# Method development for the determination of residual pesticides and heavy metals in complex samples using modern pre-concentration techniques

by

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A dissertation submitted to the Department of Chemistry, University of the Venda in
fulfilment of the requirements for the degree of
Masters in Science in **Chemistry**

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# Declaration

I, Herbert Musarurwa, declare that the entirety of the work contained therein is my own, original work and that l have not previously submitted it in its entirety or in part for any other qualification. The dissertation is being submitted for the degree of Master of Science in Chemistry at the University of Venda, Thohoyandou, Limpopo province, South Africa

Herbert Musarurwa (Student)

## List of publications

This dissertation is based on the following papers:

1. **Recent developments and applications of dispersive liquid-liquid micro-extraction technique for the analysis of pesticides in food matrices.**
   Herbert Musarurwa, Luke Chimuka, Nikita Tawanda Tavengwa (submitted)

2. **Recent developments and applications of QuEChERS based technique on food samples during pesticide analysis**
   Herbert Musarurwa, Luke Chimuka, Nikita Tawanda Tavengwa (submitted)

3. **Green pre-concentration techniques during pesticide analysis in food samples: A review**
   Herbert Musarurwa, Luke Chimuka, Nikita Tawanda Tavengwa (published)

4. **Analysis of heavy metals from vehicular emission, using vortex-assisted dispersive liquid-liquid micro-extraction, on roadside dust as well as fruits and vegetables**
   Herbert Musarurwa, Luke Chimuka, Nikita Tawanda Tavengwa (Manuscript in preparation)

5. **1,5-diphenylcarbazide based dispersive liquid-liquid micro-extraction for the speciation of chromium in fruits and vegetables**
   Herbert Musarurwa, Luke Chimuka, Nikita Tawanda Tavengwa (Manuscript in preparation)

6. **Z-sep$^+$ based QuEChERS technique for the pre-concentration of malathion in fruits**
   Herbert Musarurwa, Luke Chimuka, Nikita Tawanda Tavengwa (Manuscript in preparation)

## Contribution of the authors

**Paper I.** Principal author, involved in planning and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

**Paper II.** Principal author, involved in planning and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

**Paper III.** Principal author, involved in planning and writing of the manuscript. Co-authors revised the draft manuscript and made suggestions for improvement.

**Paper IV.** Principal author, involved in planning, performed the DLLME experiments, evaluation of the results and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

**Paper V.** Principal author, involved in planning, performed the chromium speciation experiments, evaluation of the results and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

**Paper VI.** Principal author, involved in planning and performing the QuEChERS experiments, evaluation of the results and writing of the article. Co-authors revised the draft manuscript and made suggestions for improvement.

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## Abstract

In this work, modern pre-concentration techniques, namely dispersive liquid-liquid micro-extraction (DLLME) and QuEChERS, were used to analyse pesticides and heavy metals in complex matrices. The work is divided into six papers.

In **Papers 1**, the recent developments and applications of DLLME during analysis of pesticides in food matrices were reviewed. The DLLME technique has captured the interests of many researchers in recent years. The major advantage, among others, of DLLME is miniaturisation in which the acceptor-to-donor ratio is reduced tremendously leading to high enrichment compared to other sample preparation techniques. In the present work, the different complex matrices where the DLLME technique has been employed for the analysis of pesticides are reviewed as well as the challenges associated with this technique.

**Papers II** reviewed the recent applications and developments of the QuEChERS technique during the analysis of pesticides in food matrices. QuEChERS is a versatile pre-concentration method whose application spans the whole breath of organic compounds. There are three common standard methods used during QuEChERS and these are the original QuEChERS, AOAC and the EN methods. In this paper, recent developments and applications of QuEChERS techniques in the analysis of pesticides in food samples were reviewed.

