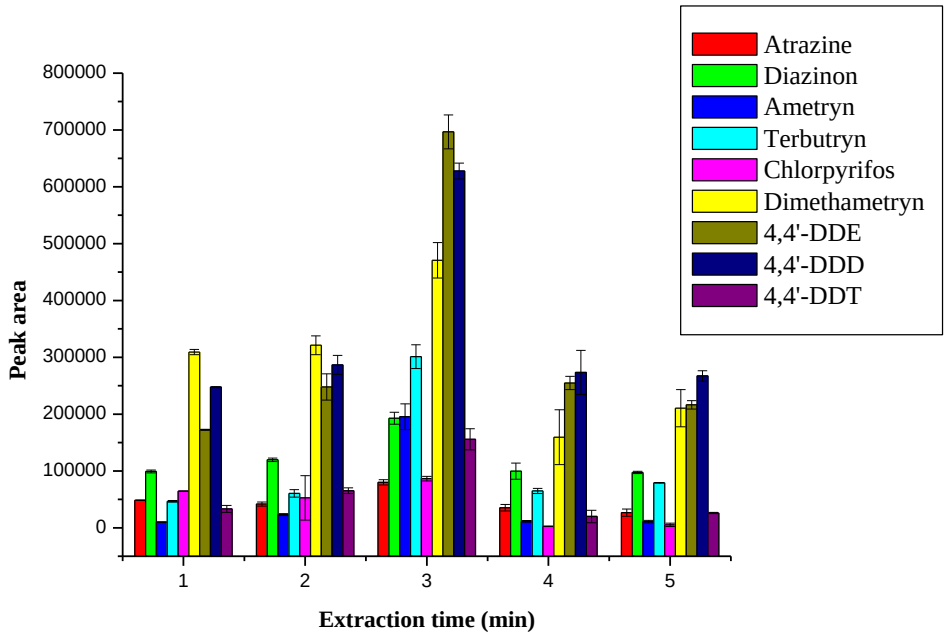


Figure 4.2.4 Effect of extraction time. Extraction conditions: 1 g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, 2 mL acetonitrile; amount of salt, 25% (w/v) $MgSO_4$; centrifugation speed, 4000 rpm; centrifugation time, 2 min; n = 3.

4.2.1.4 Effect of pH

The pH of the sample solution plays an important role in extraction of ionizable and relatively polar compounds. The important parameters governing extraction with variation of solution pH are solubility and stability of the solute due to ionization (Joarder et al. 2014). A pH of the solution should be adjusted properly so that additional selectivity can be achieved through adequate control of the pH (Jouyban et al. 2015).

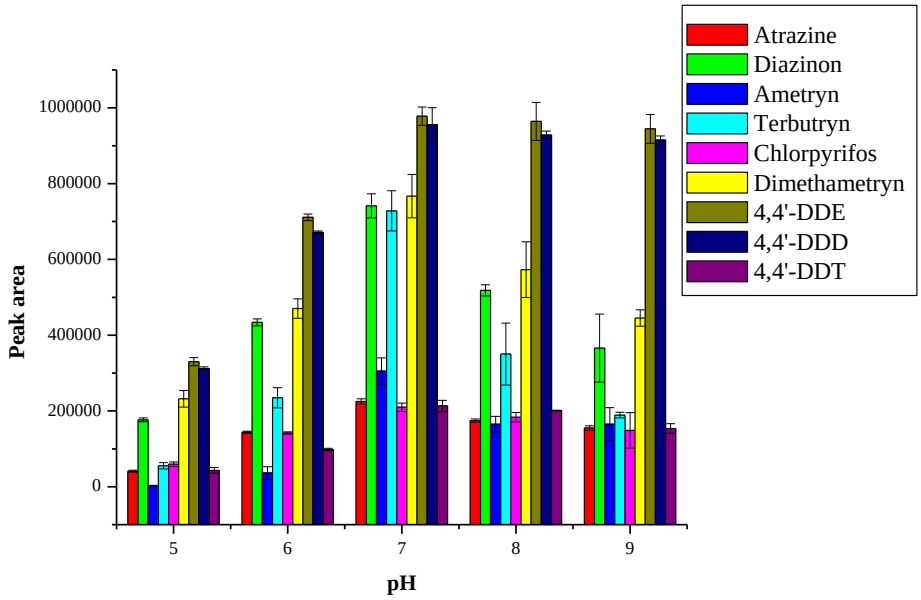


Figure 4.2.5 Effect of pH of reagent water. Extraction conditions: 1 g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, 2 mL acetonitrile; amount of salt, 25% (w/v) $MgSO_4$; centrifugation speed, 4000 rpm; centrifugation time, 2 min; extraction time, 3 min; n=3.

In this study, the effect of sample solution pH was investigated by varying from 5 to 9 using diluted HCl and NaOH solution. As can be seen in Figure 4.2.5, the highest peak areas of the target pesticides were obtained at pH 7. This may be due to the high stability of the target pesticides in the weakly acidic and weakly alkaline media, and easily degraded in strongly acidic and alkaline condition (Pandey et al. 2010). Therefore, a sample solution of pH 7 was chosen as the optimum extraction condition.

4.2.1.5 Effect of centrifugation speed and time

Centrifugation is usually used to accelerate the phase separation (Farajzadeh and Khoshmaram 2015). The effect of centrifugation speed was studied in the range of 2000 to 4000 rpm. The experimental results revealed that the highest response of all target analyte was obtained when 3000 rpm was used as centrifugation speed (Figure 4.2.6). As a result, 3000 rpm was utilized as optimum centrifugation speed for consequent experiments. Optimizing the time required for phase separation is also important analytical step, in order to obtain a clear extract (Alemayehu

et al. 2017). To this end, time of centrifugation was varied from 1 to 5 min, within 1 min interval. The experimental results confirmed that centrifugation time of 3 min to be the optimum (Figure 4.2.7) and used throughout this study.

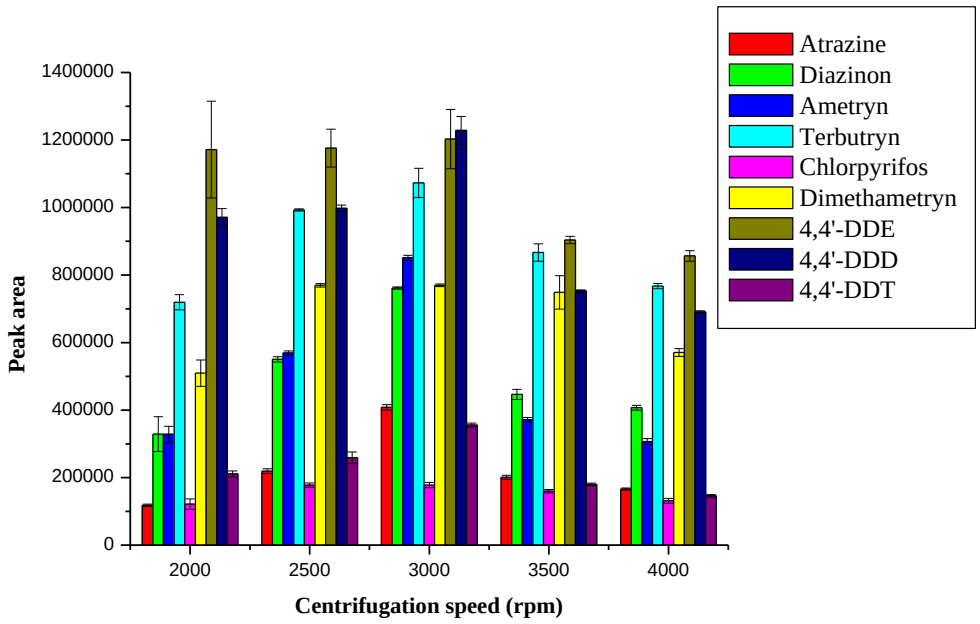


Figure 4.2.6 Effect of centrifugation speed. Extraction conditions: 1 g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, 2 mL acetonitrile; amount of salt, 25% (w/v) $MgSO_4$; centrifugation time, 2 min; pH, 7; extraction time, 3 min; n = 3.

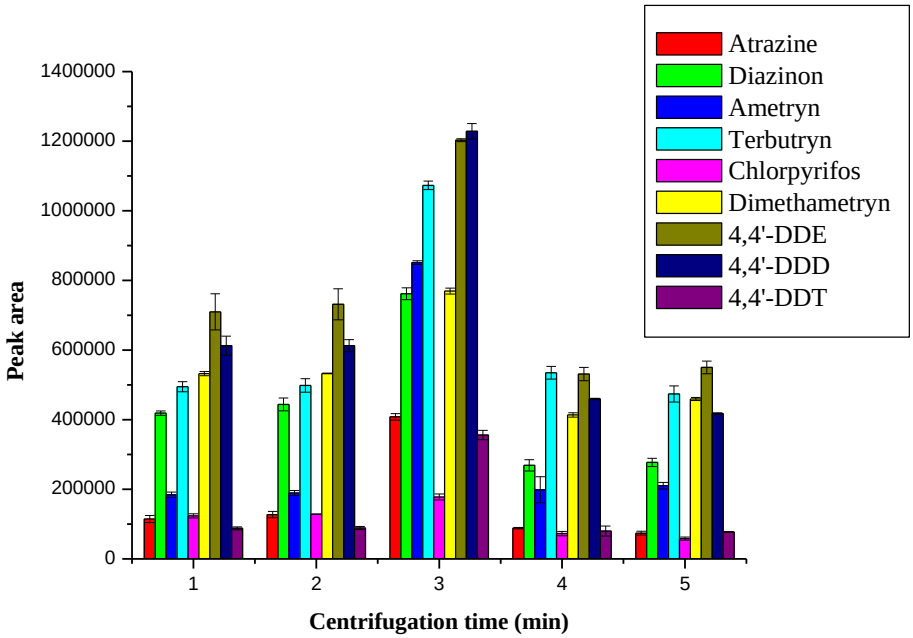


Figure 4.2.7 Effect of centrifugation time. Extraction conditions: 1 g of sugar spiked at 50 µg/kg containing the target analytes; extraction solvent, 2 mL acetonitrile; amount of salt, 25% (w/v) $MgSO_4$; centrifugation speed, 3000 rpm; pH, 7; extraction time, 3 min; n=3.

4.2.2 Enrichment factor obtained upon combining SALLE and LD-DLLME

The enrichment factor (EF) is defined as the ratio of final analyte concentration in the organic phase to the initial concentration of analyte in sample solution (Barriada-Pereira et al. 2005). In this particular work, EF is the ratio of final analyte peak area in SALLE-LD-DLLME extract to the peak area of analyte in SALLE extract. To evaluate the enrichment factor obtained, a 1 g of sugar samples spiked at 0.5 µg/g containing the target analytes was extracted under the following optimized extraction conditions: 5 mL of deionized water (pH 7), 2 mL of acetonitrile, 25% (*w/v*) of $MgSO_4$, 3 min equilibrium time and centrifuged at 3000 rpm for 3 min. First the acetonitrile extract was analyzed by GC-MS. Then the extraction was repeated and the supernatant was further enriched by LD-DLLME as described in the procedure sub-section. The concentration obtained from the acetonitrile extract before and after application of LD-DLLME was compared and the EF obtained ranged from 17.14 to 120.64 (Table 4.2.1).

4.2.3 Validation of SALLE-DLLME-GC-MS

The applicability of the proposed method was investigated for determination of target analyte, several factors including linearity, LOD, precision and accuracy were studied. The matrix matched calibration curve was linear over the concentration range from 6.25–100 µg/kg for atrazine, ametryn, terbutryn, dimethametryn and 4,4'-DDT, 2.50–100 µg/kg for diazinon, chlorpyrifos and 2,4'-DDD, and 1–100 µg/kg for 4,4'-DDE with the correlation coefficients (R^2) ranging from 0.992 to 0.999. The limits of detection (LOD) was considered as the minimum analytes concentrations yielding 3 times the signal-to-noise (S/N) ratio and found to be in the range of 0.01 to 0.3 µg/kg. Repeatability was studied by extracting and injecting spiked sugar at 12.5 and 50 µg/kg concentration levels on the same day, under the same experimental conditions. Similarly, reproducibility was performed by extracting and injecting the two spiking concentration levels in triplicate for three consecutive days. The results, expressed as relative standard deviation (RSD%) of the peak areas, are shown in Table 4.2.1 and acceptable precisions were obtained for all analytes (Gure et al. 2014).

Recoveries were calculated by comparing the average peak area for the analytes from spiked sugar and soil samples with spiked reagent water, all spiked at the same concentration level, after the application of the SALLE-LD-DLLME procedure. To investigate the accuracy of the proposed method, both sugar and soil samples were spiked at two concentration levels of 12.5 and 50 µg/kg, and extracted under the optimized conditions in triplicate. Recoveries and %RSD of each target analyte in sugar and soil samples are shown in Table 4.2.2. The recoveries of the real samples at the two spiking levels were in the range 79.03–111.45% and 87.01–104.11% respectively. Therefore, the results obtained reveals the proposed method is acceptable and also in agreement with the current EU legislation (Gure et al. 2014). Both real sugar and soil samples were subjected to the SALLE-LD-DLLME procedure and then the extracts were injected into GC-MS system for analysis. The blank samples were analyzed, but, none of these target analytes were detected in sugar while atrazine and ametryn were quantitatively detected at concentration level of 0.29 and 0.23 µg/kg in soil samples. Typical chromatograms of unspiked sugar and soil samples, and spiked soil samples with all target analyte are shown in Figure 4.2.8.

Table 4.2.1 Analytical performance of SALLE-LD-DLLME.

Analyte	**Linear range (µg/kg)**	**LOD (µg/kg)**	**Regression equation**	**R^2**	**Repeatability (%, n = 3)**		**Reproducibility (%, n = 3)**		**EF**
					12.5 µg/kg	**50 µg/kg**	**12.5 µg/kg**	**50 µg/kg**	
Atrazine	6.25–100	0.02	y = 20942x + 15652	0.999	9.3	7.3	8.2	5.2	38.5
Diazinon	2.50–100	0.08	y = 46256x + 4481	0.999	4.4	3.3	8.2	3.6	47.7
Ametryn	6.25–100	0.01	y= 10349x + 15538	0.995	7.9	7.2	7.0	5.1	42.1
Terbutryn	6.25–100	0.01	y = 13423x + 2560	0.999	8.6	4.4	9.4	1.8	74.1
Chlorpyrifos	2.50–100	0.25	y = 14217x + 1796	0.999	9.3	3.1	6.5	3.1	121
Dimethametryn	6.25–100	0.03	y = 10111x + 32706	0.999	8.7	5.8	3.5	1.5	17.1
4,4’-DDE	1.0–100	0.03	y = 11748x + 13579	0.997	3.0	1.7	7.8	5.0	63.3
4,4’-DDD	2.50–100	0.06	y = 52971x + 22176	0.992	2.1	1.5	0.88	0.87	52.9
4,4’-DDT	6.25–100	0.30	y = 10863x + 1521	0.998	6.0	3.9	2.1	1.9	30.0

Table 4.2.2 Recoveries and %RSD of spiked sugar and soil samples.