In **Paper III**, green pre-concentration techniques employed during analysis of pesticides were reviewed. Recently, the parameter of "greenness" during sample pre-concentration of pesticides in food matrices is as important as selectivity in order to avoid using large amounts of harmful organic solvents during sample preparation. Developing new green pre-concentration techniques is one of the key subjects in green chemistry in order to minimize the release of large volume of toxic organic solvents into the environment. Thus, to reduce the impact on the environment during trace analysis of pesticides in food matrices, new developments in pesticide pre-concentration have gone in three separate directions (which are reviewed in this paper): one is the search for more environmentally friendly solvents, the second one is miniaturization and the third one is the development of solvent-free pre-concentration techniques. Eco-friendly solvents such as supercritical fluids, ionic liquids and natural deep eutectic solvents have been developed for use as extraction solvents during pre-concentration of pesticides in food matrices. Also miniaturized pre-concentration techniques such as QuEChERS, dispersive liquid-liquid micro-extraction and hollow-fibre liquid phase micro-extraction have been used during trace analysis of pesticides in food samples as well as

solvent-free techniques such as solid phase micro-extraction and stir bar sorptive extraction. All these developments are geared to ensure that pesticide pre-concentration in food matrices is green and were reviewed in this paper.

The effect of vehicular emissions on the concentrations of selected heavy metals was investigated in **Paper IV**. The samples were pre-concentrated using DLLME prior to analysis with flame atomic absorption spectroscopy. Dithizone, chloroform and methanol were used as chelating agent, extraction solvent and dispersion solvent respectively during the DLLME technique. The pH of the sample was adjusted to around 8 using sulphuric acid or sodium hydroxide solution. The influential DLLME parameters, such as pH volume and type of extraction solvent, and voume of disperser solvent, were optimized prior to the application of the developed method to real samples (roadside dust, fruits and vegetables).

In **Paper V**, chromium speciation in fruits and vegetables was studied. The chromium in fruit and vegetable sample juices was pre-concentrated using DLLME prior to analysis with flame atomic absorption spectroscopy. Diphenylcarbazide (DPC) was used as a chelating agent in this study, and salting out of the complex from the aqueous medium into the organic phase was effected using sodium acetate. Chloroform and methanol were used as extraction and dispersion solvents respectively in the DLLME method for the determination of chromium (VI). For total chromium, the trivalent chromium was oxidised using acidified $KMnO_4$ to hexavalent chromium before performing the DLLME technique. The concentration of chromium (III) was determined by finding the difference between total chromium and concentration of chromium (VI). The important parameters that influence the efficiency of the DLLME technique were also optimized using the univariate approach. After optimization, the developed method was applied to real samples.

In **Paper VI**, the concentration of malathion pesticide in fruits was determined using QuECHERS for pre-concentration and UV-Vis spectrophotometry for instrumental analysis. Acetonitrile was used as the extraction solvent and Z-sep$^+$/PSA sorbent combination was used for sample clean-up. The acetonitrile extract from QuEChERS was then hydrolysed using KOH followed by reaction with acidified potassium bromate for colour development. The coloured product formed was then analysed using UV-Vis spectrophotometry. Among the fruits analysed, Oranges had no malathion residue in them. However, trace amounts of malathion, below WHO maximum allowable limits, were found in pears and apples.

## Dedications

This thesis is dedicated:

- To my wife, Anna, who supported and encouraged me when l confronted setbacks and depression during the time l was doing my project. Her patience, unconditional love and support took me through the difficult periods of my academic journey.

- To my children: Calvin, Tinotenda, Calton and Caroline, for being there for me during the time l was doing the experiments and writing the dissertation.

## Acknowledgement

- My sincere gratitude goes to my supervisors, Dr NT Tavengwa and Prof L Chimuka, for their encouragement, support, guidance and advice throughout my research. Their useful suggestions, comments and corrections after patiently reading each draft of this dissertation made it possible for me to complete the research.

- I am also grateful to lecturers, technical staff and colleagues of the Department of Chemistry for their support.

- I am indebted to the University of Venda for its material support.

- Finally, I wish to thank the almighty God for giving me life, strength and protection during the entire study period. To him, I give all the glory and honour.