Analyte	Spiked level (µg/kg)	Sugar		Soil		Amount detected (µg/kg)	
		%RR	%RSD	%RR	%RSD	Sugar	Soil
Atrazine	12.5	89.8	1.0	111	0.40	-	0.29
	50	103	5.8	102	5.8		
Diazinon	12.5	80.0	4.85	93	0.07	-	-
	50	87.0	2.1	89.0	2.1		
Ametryn	12.5	79.9	10	99.2	0.04	-	0.23
	50	96.1	0.60	94.8	0.60		
Terbutryn	12.5	95.5	7.9	96.9	0.02	-	-
	50	89.7	5.7	87.2	5.7		
Chlorpyrifos	12.5	79.0	0.2	88.9	0.13	-	-
	50	92.8	2.3	96.8	2.3		
Dimethametryn	12.5	102	3.1	88.8	0.02	-	-
	50	104	0.54	104	0.54		
4,4’-DDE	12.5	89.1	6.5	84.4	0.04	-	-
	50	87.2	0.81	87.9	0.81		
4,4’-DDD	12.5	99.7	0.76	89.8	0.06	-	-
	50	99.1	1.8	102	1.8		
4,4’-DDT	12.5	85.3	2.7	101	0.14	-	-
	50	94.7	0.62	93.9	0.62		

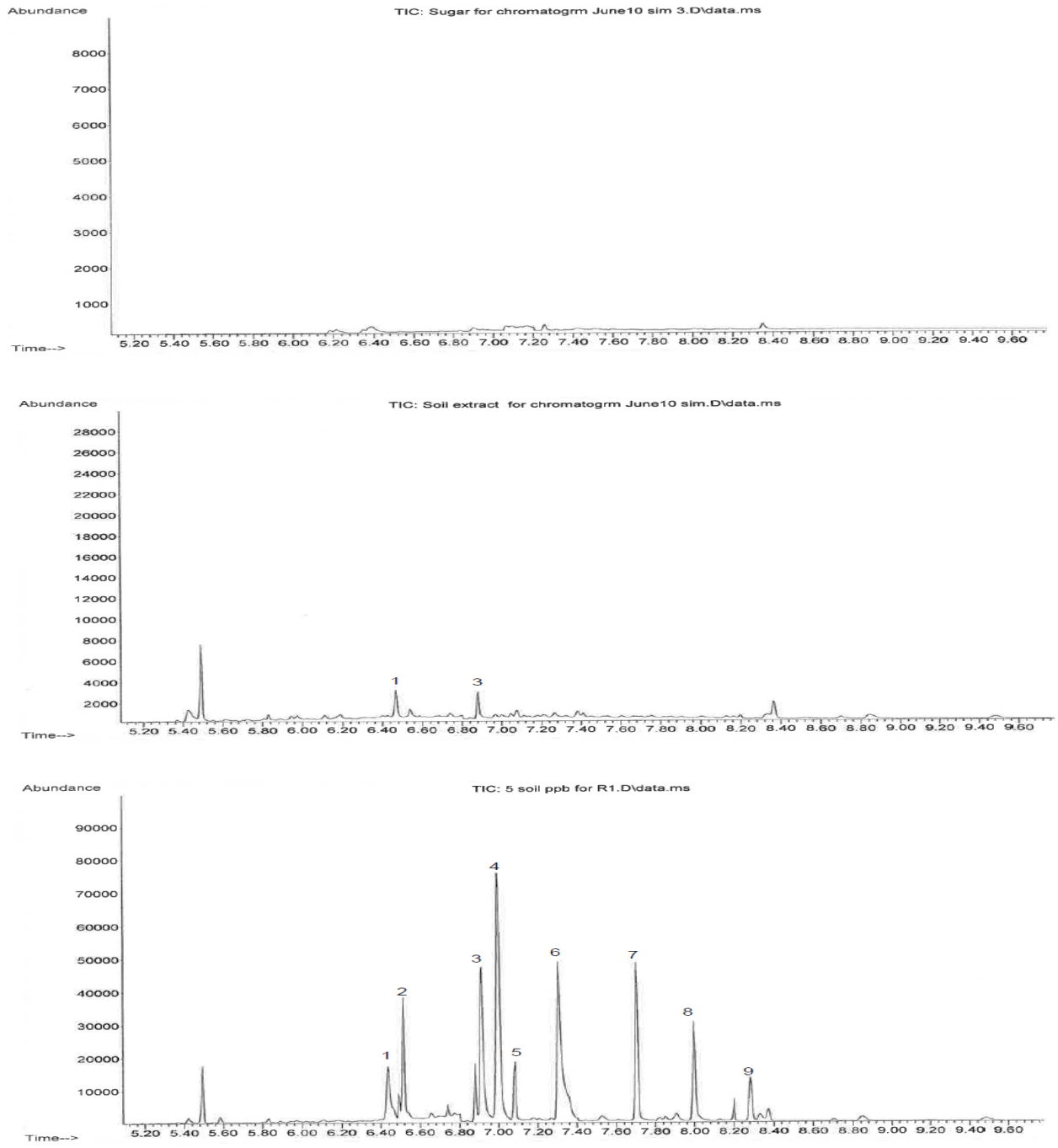


Figure 4.2.8 Representative chromatograms of blank sugar (A), soil (B) and spiked soil (C) at 12.5 µg/kg. Peaks: 1, atrazine; 2, diazinon; 3, ametryn; 4, terbutryn, 5, chloropyrifos; 6, dimethametryn; 7, 4,4'-DDE; 8, 4,4'-DDD; 9, 4,4'-DDT.

4.2.4 Comparison of SALLE-LD-DLLME-GC-MS with other reported literatures

In order to evaluate performances of the developed method, it was compared with previously reported methods including microwave assisted extraction coupled with gas chromatography mass spectrometry (MAE-GC-MS) (Merdassa et al. 2013), dispersive liquid-liquid microextraction and gas chromatography flame photometric detection (DLLME-GC-FPD) (Yang et al. 2012), dispersive solid-phase extraction combined followed by dispersive liquid-liquid microextraction combined with sweeping micellar electrokinetic chromatography (DSPE-DLLME-MEKC) (Zhang et al. 2011), DLLME-UV-Vis (Joarder et al. 2014) and Quick Easy Cheap Effective Rugged Safe coupled with gas chromatography mass spectrometry (QuEChERS-GC-MS) (Kolberg et al. 2011). The merit of the methods and that of this study are given in Table 4.2.4. The performances of the developed technique were compared with that of the previously reported techniques in terms of relative recovery, LOD and regression coefficient (R^2). Based on the findings the developed technique is found to be comparable or better.

Table 4.2.3 Comparison of the proposed SALLE method with related reported techniques.

Method	Pesticides	Matrix	Recovery (%)	LODs (µg/kg)	RSDs%	R^2	Reference
MAE-GC-MS	Organophosphorus and fungicides	Soil	93.0–104	0.10–0.12	0.2–14	0.992-0.997	(Merdassa et al. 2013)
DLLME-GC-FPD	Organophosphorus	Soil	86.7–108	0.20–0.50	2.0–6.6	0.999 -0.999	(Yang et al. 2012)
DSPE-DLLME-MEKC	Sulfonylurea	Soil	76.0–93.5	0.3–0.80	5.3–6.8	0.996– 0.998	(Zhang et al. 2011)
DLLME-UV-Vis	Neonicotinoid and *s*-triazine	Soil and water	80.59–98	0.05–0.1	-	0.992–0.998	Joarder et al. 2014
QuEChERS-GC-MS	Organochlorine, organophosphate and pyrethroid	Wheat grains, flour and bran	70.0–120	2.5	0.1–9.6	0.990–1.00	Kolberg et al. 2011
SALLE-LD-DLLME-GC-MS	Organochlorine, organophosphate and *s*-triazine	Sugar and soil	79.0–111	0.01–0.30	0.02–5.8	0.992–0.999	This study

4.3 High density sc-CO_2 extraction for quantitative analysis of *s*-triazine and organochlorine pesticides in onion samples

4.3.1 Multivariate optimization design

Designed experiment can give systematic investigation route and provide sequential steps for understanding linear interaction and more complex types of interaction. Multivariate techniques which are faster, more economical and effective and allow more than one variable to be optimized simultaneously (Martendal et al. 2007). In order to maximize the pesticides extraction efficiency, Box-Behnken response surface design using a MODDE 10.1 software was used.

Box-Behnken design (BBD) was developed by Box and Behnken in 1960 (Box and Behnken 1960). The BBD consists of a factorial design with three levels and an incomplete block design in such a way to present as a rotatable or nearly rotatable design and to avoid the extreme vertices (Sharif et al. 214). The number of experiments (N) necessary to obtain this second order equation is dependent of the number of variables, as is given by the expression: $N = 2k(k -1) + C_o$, here k is the number of variables (factor) and C_o is number of replicates at the central point (Martendal et al. 2007). BBD is useful to avoid experiments which are in extreme conditions because the highest level and lowest level combinations for every factor cannot be included in BBD. Unsatisfactory results might be avoided in BBD (Sharif et al. 214; Martendal et al. 2007). Table 4.3.1 shows the real levels of the variables, and describes the 15 runs that were carried out and recovery analysis of all analyte. In this current work, three variables including density (0.7–1 g/mL), volume (10–40 mL) and temperature (40–70 ^oC) were selected to draw Box–Behnken experimental design which was used for the optimization of the variables for efficient extraction of target analytes. The responses for each experiment were calculated as the recovery.

Table 4.3.1 Variables, surface response Box-Behnken experimental design for optimization of sc-CO_2 extraction and recovery of the analytes under study.

Expt design				**Recovery (%)**			
Exp	**Density**	**Volume**	**Temperature**	**Atrazine**	**2,4´–DDD**	**Endrin**	**4,4´–DDT**
N1	0.7	10	55	71.0	40.4	42.5	31.6
N2	1.0	10	55	102	30.1	18.6	58.7
N3	0.7	40	55	100	59.1	60.5	34.2
N4	1.0	40	55	117	86.8	65.2	72.0
N5	0.7	25	40	77.5	22.6	11.0	52.1
N6	1.0	25	40	87.6	49.4	49.1	46.3
N7	0.7	25	70	37.9	17.0	14.4	12.4
N8	1.0	25	70	48.9	44.6	46.8	33.1
N9	0.85	10	40	73.8	37.2	22.7	45.2
N10	0.85	40	40	122	51.5	32.7	68.9
N11	0.85	10	70	36.4	38.9	30.6	9.96
N12	0.85	40	70	77.6	17.1	34.2	29.4
N13	0.85	25	55	102	92.6	89.1	74.3
N14	0.85	25	55	113	114	106	82.0
N15	0.85	25	55	128	110	96.9	83.2

4.3.2 Multivariate parameter optimization

The optimization step of the sc-CO_2 extraction conditions was performed using a Box-Behnken design (Table 4.3.1). The main variables that could potentially affect the extraction efficiency were chosen: volume, density and temperature. Other parameters implicated in the extraction were kept constant, namely the amount of dried sample (1 g) and flow rate (3 mL/min). This experimental design was chosen for the optimization not only because of its reduced number of experiments (15 against 27 (3^3) experiments using full design) but also because of no loss of significant information occurs. Based on this hypothesis experiment N3 was rejected. The responses for each experiment were calculated as the recovery. The levels selected for temperature took into account a compromise between sample throughput and the range in which CO_2 is to be supercritical fluid.

Figure 4.3.1 indicates the summary of model fit for observed and predicted values of recovery analysis. The high values of R^2, Q^2, model validity and reproducibility showed that the quadratic equation can represent the system under the given experimental domain. This is also evident from the fact that the linearity plot depicted in Figure 4.3.2 indicates a satisfactory correlation between the observed and predicted values of all pesticides under investigation extraction efficiency. As seen in Figure 4.3.2, the points cluster around the diagonal line shows a good fit of the model, since the deviation between the experimental and predicted values was less.

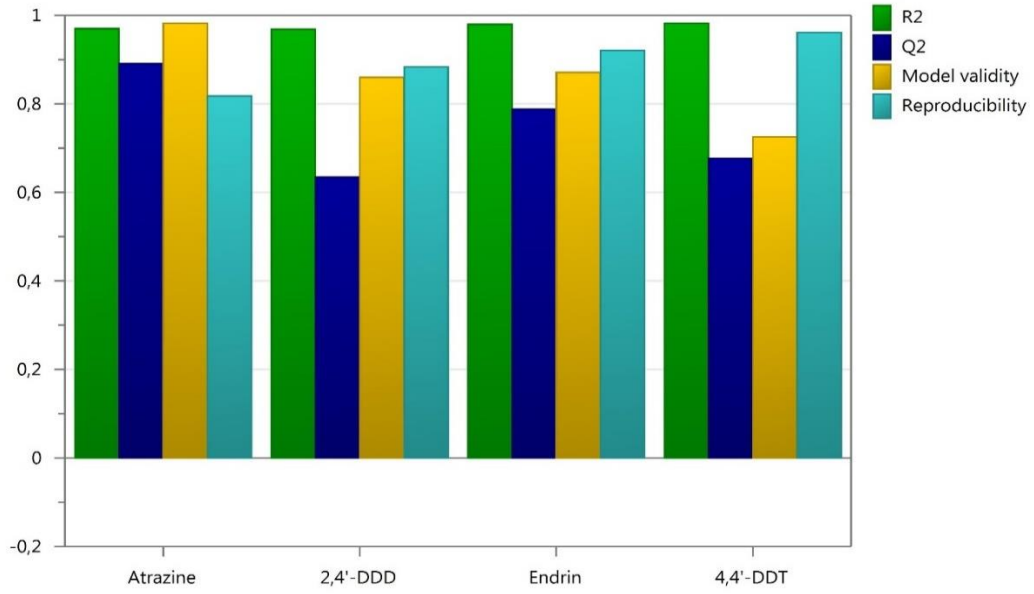


Figure 4.3.1 Summary of model fit.

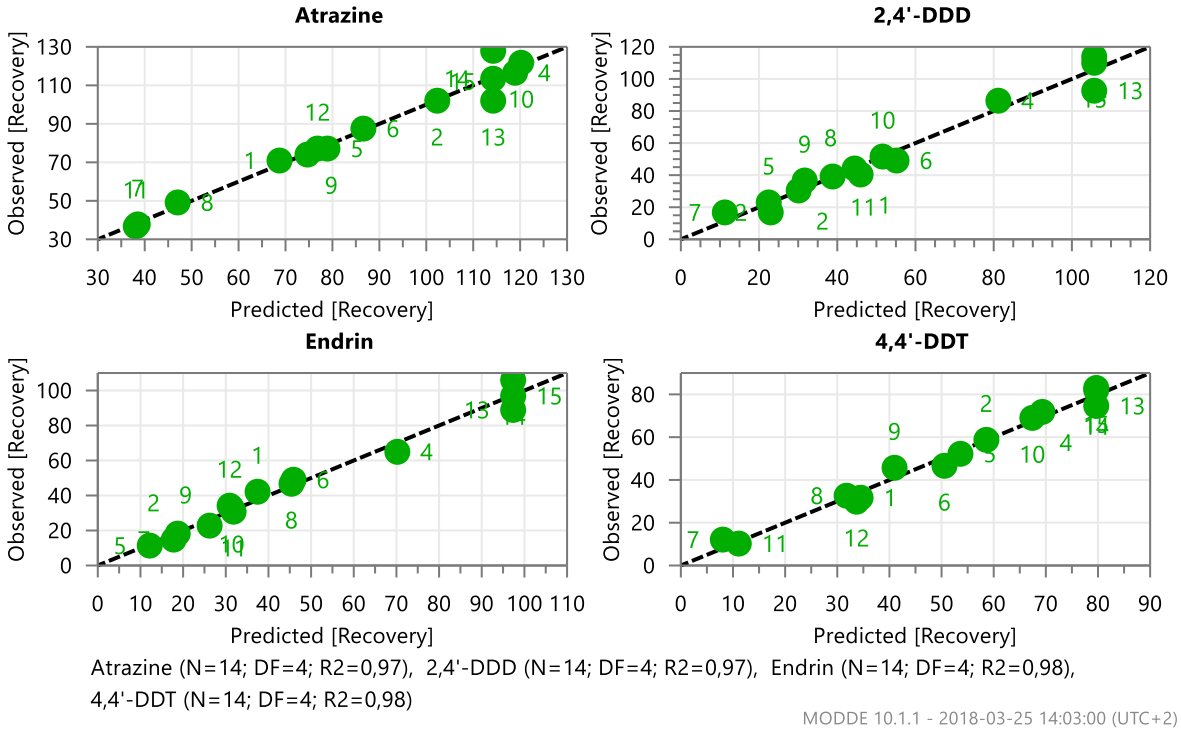


Figure 4.3.2 The linearity plot of predicted versus observed recovery.

Figure 4.3.3 shows that the extraction temperature has a significant negative effect upon the extraction efficiency of atrazine and 4,4'-DDT whereas insignificant positive effect for endrin and 2,4'-DDD. The volume of extraction has positive effect for all analytes and significant for atrazine and 4,4'-DDT. Furthermore, the density of sc-CO_2 has significant positive effect on the extraction recovery endrin and 2,4'-DDD.

According to Figure 4.3.3, the quadratic term of density and volume has positive effect on extraction of endrin and 2,4'-DDD. The interactions between density/temperature and volume/temperature also showed a significant effect on the extraction efficiency. The regression models obtained were used to calculate the response surface for each variable separately.

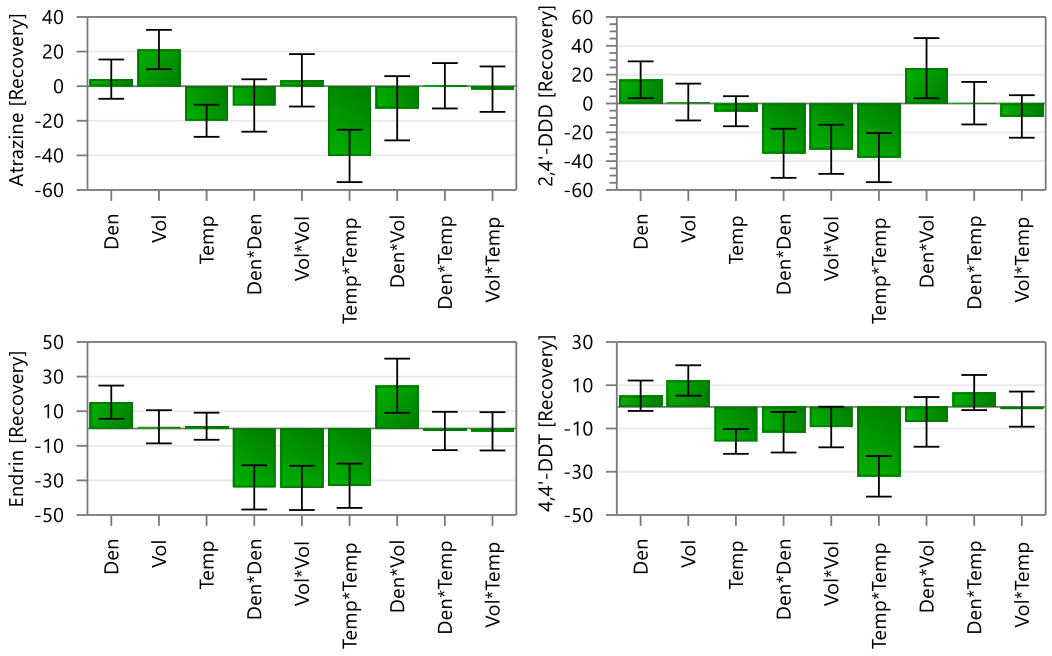


Figure 4.3.3 Effect of extraction parameters on recovery of analytes.

Figure 4.3.4 shows response surface plots for the peak recoveries. Accordingly, the plots given were used for interpreting the variation of recovery as a function of each pair of the independent variables graphically. From the graph it can be seen a significant interaction between volume and density for all analyte indicating that higher density values are optimal for the extraction process. Lower extraction efficiencies were obtained for atrazine at lower volume and density. In Figure 4.3.4 A, B and C, the interaction between volume and density, temperature and density and volume and temperature is demonstrated, respectively revealing that the higher extraction efficiency is obtained at lower temperature and high densiy.

From the response surface plot of interaction between volume and density the peak area of atrazine and 4,4'-DDT is more affected by volume whereas density is more pronounced for endrin and 2,4'-DDD. In the interaction of temperatre and density, the extracability of the endrin is assisted by increasing temperature and density has positive effect for the others. In the same way in the volume and temperature interaction volume has significant effect and increasing temperature decreases the extractability of all the analyte under study.

The increase in temperature may increase the extraction efficiency because the solubility of many organic compounds can dramatically increase as the the solute's vapor pressure increase with temperature (Hauthal 2001). On the other hand, increasing temperature will decrease the fluid density which will decrease the solvation power of the sc-CO_2 fluid (Hawthorne 1995). The high density of these fluids gives them a high solvation power. Its high diffusion and low viscosity allows to penetrate in to the solid matrix. This characteristic promotes higher solubility of the compounds in supercritical fluids when compared with organic solvents (Machado et al. 2013). According to the overall results of the optimization study, the following experimental conditions were chosen: density, 0.9 g/mL; volume, 29 mL and temperature, 53 ^{o}C.

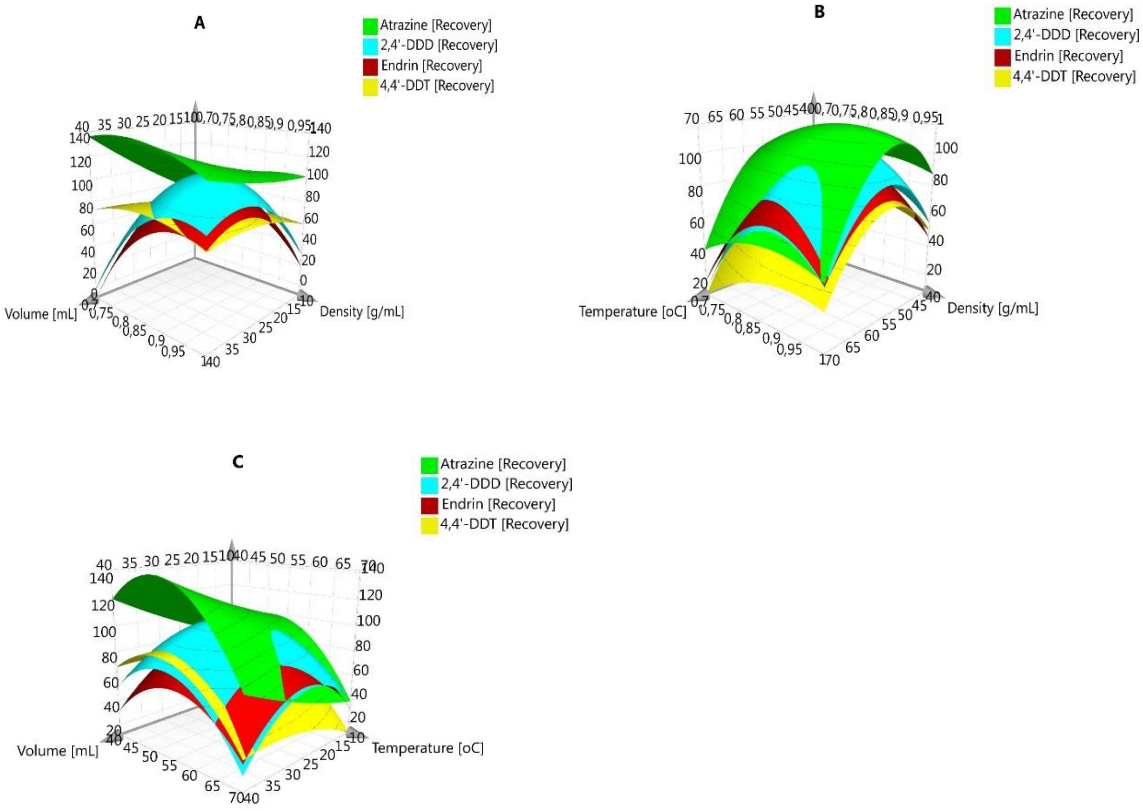


Figure 4.3.4 Response surface plot for parameter optimization and recovery obtained.

4.3.3 Analytical performance and application of proposed method to real onion samples

Matrix-matched calibration curves were established in onion samples fortified at five different concentration levels (7.8, 125, 250, 500 and 2000) for all analytes except endrin (31.25, 125, 250, 500 and 1000 μg/kg). Three spiked onion samples per level were treated following the sc-CO_2 procedure. A blank onion sample was analyzed and matrix interferences were not found at any analyte retention time. Peak area of each compound was considered as function of analyte concentration on the sample. LODs of the method were calculated as the minimum analyte concentration yielding a S/N equal to three. Table 4.3.2 shows that LODs ranging between 0.2 and 2 μg/kg were achieved. The obtained LODs fulfil with the detection levels recommended for each pesticides by EURLs.

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and reproducibility (inter-day precision) of the proposed sc-CO_2-GC-MS method. Repeatability and reproducibility were assayed by analyzing spiked samples at 500 μg/kg concentration levels. Results expressed as recovery (%RSDs) are shown on Table 4.3.2. In all the precision, %RSDs, were lower than 11%.

In order to check the trueness of the proposed method, recovery experiments were performed using spiked onion sample at 500 μg/kg concentration level. Moreover, a blank of onion sample was analyzed and no matrix interferences were observed at each of pesticides under investigation retention times. Figure 4.3.5 shows a blank and a spiked onion sample treated and analyzed according to the proposed procedure. Recoveries were estimated by the comparison of the obtained signal for each analyte with the signal obtained for a blank sample spiked after the sample treatment and prior to its analysis. Recovery results obtained for each analyte of are shown on Table 4.3.2. As can be seen, recoveries ranges from 80.3 to 103%, demonstrating the convenience of the proposed sample treatment for quantitative and qualitative analysis of pestcides studied in onion samples. Figure 4.3.5 shows the typical chromatogram of unspiked and spiked onion sample.

Table 4.3.2 Analytical performances of the proposed sc-CO_2 method for onion samples.

Pesticides	**Linearity (µg/kg)**	**Regression equation**	**(R^2)**	**Recovery**	**Repeatablity (%RSD)**	**Reproducibility (%RSD)**	**LOD (µg/kg)**
Atrazine	7.8-2000	y = 9602.6x + 47344	0.999	92.9	3.8	1.8	0.2
2,4'-DDD	7.8-2000	y = 12368x + 99235	0.998	93.9	0.9	3.6	0.4
Endrin	31.25-1000	y = 511.08x + 12.796	0.998	103	10	8.3	2.0
4,4'-DDT	7.8-2000	y = 1482.6x + 9754.7	0.994	80.3	4.4	2.9	0.6

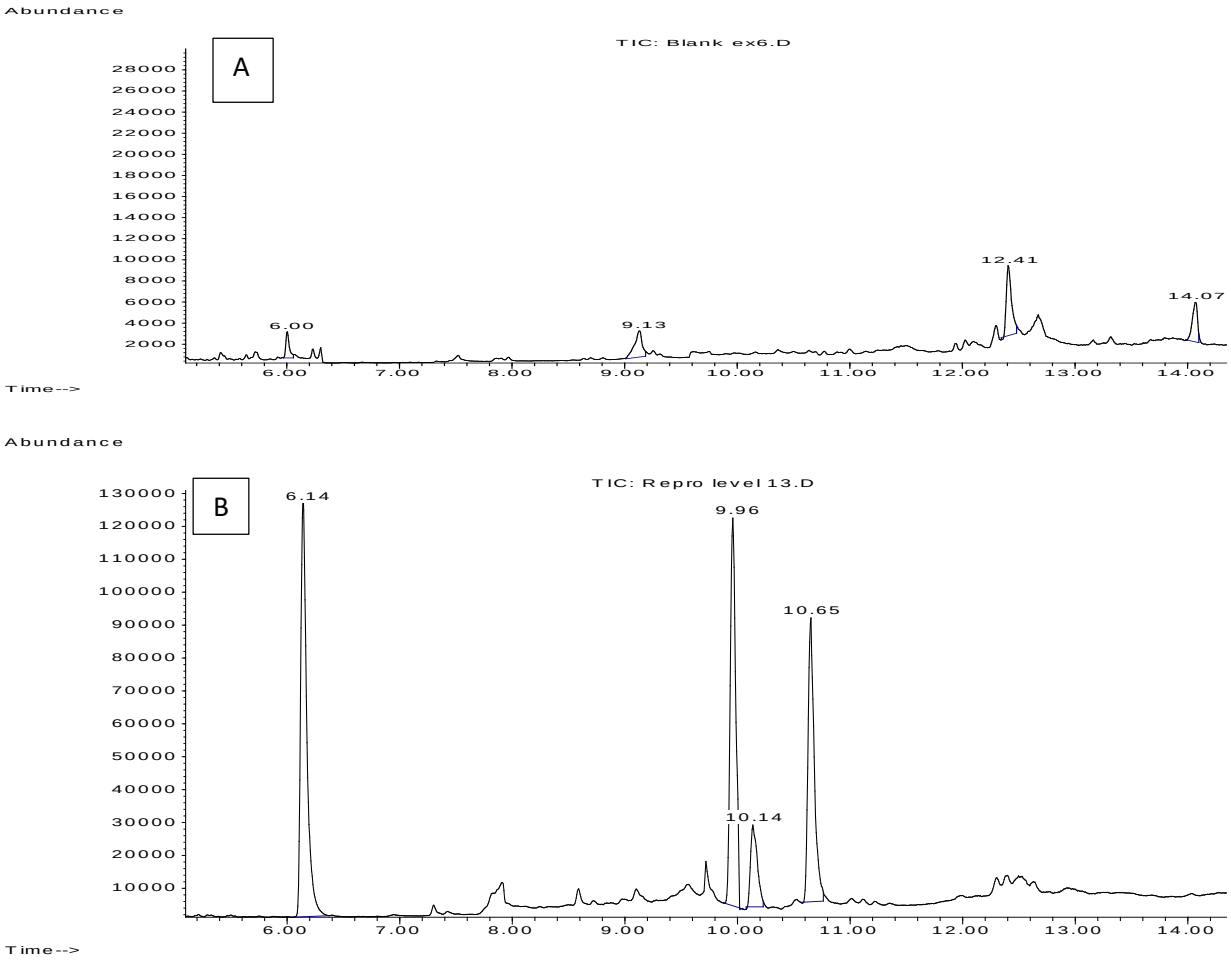


Figure 4.3.5 Typical chromatograms of unspiked (A) and spiked (B) onion extract. Peaks: 6.14, atrazine; 9.96, 4,4'-DDE; 10.14, endrin; 10.65, 2,4'-DDT.

4.3.4 Comparison of sc-CO_2-GC-MS with other reported literatures

The currentely proposed extraction method, sc-CO_2-GC-MS, was compared with SPE-GC-ECD (Sharif et al. 2006), QuECheRS-GC-FPD (Ueno et al. 2003), QuEChERS- MWCNTs combined with GC-MS (Zhao et al. 2012) and QuEChERS-GC-MS (Kolberg et al. 2011). The summary of the results obtained are given in Table 4.3.3. When compared to these methods, the proposed method provided the highest recoveries, lower LOD and better or comparable coffiencet of determination. Therefore, the proposed method demonstrated is more efficient for extraction of target analyte and other pesticides having similar physical and chemical properties.

Table 4.3.3 Comparison of the proposed sc-CO_2-GC-MS with related reported techniques.