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## List of abbreviations

| | |
|---|---|
| AAS | Atomic absorption spectroscopy |
| AchE | Acetylcholinesterase |
| APDC | Ammonium pyrrolidine dithiocarbamate |
| ATP | Adenosine triphosphate |
| CE | Capillary electrophoresis |
| DAD | Diode array detector |
| DDT | Dichlorodiphenyltrichloroethane |
| DDTC | Diethyldithiocarbamate |
| DDTP | Diethyldithiophosphate |
| DI-SPME | Direct immersion solid phase micro-extraction |
| DLLME | Dispersive liquid-liquid micro-extraction |
| DMF | Dimethylformamide |
| d-SPE | Dispersive solid phase extraction |
| DVB | Divinylbenzene |
| EA | Effervescence assisted |
| ECD | Electron capture detector |
| GC | Gas chromatography |
| GCB | Graphitised carbon black |
| HF-LPME | Hallow fibre liquid phase micro-extraction |
| HPLC | High-performance liquid chromatography |
| HS-SPME | Headspace solid phase micro-extraction |
| IL | Ionic liquid |
| LLE | Liquid-liquid extraction |
| LOD | Limits of detection |
| LOQ | Limits of quantification |
| MeHg | Methylmercury |
| MIPs | Molecularly imprinted polymers |
| MIT | Molecularly imprinted technology |
| MRL | Maximum residue levels |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MSPD | Matrix solid phase dispersion |
| MWCNTS | Multiwalled carbon nanotubes |

| | |
|---|---|
| Na-DDTC | Sodium diethyldithiocarbamate |
| NB | Narrow bore |
| POPs | Persistent organic pollutants |
| PSA | Primary and secondary amines |
| PTV | Programmable temperature vaporiser |
| QuEChERS | Quick, easy, cheap, effective, rugged and safe |
| REPSM | Reversed-electrode polarity stacking mode |
| ROS | Reactive oxygen species |
| RSD | Relative standard deviation |
| SDS | Sodium dodecyl sulphate |
| SERS | Surface enhanced raman spectroscopy |
| SPE | Solid phase extraction |
| SPME | Solid phase micro-extraction |
| UA | Ultrasound assisted |
| UNEP | United Nations Environment Programme |
| VA | Vortex assisted |
| WHO | World Health Organization |
| $\mu$ -SPE | Micro-solid phase extraction |

# Chapter 1

# Introduction and background

This chapter gives the introduction and background to the study as well as the problem statement. It concludes by giving the outline on how the work is presented in this dissertation

# 1. Introduction

## *1.1. Background of study*

Anthropogenic activities such as mining, farming and industrial processes have contributed to serious pollution of the environment. Such activities introduce pollutants like pesticides and heavy metals into the environment. Most pesticides and heavy metals are persistent in the environment and are not biodegradable. As a result, they undergo bio-accumulation and bio-magnification in the food chains causing adverse effects to fauna and flora.

Pesticides such as organochlorines are nervous system disruptors and they cause convulsions and paralysis of living organisms, eventually leading to death (Thomas et al., 2017). Organophosphates also negatively affect the ecosystem. For instance, they cause unconsciousness and twitching of muscles of living organisms resulting in death (Sapahin et al., 2014). Heavy metals, on the other hand, are highly carcinogenic and can cause various psychological disorders and damage the immune system (Cherfi et al., 2014; Porova et al., 2014; Sharma and Prasad, 2010). The toxicities of heavy metals and pesticides are reviewed in detail in Chapter 2.

The presence of pollutants, such as pesticides and heavy metals, in the environment should not be allowed to continue unchecked. Thus, some green technologies, reviewed in detail in Chapter 2, have been developed to alleviate pollution problems from pesticides and heavy metals. Such technologies include phytoremediation and biosorption. Phytoremediation involves the use of plants to rid the environment of contaminants whereas biosorption involves the use of biosorbants to clean polluted environments.