Method	**Pesticides**	**Matrix**	**Recovery (%)**	**LODs (µg/kg)**	**R^2**	**References**
SPE-GC-ECD	Organochlorine and pyrethroid	Fruit and vegetables	54.0–104	0.3–15	0.998 –0.999	(Sharif et al. 2006)
QuECheRS-GC-FPD	Organophosphoru	Onion	61.0–105	2.0–10	-	(Ueno et al. 2003)
QuEChERS-MWCNTs-GC-MS	Multiclass pesticides	Leek, onion, ginger and garlic	78.0–110	2.0–20	> 0.990	(Zhao et al. 2012)
QuEChERS-GC-MS	Organochlorine, organophosphate and pyrethroid	Wheat grains, flour and bran	70.0–120	> 2500	0.990–1.0	(Kolberg et al. 2011)
SC-CO_2-GC-MS	Organochlorine and *s*-triazine	Sugar and soil	80.3-103	0.2–2	0.998–0.999	This study

4.4 Multivariate optimization of combined static and dynamic mode sc-CO_2 for trace analysis of pesticides pollutant in honey

4.4.1 Multivariate optimization design

Method development became more effective using multivariate optimization because more information is obtained about the interaction among the variables, sometimes undetected when univariate approach is used (Meneghini et al. 2014). For analytical purposes, Box-Behnken response surface design using a MODDE 10.1 software (Sartorius Stedim Biotech, Malmö, Sweden) has shown to be the most adequate kind of design and can be applied to response surfaces with a good estimation of the parameters of the quadratic mathematical model, allowing the study of independent variables, at different levels (Bezerra et al. 2008). In this study a multifactor optimization strategy has been selected to simultaneously take into account the main factors and their interactions influencing the sc-CO_2 extraction of atrazine, diazinon, chlorothalonil and deltametryn. The parameters studied includes static time (5–15 min), pressure (200–700 bar) and temperature (45–70 ^{o}C). Table 4.4.1 indicates the levels of the variables, and describes the 15 runs conducted and corresponding peak areas of all analyte.

Table 4.4.1 Variables and surface response Box-Behnken experimental design for optimization of static and dynamic sc-CO_2 extraction and peak areas obtained for the analytes under study.

Exp No.	Static time (min)	Pressure (bar)	Temperature (°C)	Peak area (mAU)			
				Atrazine	Diazinon	Chlorothalonil	Deltametryn
N1	5	200	57.5	8.77	18.8	3.81	3.35
N2	15	200	57.5	7.37	9.19	1.60	3.11
N3	5	700	57.5	8.04	7.81	1.60	3.17
N4	15	700	57.5	8.99	10.9	2.97	4.38
N5	5	450	45.0	9.58	16.8	6.72	3.70
N6	15	450	45	8.16	12.5	3.35	4.18
N7	5	450	70	12.3	19.2	3.99	2.63
N8	15	450	70	14.0	18.2	5.62	4.87
N9	10	200	45	10.5	18.5	3.39	3.61
N10	10	700	45	10.5	8.67	3.92	5.73
N11	10	200	70	13.5	21.4	4.38	5.16
N12	10	700	70	12.6	14.9	4.55	3.30
N13	10	450	57.5	12.8	6.56	4.39	3.42
N14	10	450	57.5	13.0	7.87	5.34	3.22
N15	10	450	57.5	11.8	6.64	5.37	3.18

4.4.2 Optimization of qualitative parameters

4.4.2.1 Effect of solid support on extractability of pesticides

Honey is a sticky semisolid matrix. To reduce error during quantitative transfer of the sample from weighing bottles to extraction cell and to eliminate blockage of sc-CO_2 line, it requires the solid support. The solid support should not absorb the analyte and inert under the extraction conditions. Based on this fact two sample supporting, glass beads (1 g) and diataomaceous Earth (0.2 g), materials were avaluated. As shown in the Figure 4.4.1, the use of diatomaceous Earth results in the decrease of extraction efficiency of all pesticides under study. Therefore, glass beads were used for further experiments.

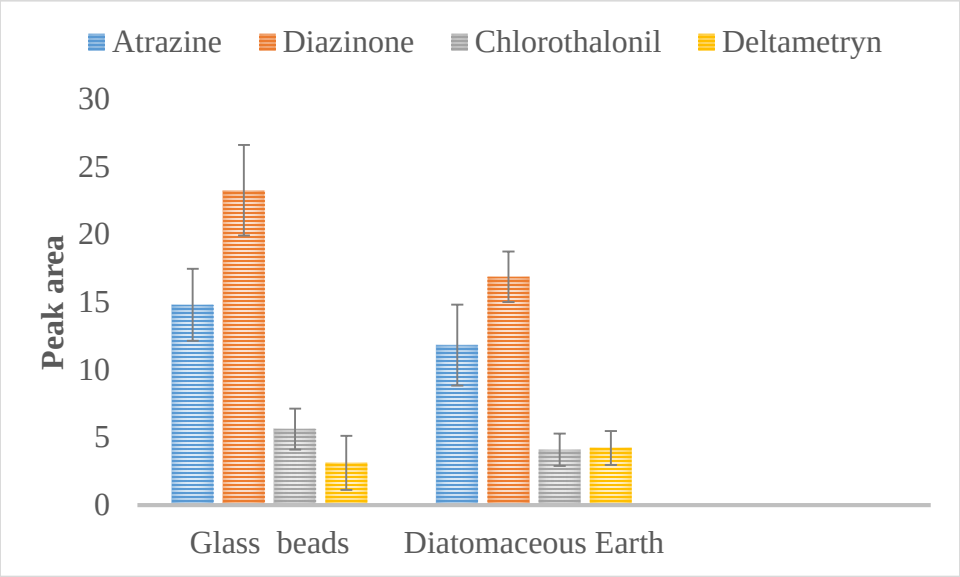


Figure 4.4.1 Effect of addition of solid support. Extraction conditions: Sample amount; 2 g, spiking level; 500 µg/kg, modifier; 2 mL of methanol, tempemperature; 45 ^{o}C, pressure; 200 bar, static time; 5 min, volume of sc-CO_2; 30 mL.

4.4.2.2 Selection of modifier solvent

The use of pure sc-CO_2 in the simultaneous analysis of polar and nonpolar pesticide residues is limited because sc-CO_2 is considered as a nonpolar solvent having a liquid solubility equal to that of hexane (Lanças et al. 2000). For this reason, acetone, acetonitrile and methanol were evaluated for quantitative extraction of moderately polar (atrazine and diazinon) and non polar pesticides

(chlorothalonil and deltametryn). A satisfactory result was obtained when acetonitrile was used as a modifier solvent (Figure 4.4.2). A modifier may (Lanças et al. 2000):

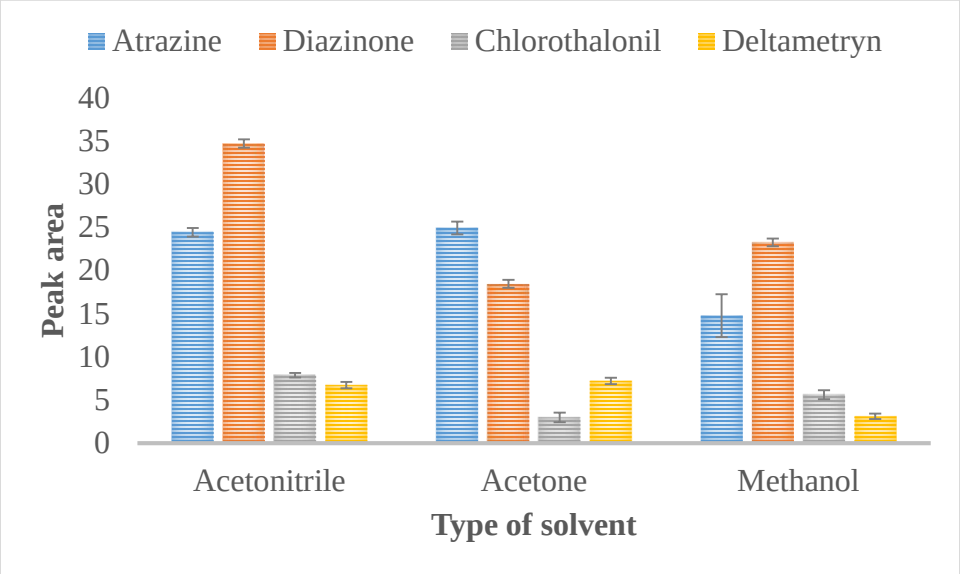


Figure 4.4.2 Effect of type of modifier solvent. Extraction conditions: Sample amount; 2 g, spiking level; 500 µg/kg, volume of modifier; 2 mL, temperature; 45 ^{o}C, pressure; 200 bar, static time; 5 min, volume of sc-CO_2; 30 mL.

a) Increasing the analyte's solubility in the supercritical fluid, as a result of analyte-modifier interactions in the fluid phase.
b) Facilitating the analyte desorption-the molecules of polar modifiers are able to interact with the matrix and compete efficiently with the analytes for the active sites in the matrix.
c) Distorting the matrix-analyte diffusion and penetration of the supercritical fluid inside the matrix are favored when the modifier swells the matrix.

4.4.3 Parameter optimization

A multivariate experimental design and optimization was carried out to find the optimal experimental conditions for static and dynamic sc-CO_2 extraction process. Three factors including static time, pressure and temperature were selected in this study. The design matrix and response data (peak area) obtained for honey smaples spiked at 500 µg/kg are given in Table 4.4.1. Figure 4.4.3 indicates the summary of model fit for observed and predicted values of peak area of target analytes. A good model representing the quadratic equation has $R^2 > 0.5$, $Q^2 > 0.1$; which estimate

the future prediction precision, model validity > 0.25 and reproducibility > 0.5. Therefore, for this study the quadratic equation can represent the extraction system under a given experimental condition based on the values of R^2, Q^2, model validity and reproducibility. The linearity plot between observed and predicted peak area (Figure 4.4.4) also indicate the model can represent the extraction system under given experimental domain, because the deviation between the experimental and predicted values was less and the points cluster around the diagonal line which shows a good fit of the model.

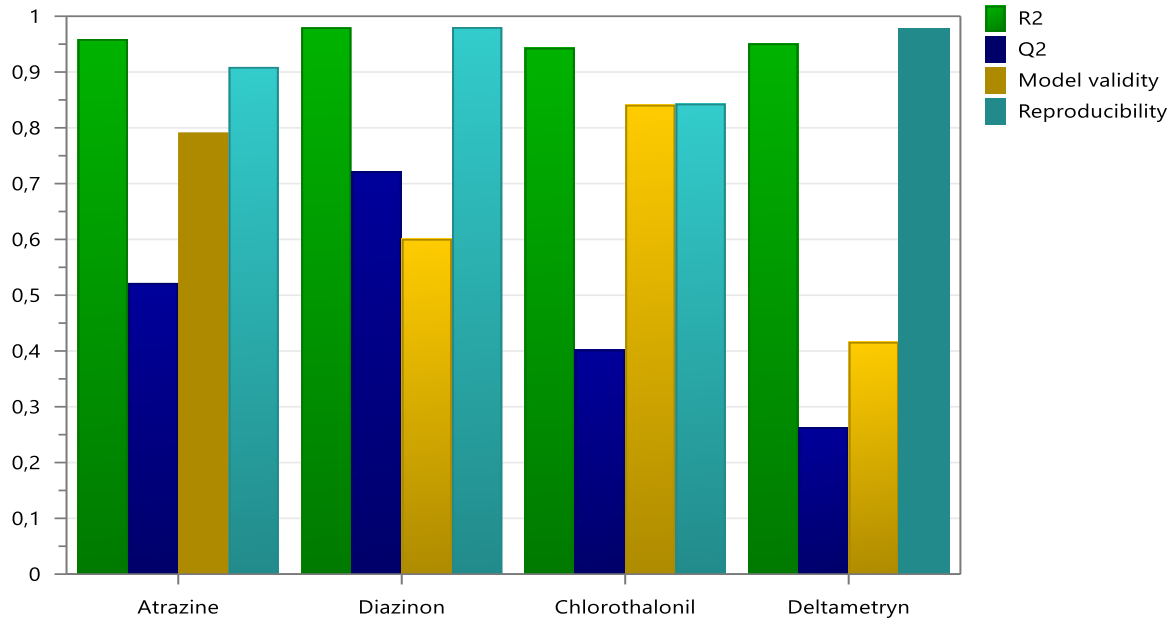


Figure 4.4.3 Summary of model fit.

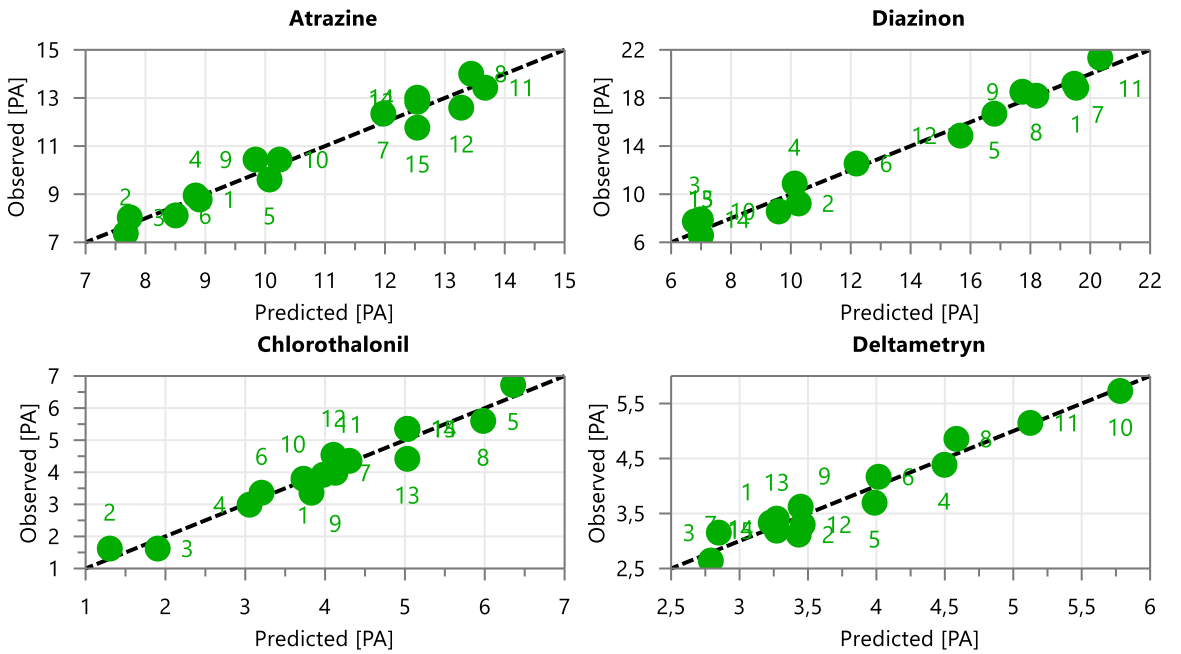


Figure 4.4.4 The linearity plot of predicted versus observed peak areas of target pesticides.

As shown in Figure 4.4.5 the effect of static time on extraction efficiency is significant only for diazinon (negative) and deltametryn (positive). Pressure is significant only for extraction effeiciency of diazinon (negative). On the other hand, temperature has positive significant effect for extraction of atrazine and diazinon. The combined effect of static time and pressure which is positive significant effect for diazinone and chlorothalonil. The interaction between static time and temperature is significant only for chlorothalonil and its effect is positive. Pressure and temperature interaction also showed a significant effect on the extraction efficiency for deltametryn and its effect is negative.

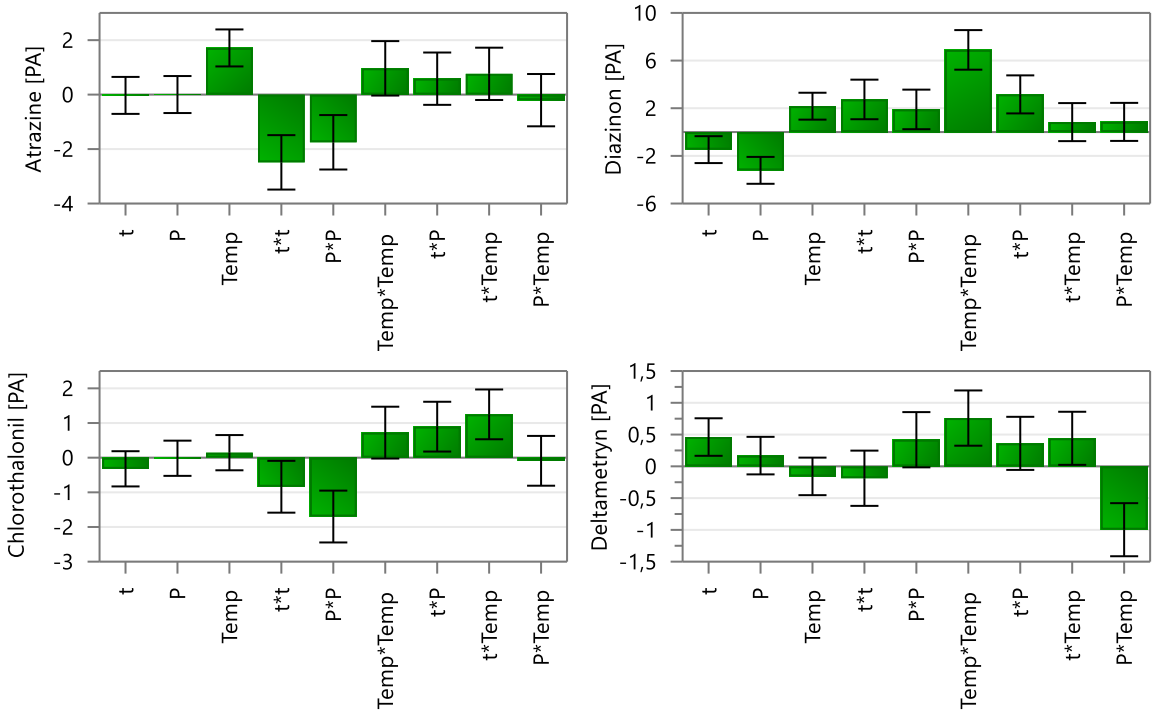


Figure 4.4.5 Effect of extraction parameters on peak area of analytes.

The regression models obtained were used to calculate the response surface for each variable separately. The interactions temperature-pressure (TempP), static time-pressure (tP) and static time-temperature (tTemp) were significant for all compounds, except deltamethrin. Charts showing the simultaneous two-factor effects (TempP, tP and tTemp) are presented in Figure 4.4.6, the pink color indicate the region of highest responses in terms of peak area, for all pesticides under study.

The TempP interaction (Figure 4.4.6 A) is clearly indictaed the importance of temperature for extractability of alla anlytes except atrazine and deltametryn. The region where high response observed is indicated by pink color. Increasing temperature results high peak area of the analyte. The extractability of the atrazine and deltametryn is siginificant at lower temperature and higher pressure. The combined effect of tP is shown in Figure 4.4.6 B. It can be seen that the tP has significant effect on extraction efficiency of all pesticides except diazinon. The extraction of diazinon is favorable at lower static time and pressure. Figure 4.4.6 C also shows the interaction

between the extraction static time and temperature, better results were obtained when both parameters are increased except deltametryn.

The length of extraction time influenced the extraction efficiency and it is important to maximize the contact of the sc-CO_2 with the sample material because mass transfer is time dependent. The extraction could be incomplete with shorter extraction time and longer extraction with heating will affect the stability of the analytes (Pourmortazavi et al. 2007). The fluid density is proportional to the pressure, so that increasing the pressure is beneficial to the solubility of analytes into the fluid (Karale et al. 2011). On the other hand, at higher pressure compaction may occurs, and produces a lower extraction of the heavier compounds. Furthermore, increasing temperature will increase solubility of the analyte and decreses solvation power of sc-CO_2 (Sartori et al. 2017). Based on optimization results, the experimental conditions giving the highest responses were static time 11.5 min, pressure 252 bar and 70 ^{o}C.

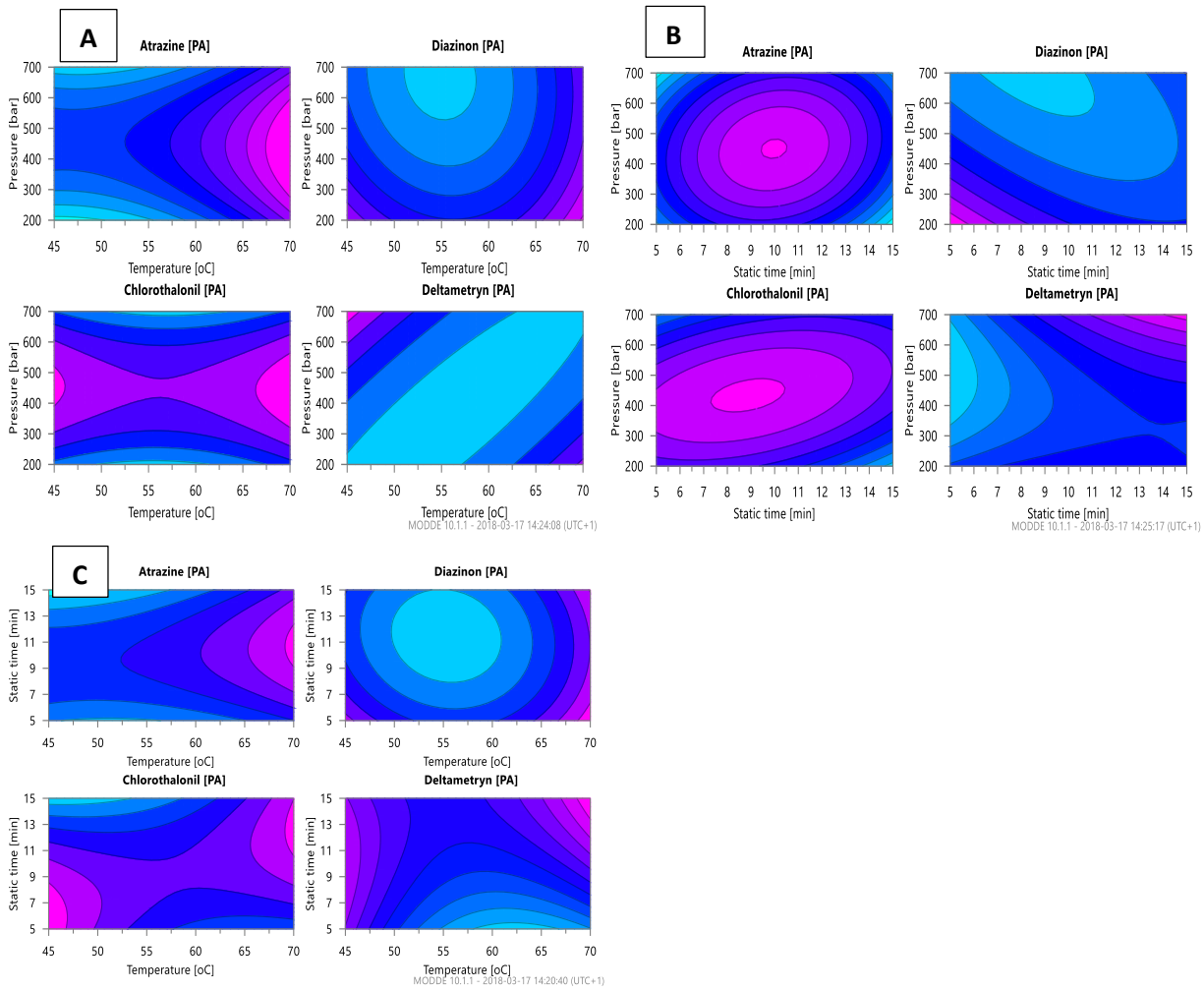


Figure 4.4.6 Response contour plot for the geometric mean of the peak areas of pesticides.

4.4.4 Analytical features of the proposed method and its application to honey samples

The merit of the proposed method was obtained under the optimized conditions. Some quantitative parameters including linear range of calibration curve, coefficient of determination (R^2), LOD and precision int erms of relative standard deviation (RSD%) are calculated and summarized in Table 4.4.2. good linearty was obtained for the calibration graphs with R^2. Precision of the method was determined by analyzing the spiked 2 g of honey sample at concentration level of 250 and 1000 µg/kg at the same day and at three different days. RSDs % are in the ranges of 2.3–4.2% for intra-day (n = 3) and 3.9–8.0% for inter-day precisions (n = 3) which indicate that the method is repeatable. LODs were calculated from standard deviation of seven replicate of spiked honey samples extract at 70 µg/kg based on measurements at a signal-to-noise ratio (S/N) of 3.

To evaluate performance of the presented method 2 g of honey samples were spiked at two different concentration levels, 250 and 1000 µg/kg and extracted under optimum condition and analysed in triplicate. To determine the pesticide residues contents of the samples, standard addition method was used. The matrix effects of the selected samples were investigated and the obtained results are summarized in Table 4.4.2. Typical HPLC chromatograms are shown in Figure 4.4.7. It should be noted that all pesticides under study were not found in honey samples analyzed.

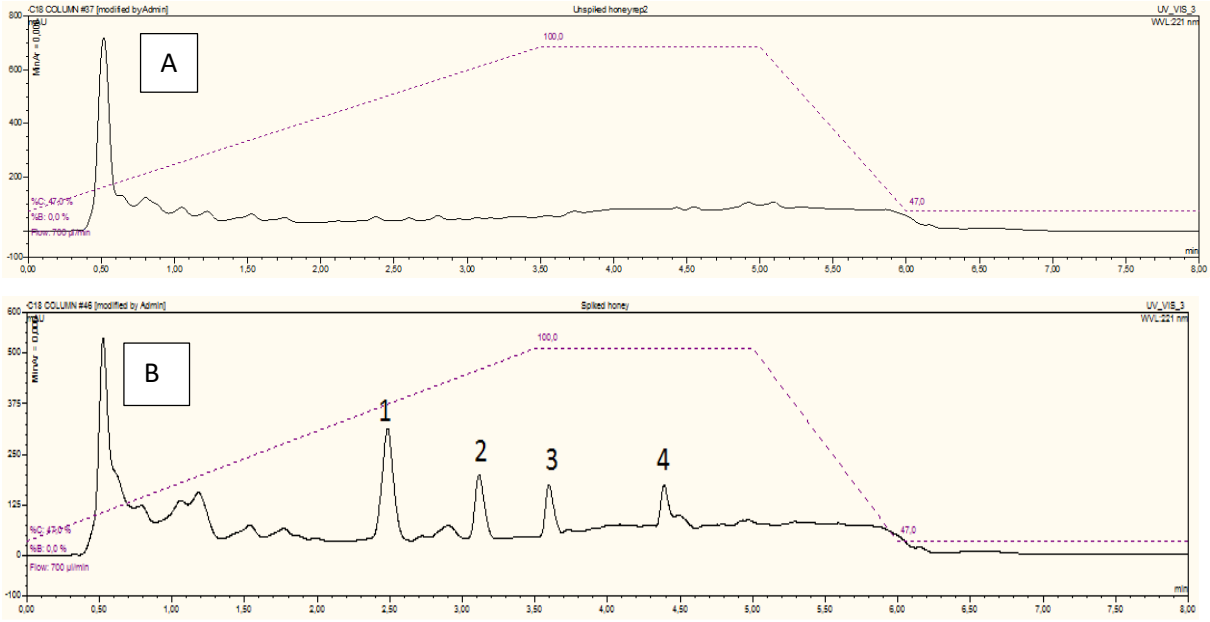


Figure 4.4.7 Typical chromatograms of a unspiked (A) and spiked (B) honey sample. Peaks 1; atrazine, 2; diazinon, 3; chlorothalonil, 4; delthametryn

Table 4.4.2 Performance characteristics of the proposed analytical method.

Analyte	**Linear range (µg/kg)**	**Regression equation**	**R^2**	**LOD (µg/kg)**	**Recovery (%RSD, n = 3)**		**Repeatability (%RSD, n = 3)**		**Reproducibility (%RSD, n = 3)**	
					250 µg/kg	**1000 µg/kg**	**250 µg/kg**	**1000 µg/kg**	**250 µg/g**	**1000 µg/kg**
Atrazine	70-8000	y = 17.446x + 0.6109	0.999	5.0	73.9(3.9)	83.4(1.5)	3.2	1.1	8.0	3.4
Diazinone	70-8000	y = 9.1251x + 0.8334	0.998	8.0	87.5(2.0)	96.6(1.3)	2.3	1.5	6.3	3.6
Chlorothalonil	70-8000	y = 5.3114x + 0.7659	0.999	7.0	95.6(6.0)	111(1.8)	4.2	2.1	3.9	2.1
Deltametryn	70-8000	y = 4.4853x + 0.5128	0.999	9.0	73.4(3.8)	79.8(1.9)	3.8	2.3	6.9	4.7

4.4.5 Comparison with other reported analytical methods

The proposed method, multivariate optimization of combined static and dynamic sc-CO_2-HPLC-DAD, was compared with preveously reported extraction methods in terms of limit of detection, recovery and cofficient of determination. The results obtained are summarized in Table 4.4.2. When compared to LLE-GC-ECD (Mukherjee 2009), LLE-GC-MS (Rissato et al. 2007), LLE and sc-CO_2-GC-ECD (Rissato et al. 2004) and SPE-GC-ECD (Sharif et al. 2006) techniques, static and dynamic sc-CO_2-HPLC-DAD provided the highest recoveries, better or comparable LOD and good cofficient of determination. The proposed analytical method is also greener than the others. Therefore, the proposed method demonstrated superior extraction capabilities for relatively polar and non-polar pesticides in honey sample.

Table 4.4.3 Comparison of the proposed method with different sample preparation techniques.

Method	**Pesticides**	**Matrix**	**R (%)**	**LODs (µg/kg)**	**R^2**	**References**
LLE-GC-ECD	Organochlorine and pyrethroid	Honey	60.0–90.6	1.0–50	> 0.995	(Mukherjee 2009)
LLE-GC-MS	Multiclass pesticides	Honey	76.0 – 95.0	0.2–4.0 (mg/L)	0.997	(Rissato et al. 2007)
LLE and SC-CO_2-GC-ECD	Multiclass pesticides	Honey	75.0–94.0	5.0–10	> 0.990	(Rissato et al. 2004)
SPE-GC-ECD	Organochlorine and pyrethroid	Fruit and vegetables	54.0–104.0	0.3–15	0.998 – 0.999	(Sharif et al. 2006)
Static and dynamic SC-CO_2-HPLC-DAD	Organochlorine, *s*-triazine and pyrethroid	Honey	73.4-111	5.0–9.0	0.998– 0.999	This study

4.5 *Typha latifolia* plant parts as low cost adsorbent for removal of selected multi pesticide residues in contaminated aqueous samples

Typha latifolia plants are among the most common of all aquatic plants. They are often a nuisance but also perform an important function in keeping a lake healthy by filtering the runoffs. *Typha latifolia* plant form dense monocultures when there is a wetland disturbance. They can reach up to 2 or 3 meters and grow fast from thick underground rhizomes. Efficiency of the biosorbents (stem, leaves and flower powder) of *Typha latifolia* plant were tested for the removal of atrazine, diazinon, chlorothalonil, ametryn, malathion, chloropyrifos and dimethametryn in water. The factors that affect pesticides sorption capacity of biosorbents including pH, contact time, centrifugation speed, dose of biosorbent and initial pesticides concentration were investigated. All the experiments were repeated in triplicate to confirm the results and average values are presented.

4.5.1 Fourier transform infrared spectroscopic (FTIR) analysis

FTIR spectra of powdered stem, leaf and flower for pesticide adsorption are shown in Figures 4.5.1. As can be seen from the FTIR of spectra of stem in Figure 4.5.1, the presence of broad band at 3412 cm^{-1} indicate hydrogen bonded OH stretch (alcohol), alkyl C-H stretch at 2923.5 cm^{-1}, C=C stretch at 1603 $cm^{-1}$and C-O stretch at 1039.5 cm^{-1}. The FTIR of leaf are also due to the vibrational bands at 3423, 2921, 2852, 1615 and 1036 cm^{-1}indicating hydrogen bonded OH stretch (alcohol), alkyl C-H, alkenyl C-H stretch, C=C stretch and C-O stretch, respectively. The bands in the FTIR spectrum of flower at 3410.5 cm^{-1}indicate hydrogen bonded OH stretch (alcohol), alkyl C-H at 2918.5 cm^{-1}, alkenyl C-H stretch at 2850.5 cm^{-1}, C=C at 1606 cm^{-1} and C-O bond stretch at 1039.5 cm^{-1}.