Plants that have been successfully used to remediate contaminated environments include popula, acacia, ragweed, water hyacinth and Pistia stratides (Abubakar et al., 2014; Aisien et al., 2010; Čudić et al., 2016; García et al., 2018). Biological materials are used as sorbents during biosorption. Bacteria and algae have been successfully employed as biosorbents to alleviate pollution of various environments (García et al., 2016; Nwidi and Agunwamba, 2015; Utomo et al., 2016)

The remediation technologies are not 100% efficient. Some trace amounts of pollutants are found in the environment even after application of remediation technologies. Pollutants such as pesticides and heavy metals can adversely affect flora and fauna even at trace levels. It is therefore imperative that effective techniques should be used to pre-concentrate and monitor levels of pesticides and heavy metals, at trace concentrations, in the environment.

Many techniques have been used to pre-concentrate pesticides and heavy metals form different matrices. These pre-concentration techniques include liquid-liquid extraction (LLE) (Asl et al., 2017; Bouabdallah et al., 2006; Song, 2016), solid phase extraction (SPE) (Alqadami et al., 2013; Babazadeh et al., 2015; Pelajić et al., 2016; Shamsipur et al., 2016), QuEChERS (Abbas et al., 2017; Ferreira et al., 2016; Singh et al., 2018; Viera et al., 2017), dispersive liquid liquid micro-extraction (DLLME) (Martins et al., 2014; Pourreza et al., 2015; Rahmani et al., 2016; Seeger et al., 2017), solid phase micro-extraction (SPME) (Carro et al., 2002; Jafari et al., 2016a; W. Liang et al., 2017; Merkle et al., 2015) and hollow fibre liquid phase micro-extraction (HF-LPME) (Bahrami et al., 2017; Gjelstad and Pedersen-Bjergaard, 2013; Pinto et al., 2010; Sun et al., 2011). A general review of these pre-concentration techniques is given in Chapter 2.

The pre-concentration techniques can be divided into classical pre-concentration methods such as LLE and SPE, and modern pre-concentration methods such as QuEChERS and DLLME. The use of the classical methods has some drawbacks such as the use of large volumes of toxic organic solvents and the fact that they are time consuming. Consequently, in recent years, priority of use has been skewed towards the modern techniques such as DLLME and QuEChERS that cause less pollution of the environment. These modern techniques were used in this study. Papers I and II give detailed reviews of the recent applications of DLLME and QuEChERS respectively during the pre-concentration of pesticides in food samples. In papers IV and V, the DLLME technique was used to pre-concentrate heavy metals in roadside dust samples exposed to vehicular emissions, and speciation of chromium in fruit samples respectively. Paper VI involves the use of QuEChERS to pre-concentrate malathion pesticide in fruits.

Recently, the major thrust in the analytical world is the use of cleaner methods that conform to the dictates of green chemistry. The popularity of a pre-concentration technique hinges, among other things, on the "greenness" of the method. Thus, miniaturisation is a key feature among modern pre-concentration and there is a conscious effort to develop solvent-free pre-concentration techniques such as SPME. Paper III gives a brief review of the green pre-concentration techniques that are currently being applied to pre-concentrate pesticides in food samples.

### 1.2. Problem statement

Pollution of environmental media (air, water and soil) as well as foodstuffs by pesticides (Bernardi et al., 2016; N. Liang et al., 2017; Shamsipur et al., 2016; Tette et al., 2016; Zhou et al., 2018) and heavy metals (Chen et al., 2018; Du et al., 2018; Elik et al., 2017; Sager et al., 2018; Zhang et al., 2018) have rapidly increased worldwide recently. This can be attributed to, among other things, rapid industrialization, urbanization and increased agricultural production.

The use of pesticides in agricultural applications provides a wide range of beneficial effects. However, their extensive and excessive use has been a concern because of their potential harm to the environment and known or suspected toxic effects for humans. The detrimental effects of pesticides to human health include acute neurological toxicity, neuro-developmental impairment, and possible dysfunction of the immune, reproductive and endocrine systems, cancer, chronic kidney diseases and other potential diseases (Pastor-Belda et al., 2015; Pourreza et al., 2015). The heavy metals, on the other hand, are equally toxic to humans. Most heavy metals are carcinogenic and can cause damages to the kidneys and the reproductive system (Haber et al., 2017; Taylor et al., 2017). Due to these concerns, the monitoring of heavy metals and pesticide residues in food products and the environment at large should be extensively pursued.