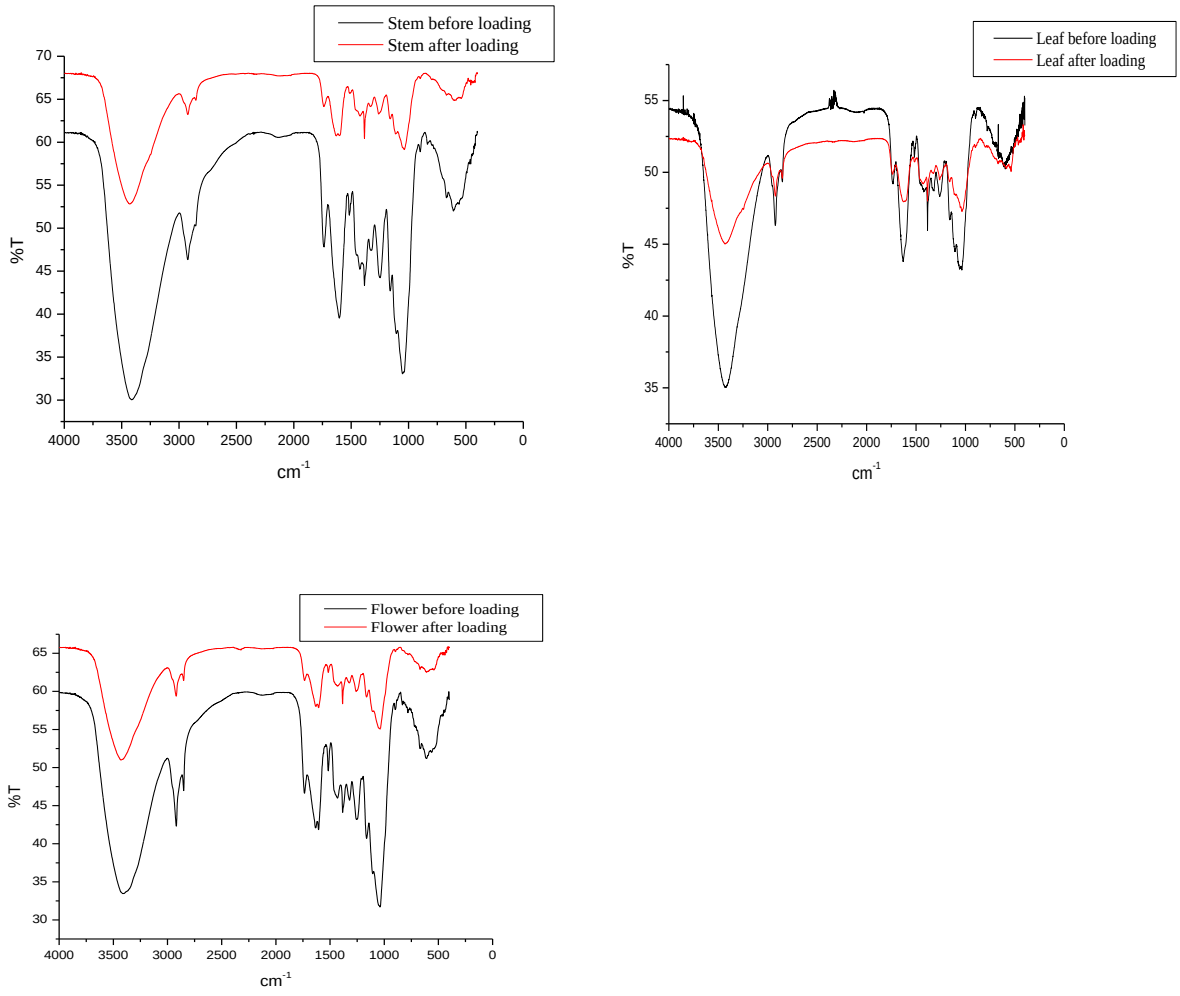


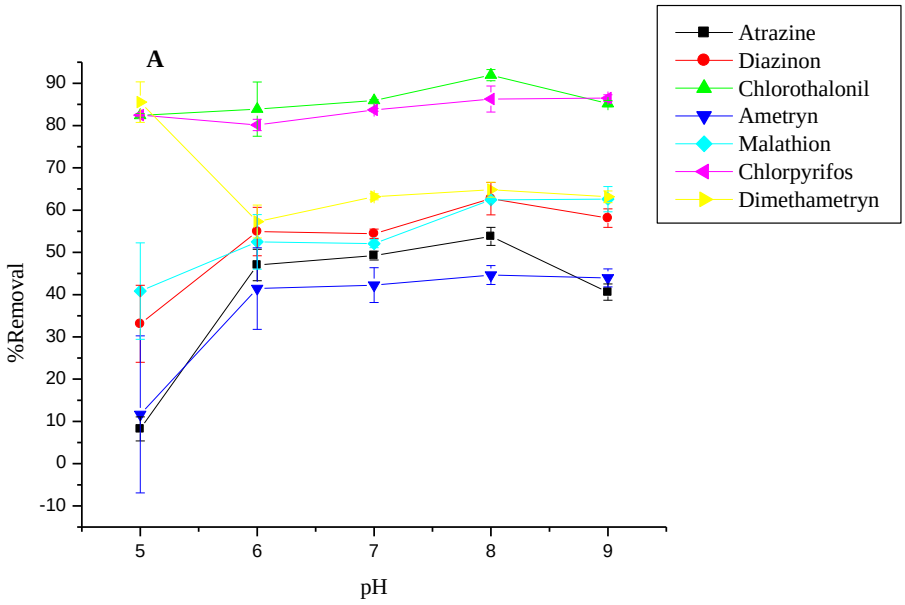
Figure 4.5.1 FTIR spectra of the biosorbents (stem, leaf and flower) before (black) and after (red) sample loading.

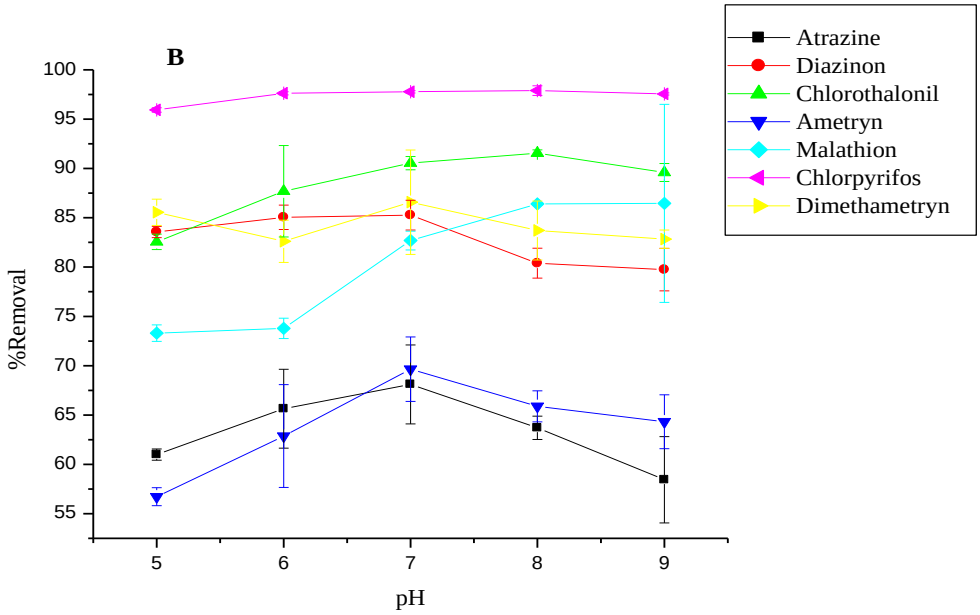
4.5.2 Optimization of adsorption parameters

4.5.2.1 Effect of pH

Solution pH is an important monitoring parameter influencing the sorption behavior of adsorbate onto biosorbent because it controls concentration of the counter ions on the functional groups of the adsorbent and the degree of ionization of the adsorbate during reaction (Gangadhar et al. 2016). In the present study, the effect of pH on biosorption of pesticide was studied over different pH range (Figure 4.5.2). The amount of pesticide removed at equilibrium increases with increasing

pH, appreciably up to pH, 8, pH 7and pH 6 for stem, leaf and flower of *Typha latifolia*, respectively. With further increase in pH, there is decrease or no significant increase in the amount of pesticide removed. Hence, all further experiments were carried out at pH 8, 7 and 6 for stem, leaf and flower respectively.





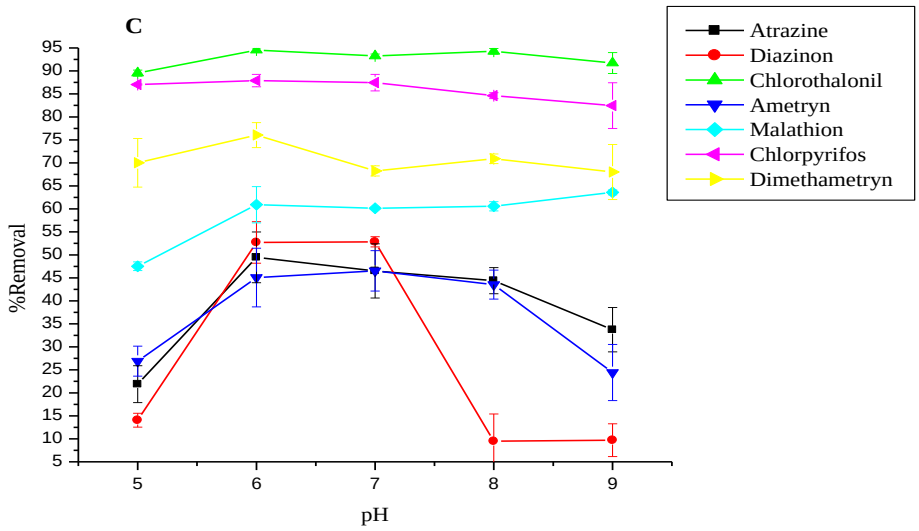
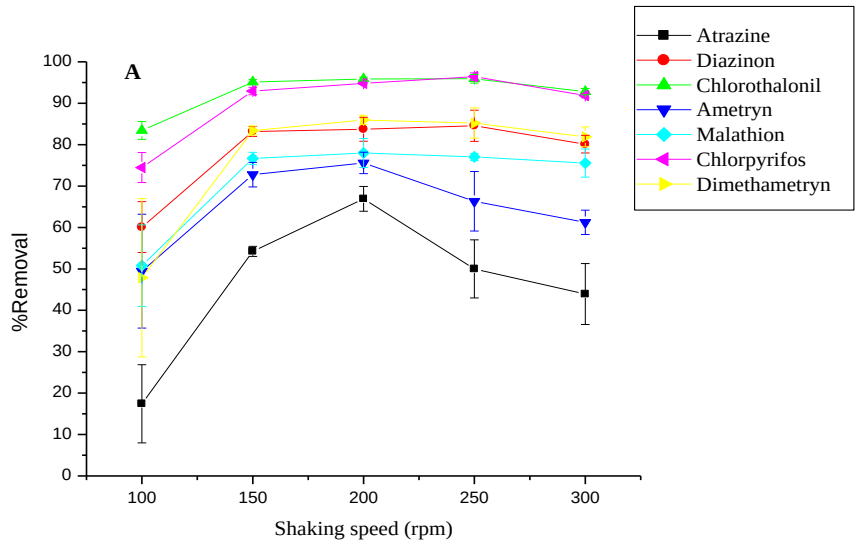
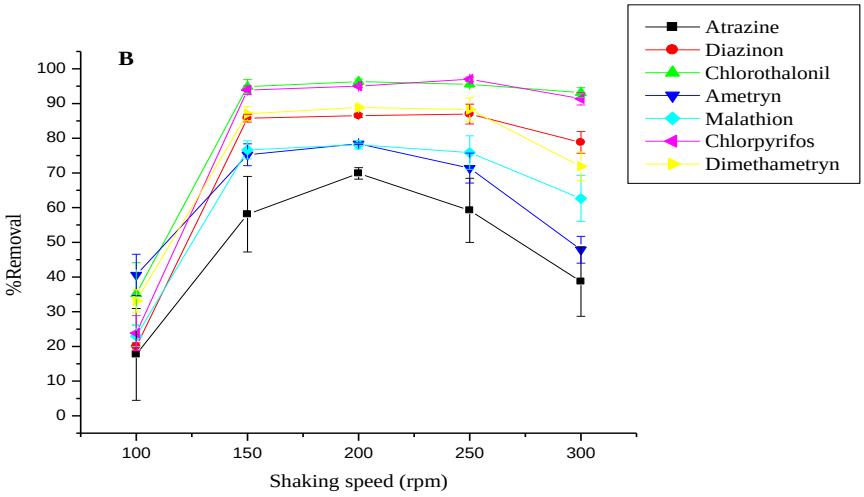


Figure 4.5.2 Effect of pH on uptake of pesticides by stem (A), leaf (B) and flower (C) of *Typha latifolia* plant. Experimental conditions: sample size, 50 mL; spiked concentration, 5 µg/L; shaking speed, 150 rpm, contact time, 40 min; adsorbent dose, 0.5 g.

4.5.2.2 Effect of shaking speed

The removal efficiency of the adsorbent for a given sorbate generally increases with increasing agitation speed. Increasing speed of agitation decreases the time required for the adsorbate to equilibrate, by decreasing the boundary layer resistance to mass transfer and hence increases the diffusion rate of the adsorbate from the bulk into the adsorbent particles (Tadesse et al. 2015).


A
%Removal
Shaking speed (rpm)
Atrazine
Diazinon
Chlorothalonil
Ametryn
Malathion
Chlorpyrifos
Dimethametryn


B
%Removal
Shaking speed (rpm)
Atrazine
Diazinon
Chlorothalonil
Ametryn
Malathion
Chlorpyrifos
Dimethametryn

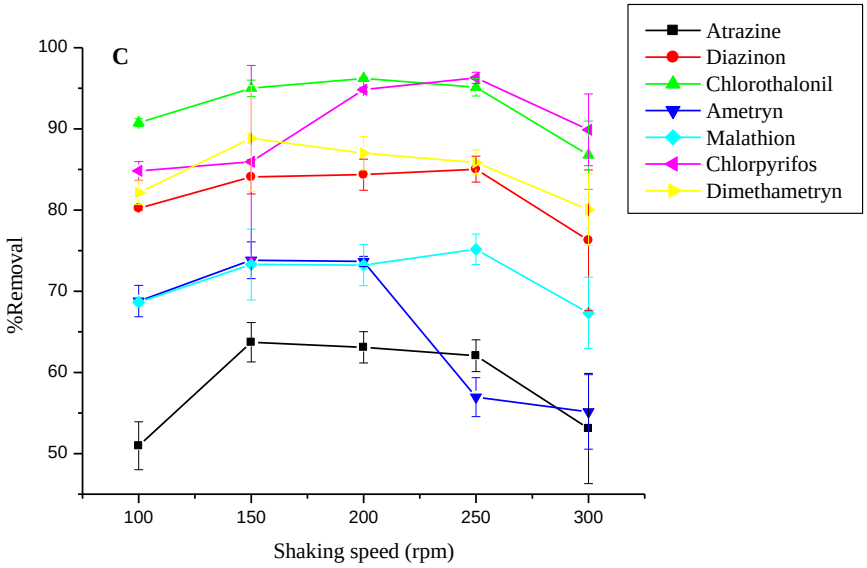


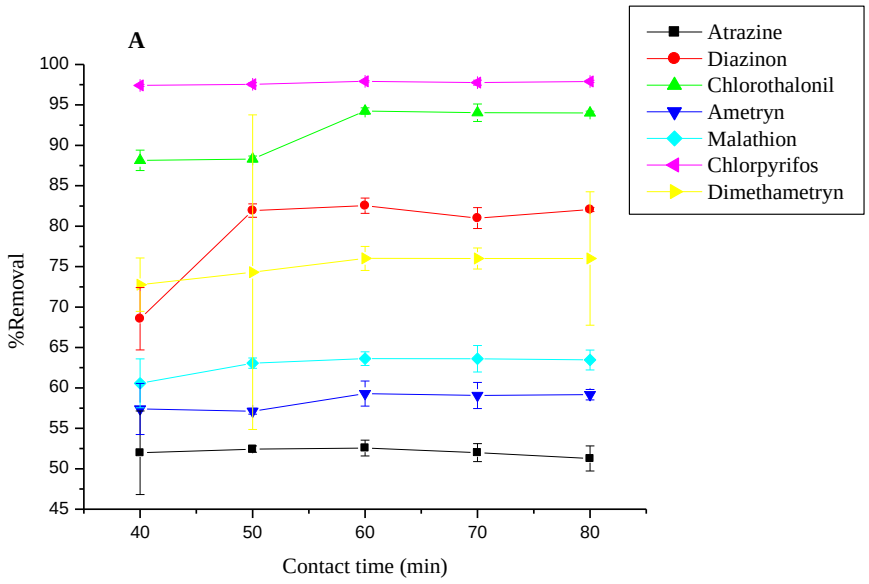
Figure 4.5.3 Effect of shaking speed on uptake of pesticides by stem (A), leaf (B) and flower (C) of *Typha latifolia* plant. Experimental conditions: sample size, 50 mL; spiked concentration, 5 µg/L; pH, 8 for stem, 7 for leaf and 6 for flower; contact time, 40 min; adsorbent dose, 0.5 g.

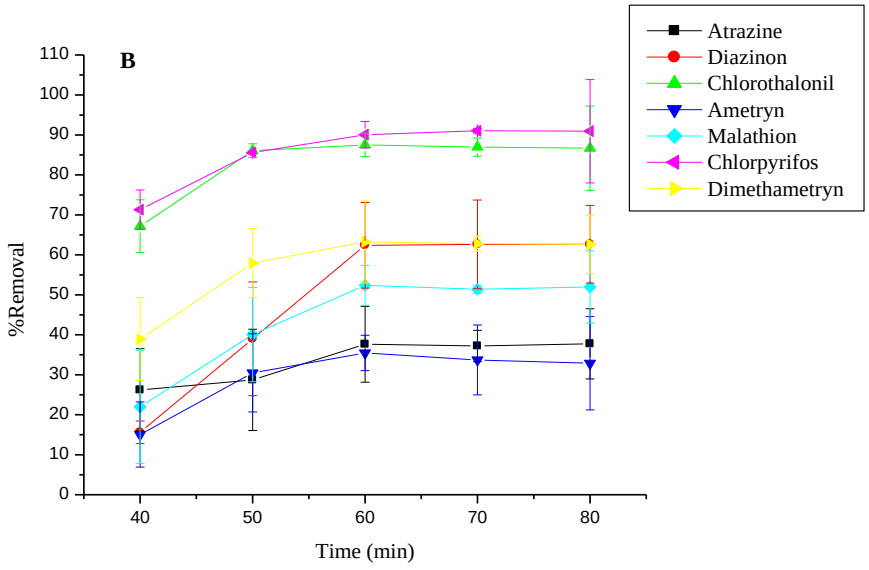
Sorption of target pesticide as a function of shaking speed was studied in the range of 100-300 rpm for stem, leaf and flower parts of *Typha latifolia* plant. As shown in Figure 4.5.3 it was found that removal efficiency increases with increasing shaking speed and attains a maximum sorption at 200 rpm for stem while 150 rpm for leaf and flower, and then declined with increasing shaking speed. Beyond this optimum value the removal efficiency increase slightly for some of analyte and decreases for others. Therefore, 200 rpm for stem and 150 rpm for leaf and flower were selected as optimum shaking speed for further study.

4.5.2.3 Effect of contact time

The equilibrium time required for the adsorption of pesticides, of the three biosorbents was tested at different time intervals. The removal rate of all analytes considered in this study increases with increasing the adsorption time. However, it remains constant after an equilibrium time, 60 min for stem and leaf. The same results obtained at 120 min. for flower powder, which indicates that the adsorption tends towards saturation. This phenomenon was due to the fact that many of the vacant surface sites were available for adsorption during the initial stage and after that the other vacant

surface sites were difficult to be absorbed due to repulsive forces between the adsorbate molecules on the adsorbent (Rahmanifar et al. 2014; Verma et al. 2006). The equilibrium time of 60 min. for stem and leaf, and 120 min. for flower powder was selected as optimum contact time (Figure 4.5.4).





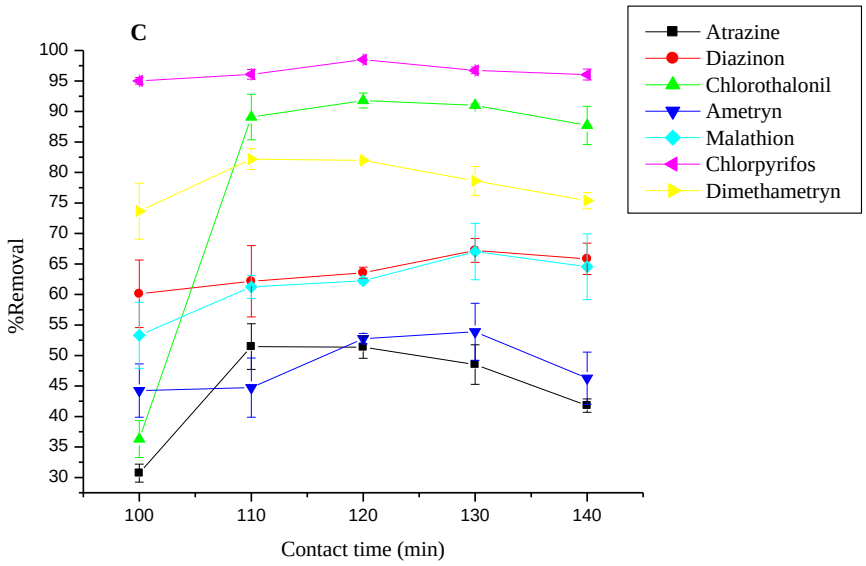
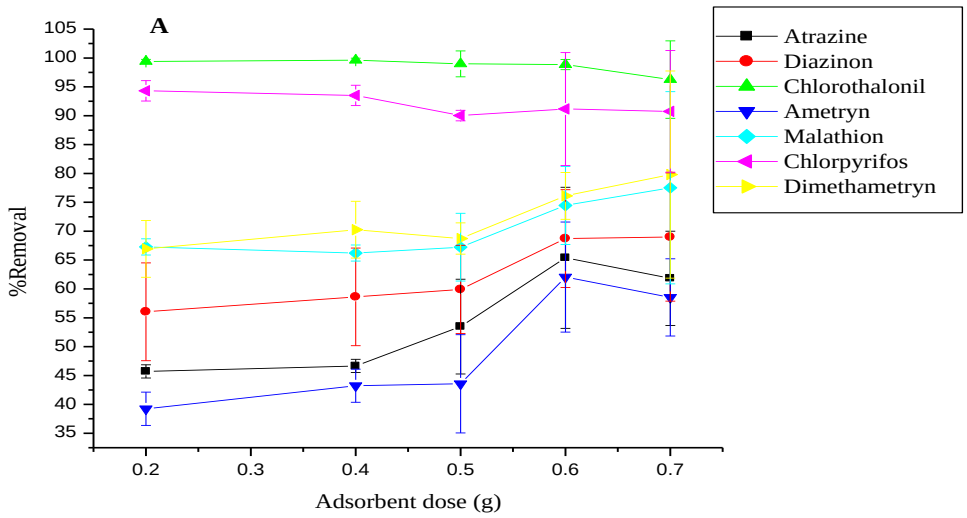


Figure 4.5.4 Effect of contact time on uptake of pesticides by stem (A), leaf (B) and flower (C) of *Typha latifolia* plant. Experimental conditions: sample size, 50 mL; spiked concentration, 5 µg/L; pH, 8 for stem,7 for leaf and 6 for flower; shaking speed, 200 rpm for stem and 150 rpm for leaf and flower; adsorbent dose, 0.5 g.

4.5.2.4 Effect of adsorbent dose

The effect of adsorbent dose on the removal of pesticides was studied by varying the dose of adsorbent from 0.2–0.7 g for stem and leaves, and 0.2–0.6 g for flower. From Figure 4.5.5, it is evident that adsorbent dose significantly influences the amount of pesticide adsorbed. Initially, the amount of pesticide adsorbed was found to be increased from 0.2 to 0.6 g for stem, 0.2–0.5 g for leaf and flower part of *Typha latifolia* plant. Adsorbent dose has little effect on the adsorption of chlorothalonil and chlorpyrifos by all parts of this plant. Further increase of adsorbent dose resulted in very less increase in adsorption. The initial rise in adsorption with adsorbent dose is probably due to a stronger driving force and larger surface area. With a rise in adsorbent dose, there is a less commensurate increase in adsorption resulting from lower adsorptive capacity utilization of adsorbent (Gupta et al. 2011). Therefore, the optimum dose 0.6 for stem and 0.5 g for leaf and flower, were used for further experiment.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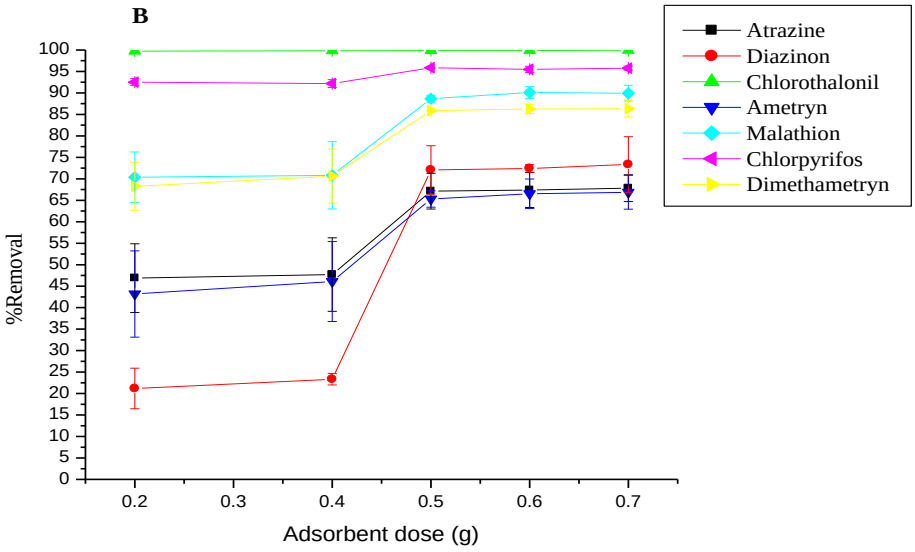
%Removal
Adsorbent dose (g)
Atrazine
Diazinon
Chlorothalonil
Ametryn
Malathion
Chlorpyrifos
Dimethametryn


B
%Removal
Adsorbent dose (g)
Atrazine
Diazinon
Chlorothalonil
Ametryn
Malathion
Chlorpyrifos
Dimethametryn

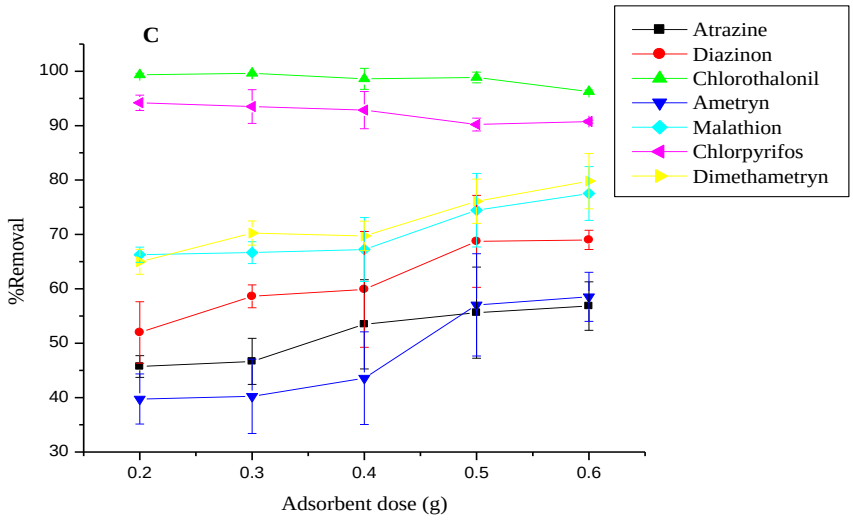
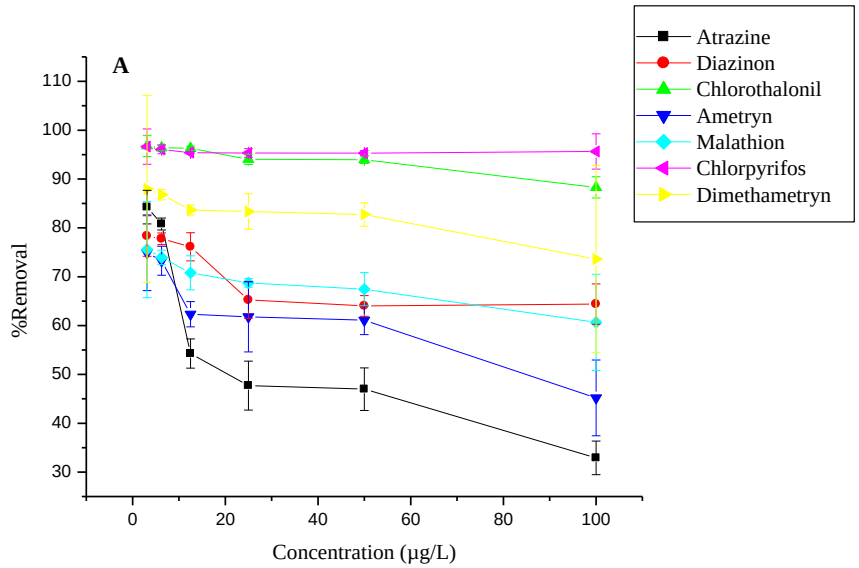


Figure 4.5.5 Effect of adsorbent dose on uptake of pesticides by stem(A), leaf (B) and flower (C) of *Typha latifolia* plant. Experimental conditions: sample size, 50 mL; spiked concentration, 5 µg/L; pH, 8 for stem, 7 for leaf and 6 for flower; shaking speed, 200 rpm for stem and 150 rpm for leaf and flower; contact time 60 min for stem and leaf, and 120 min for flower powder.

4.5.2.5 Effect of initial concentration of pesticides

Effect of initial concentration of atrazine, daizinon, chlorothalonil, ametryn, malathion, chloropyrifos and dimethametryn over the range of 0.32-100 µg/L, on their own uptake by optimum adsorbent dose/50 mL of sorbate solutions were examined under optimized conditions.


A
%Removal
Concentration (µg/L)
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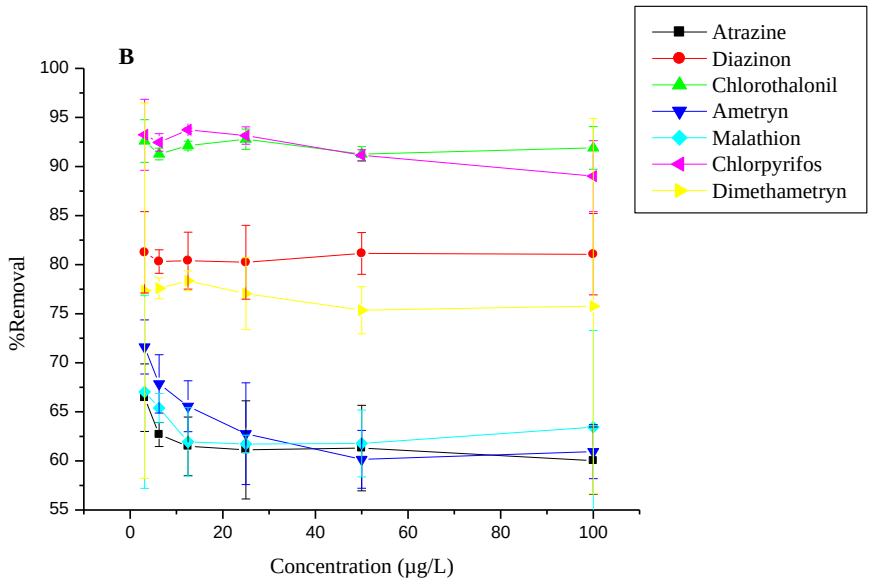
Diazinon
Chlorothalonil
Ametryn
Malathion
Chlorpyrifos
Dimethametryn


B
%Removal
Concentration (µg/L)
Atrazine
Diazinon
Chlorothalonil
Ametryn
Malathion
Chlorpyrifos
Dimethametryn

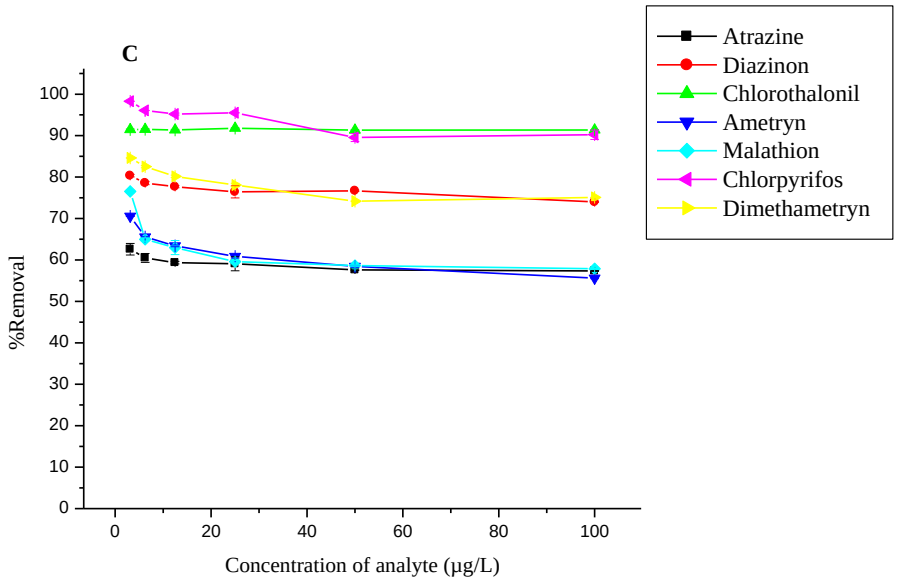


Figure 4.5.6 Effect of initial concentration on uptake of pesticides by stem (A), leaf (B) and flower (C) of *Typha latifolia* plant. Experimental conditions: sample size, 50 mL; spiked concentration, 5 µg/L; pH 8 for stem, 7 for leaf and 6 for flower; shaking speed, 200 rpm for stem and 150 rpm for leaf and flower; contact time 60 min for stem and leaf, and 120 min for flower powder.

As indicated in Figure 4.5.6, with an increase in sorbate concentrations, a corresponding decrease in the removal efficiency was observed exhibiting limiting number of sorption sites available for sorption at higher concentration of sorbate molecules. But, the actual amount of pesticides adsorbed per unit mass of the adsorbent was increased with increase in the initial pesticides concentration. This may also be deduced that at low sorbate/sorbent ratios, sorbate sorption involves the higher energy sites. As the sorbate/sorbent ratio increases, the higher energy sites are saturated and sorption begins on lower energy sites, resulting in a decrease in percent removal (Memon et al. 2007). From the xperimental resuls, it is observed that the removal efficiency of atrazine, ametryn, malathion and diazinon by stem part of *Typha latifolia* is highly affected by concentration. The same results have been observed for atrazine, ametryn and malathion uptake by leaf part of the plant.

4.5.3 Adsorption isotherms and sorption kinetics

4.5.3.1 Adsorption isotherms

Experiments for adsorption isotherms were carried out with a fixed adsorbent dose and varying adsorbate concentrations and applicability of the data to the Langmuir and Freundlich adsorption were evaluated at room temperature (Abdullah and Prasad 2009; Gupta and Babu 2006). The separation factor (R_L) values for the adsorption of all pesticides onto the adsorbents were calculated using equation 2.9. All the obtained results were in the range between 0 and 1 which implies a favorable adsorption. Higher R_L values at lower concentrations showed that adsorption was more favorable at lower concentrations (Abdullah and Prasad 2009).

The Langmuir parameters, q_{max} and K_L, were calculated from the intercepts and slopes, respectively, of the linear plot of $1/q_e$ versus $1/C_e$ using equation 2.8. Similarly, Freundlich parameters (n, k_f) were calculated from slope and intercept, respectively, of the linear plot of log q_e versus log C_e based on equation 2.11. The results indicate that there is a linear relationship between the amounts (mg) of pesticides sorbed per unit mass (g) of the biosorbents against the concentration of pesticides remaining in solution (mg/L). The values of the regression coefficients obtained from these models were used as the fitting criteria to find out these isotherms.

Table 4.5.1 Langmuir and Freundlich adsorption parameters.

	Analyte	Langmuir parameters			Freundlich parameters		
		q_{max} (mg/g)	R_L (L/mg)	R^2	K_f ((mg/g)/(mg/L)$^{1/n}$)	1/n	R^2
Stem	Atrazine	15.9	0.44	0.938	3.78	0.50	0.960
	Diazinon	58.8	0.83	0.997	3.53	0.78	0.990
	Chlorothalonil	71.4	0.42	0.997	17.9	0.71	0.987
	Ametryn	33.3	0.77	0.988	2.93	0.72	0.985
	Malathion	62.5	0.86	0.997	3.03	0.83	0.998
	Chloropyrifos	76.9	0.89	0.993	22.0	0.92	0.995
	Dimethametryn	62.5	0.72	0.995	6.27	0.79	0.992
Leaf	Atrazine	125	0.96	0.989	1.85	0.94	0.998
	Diazinon	333	0.96	0.999	4.17	1.0	0.999
	Chlorothalonil	200	0.84	0.992	11.6	0.98	0.995
	Ametryn	47.6	0.85	0.992	2.36	0.87	0.998
	Malathion	76.9	0.97	0.997	1.92	0.95	0.997
	Chloropyrifos	333	0.88	0.995	12.4	0.87	0.989
	Dimethametryn	111	0.99	0.994	3.51	0.96	0.999
Flower	Atrazine	125	0.98	0.999	1.40	1.0	0.961
	Diazinon	111	0.90	0.997	3.42	0.98	0.903
	Chlorothalonil	100	0.97	0.999	10.3	0.96	0.997
	Ametryn	40.0	0.84	0.988	1.72	0.92	0.664
	Malathion	22.2	0.68	0.936	1.99	0.61	0.476
	Chloropyrifos	25.0	0.11	0.918	4.98	0.18	0.111
	Dimethametryn	58.8	0.77	0.995	3.75	0.87	0.568

The values of 1/n obtained from Freundlich model were ≤ 1.0 indicating favorable biosorptions (Abdullah and Prasad 2009; Abdullah and Prasad 2010). However, close evaluation of the values of the regression coefficients, (R^2), depicted in Table 4.5.1, the equilibrium data seemed to fit better to the Langmuir isotherm model than the Freundlich adsorption model indicating monolayer homogeneous surface conditions for most analytes in all adsorbents. Atrazine, malathion and chloropyrifos for stem and atrazine, ametryn, chlorothalonil and dimethametryn for leaf powder as adsorbent fit more to Freundlich adsorption model indicating monolayer sorption with a heterogeneous energetic distribution of active sites, accompanied by interactions between adsorbed molecules (Oliveira et al. 2008).

4.5.3.2 Sorption kinetics

The kinetics of the parameters for adsorption process were studied from variation of the contact times and analyzed using two simple kinetic models; *viz.*, the pseudo-first order and pseudo-second order models (Rozaini et al. 2010; Vinodhini and Das 2010). The first-order rate expression of Lagergren was calculated using equation 2.12. A graph was drawn by plotting log (q_e-q_t) versus 't'. The slope and intercept of this graph were used to obtain the first-order rate constant k_1 and equilibrium adsorption capacity, q_e, while for parameters calculation for the second order kinetics equation 2.13 was utilized. For all the biosorbents used in the current study, the plots of t/q_t against t displayed linear relationships.

The intercept and slope of the plot were used to determine the numerical values of the rate constant, k_2, and the equilibrium adsorption capacity, q_e, respectively. Based on the determined experimental values indicated in Table 4.5.2, for various parameters, it has been observed and concluded that pseudo-second order equation which exhibited significantly better regression coefficient (R^2) and also noted to be in good agreement with the calculated q_e values, suggest that chemisorption is the rate determining step in the adsorption of all pesticides on the biosorbents (Kumar and Kirthika 2009; Rozaini et al. 2010).

Table 4.5.2 Pseudo first and second order parameters.

Adsorbent	Analyte	Pseudo-1st order				Pseudo-2nd order		
		qe*(mg/g)	qe (mg/g)	k_1 (min^{-1})	R^2	qe (mg/g)	k_2 (g/mg min)	R^2
	Atrazine	2.6	0.09	0.009	0.163	2.6	0.29	0.999
Stem	Diazinon	4.1	3.9	0.062	0.649	4.7	0.019	0.989
	Chlorothalonil	4.7	2.4	0.071	0.865	5.0	0.038	0.998
	Ametryn	3.0	0.76	0.051	0.828	3.1	0.13	0.999
	Malathion	3.2	0.59	0.053	0.181	3.3	0.15	0.999
	Chloropyrifos	4.9	2.2	0.053	0.338	5.0	0.13	0.999
	Dimethametryn	3.8	2.5	0.081	0.503	3.9	0.081	0.999
	Atrazine	2.9	1.9	0.007	0.930	2.4	0.018	0.997
Leaf	Diazinon	3.1	7.1	0.053	0.856	3.4	0.038	0.998
	Chlorothalonil	4.4	7.8	0.058	0.930	5.2	0.011	0.999
	Ametryn	1.8	0.67	0.018	0.980	1.8	0.055	0.999
	Malathion	2.6	12	0.074	0.927	3.8	0.007	0.959
	Chloropyrifos	4.5	4.0	0.069	0.036	4.8	0.009	0.998
	Dimethametryn	3.2	8.6	0.062	0.917	4.1	0.036	0.997
	Atrazine	2.6	5.6	0.023	0.624	3.3	0.006	0.988
Flower	Diazinon	3.4	0.72	0.007	0.464	3.3	0.024	0.991
	Chlorothalonil	4.6	0.04	0.007	0.072	4.5	0.074	0.990
	Ametryn	2.9	3.4	0.014	0.278	3.3	0.004	0.999
	Malathion	3.4	1.6	0.007	0.976	3.5	0.009	0.998
	Chloropyrifos	4.9	0.46	0.007	0.391	5.0	0.051	0.998
	Dimethametryn	4.1	0.89	0.007	0.684	4.1	0.021	0.996

q*, experimental value

5 CONCLUSIONS

This study has focused on the potential applications of one of the miniaturized analytical techniques; i.e., a high density solvent based DLLME, utilized for selective and quantitative extraction of trace quantities of multiclass pesticide residues from different environmental samples. During method development, various parameters affecting the chromatographic separation and extraction efficiencies of the target analytes were evaluated and the optimum conditions were established. Under these conditions, the method was found to be linear over wide concentration ranges with coefficient of determination of 0.991 or better; LOD varied in the range of 0.005–0.02 µg/L; exhibited acceptable precision (%RSD $\leq$ 8.74) and satisfactory relative recoveries ranging from 80.4–114%. Employing the optimized experimental parameters, trace level extraction followed by GC-MS determination of the target analytes in the water samples collected from Hawassa Lake and Wonji Shoa sugarcane irrigation as well as sugarcane juice samples were successfully achieved. The results indicated that ametryn was detected in water from Hawassa Lake and sugarcane juice samples at concentration level of 1.5 and 4.1 µg/L, respectively. Furthermore, atrazine and ametryn were detected in Wonji Shoa sugarcane irrigation water at concentration levels of 4.8 µg/Land 7.1 µg/L, respectively. Based on the experimental findings and the inference from the comparison, it can be generalized that the currently developed method is simpler, cheap, rapid and reliable for selective and quantitative extraction of trace level multiclass pesticide residues and other chemical pollutants, possessing similar physicochemical properties from contaminated samples of different origins.

In the present work, SALLE combined with LD-DLLME procedure was also developed for the extraction of a pesticides of different physicochemical properties from sugar and soil. LODs obtained were in the low concentration range. In general, the method provides very good analyte recovery (79.0–111%), coefficient of determination (0.992–0.999), LODs (0.01–0.3 µg/kg) and yielding extracts that were clear avoiding the need for further cleanup. The current method was successfully applied for the determination of target analytes in sugar and soil samples. The results indicated that none of target analyte detected in sugar while soil is contaminated by atrazine and ametryn at concentration level of 0.29 and 0.23 µg/kg, respectively. The experimental findings revealed that the currently developed method is simpler, cheap, rapid and reliable for selective and

quantitative extraction of trace level multiclass pesticide residues from contaminated matrices of different origins.

A rapid, selective and simple sc-CO_2 coupled with GC-MS method was developed for the determination of four pesticide residues in onion samples. The optimized method was successfully applied to the analysis of atrazine, 4,4'-DDE, endrin and 2,4'-DDT pesticide residues in onion samples. The experimental results obtained confirms that the proposed method is green and easy sample treatment. The variables involved in the sc-CO_2 procedure were optimized by Box-Behnken design, using by MODDE 10.1 soft war, achieving satisfactory analyte recoveries. LODs at lower µg/kg levels, good recovery and precision were obtained. The results also indicate that parameters have a marked influence on the extraction process. The onion sample tested is either free of pesticides investigated or below detection limit of the method.

Another green analytcical method for trace analysis of atrazine, diazinon, chlorothalonil and deltametryn in the honey samples was developed and validated without any laborious pretreatment of the samples. The multivariate optimization based on the response surface methodology showed to be very suitable for the optimization of static time, pressure and temperature, providing optimum conditions for the measurement of pesticides residue level by HPLC-DAD free of interferences. The employment of the three level of the Box-Behnken design for the multivariate optimization allowed a fast and efficient optimization process and supplied important information regarding the interactions among the variables. The method, combined static and dynamic mode sc-CO_2 for trace analysis of the residue of pesticides in honey, provides a good linearity, precision, selectivity and recoveries.

The adsorption capacity of *Typha latifolia* plant parts has also been studied. The studied biosorbents has admirable adsorption capacities for the removal of atrazine, diazinon, chlorothalonil, ametryn, malathion, chlorpyrifos and dimethametryn. It was found that the adsorption process in the current study was dependent on pH, shaking speed, contact time, adsorbent dose and initial pesticide concentration. The adsorption of most analytes was found to fit the Langmuir isotherm suggesting monolayer coverage of the adsorbent surface. But atrazine, malathion and chloropyrifos removal by stem of *Typha latifolia* and the reoval of atrazine, ametryn, chlorothalonil and dimethametryn by leaf part of *Typha latifolia* as adsorbent fit more to Freundlich adsorption model indicating monolayer

sorption with a heterogeneous energetic distribution of active sites. The kinetic study also revealed that the adsorption process in the present study obeyed pseudo-second order model indicating chemi-sorption as the rate limiting step. Finally, this plant is more effective, efficient, economic adsorbent and can be used for the removal of reported and other pesticides having the relatively same physico-chemical properties from contaminated water.

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