Heavy metals and pesticides have negative impacts on the environment even at trace levels. Thus, effective pre-concentration techniques such as QuEChERS (Paper II and VI) and DLLME (Paper 1, IV and V) are required to pre-concentrate and facilitate the detection of these trace pollutants. Some pre-concentration techniques such as solid phase extraction (SPE) use large volumes of toxic organic solvents and thereby polluting the environment with organic pollutants. It is therefore imperative that the selection of the pre-concentration techniques during heavy metal and pesticide analysis should be geared towards green chemistry (Paper III) to minimize pollution of the environment.

## 1.4. Outline of the dissertation

The outline of the dissertation (comprising of five chapters) is presented as follows:

**Chapter 1**: A general introduction to environmental pollution by heavy metals and pesticides is given. This chapter also spells out the problem statement that brings out the motivation of carrying out the research.

**Chapter II**: A literature survey of two priority pollutants of the environment, namely pesticides and heavy metals, is given.

**Chapter III**: The research objectives are provided in this section.

**Chapter IV**: This chapter lists manuscripts (Papers I-VI) presented for my MSc examination. The work carried out, results and discussion are presented in each paper.

**Chapter V**: General conclusions and future work based on experimental findings are discussed in this section.

**References**: The references arising from the introduction and literature review (Chapter 1 and 2) are listed at the end of the dissertation.

**Appendix:** The Appendix has supplementary information of the results of the experiments performed

# Chapter 2

# Literature review

This chapter gives the literature survey of two priority pollutants, heavy metals and pesticides. Particular emphasis was placed on the modern sample preparation techniques for each group as well as the possible remediation technologies.

## 2. Pesticides and heavy metals

Environmental pollution by pesticides and heavy metals is of great concern nowadays (Rahmani et al., 2016). Heavy metals and pesticides cause serious ecological problems in many parts of the world (Mohmand et al., 2015). Industrialization, agricultural production and urbanization are the principal reasons of contamination of air, water and other environmental sources with pesticides and heavy metals (Filipiak-Szok et al., 2015).

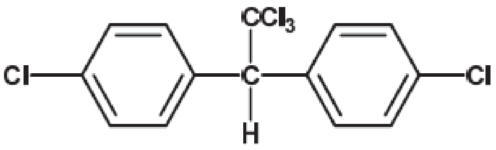
### 2.1.Pesticides

A pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during the production, processing, storage or marketing of food, agricultural commodities, wood and wood products or animal food stuffs (Villaverde et al., 2016). The term includes chemicals used as growth regulators, defoliants, desiccants, fruit thinning agents, or agents for preventing the premature fall of fruits, and substances applied to crops either before or after harvest to prevent deterioration during storage or transport. The term, however, excludes such chemicals used as fertilizers, plant and animal nutrients, food additives and animal drugs. The term pesticide is also defined by Food and Agriculture Organisation in collaboration with UNEP (1990) as chemicals designed to combat the attacks of various pests and vectors on agricultural crops, domestic animals and human beings. The definitions above imply that, pesticides are toxic chemical agents (mainly organic compounds) that are deliberately released into the environment to combat crop pests and disease vectors. The intended purpose of pesticides is, therefore, to prevent the activity of organisms that affect negatively the activities of human beings. Pesticides can be classified in many different ways and one of the most common way of classifying them is based on chemical structure.

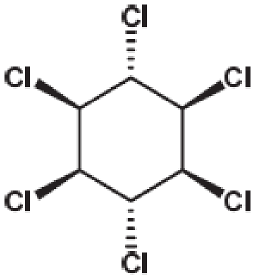
#### *2.1.1. Classification of pesticides according to chemical structures*

#### *2.1.1.1.Organochlorines*

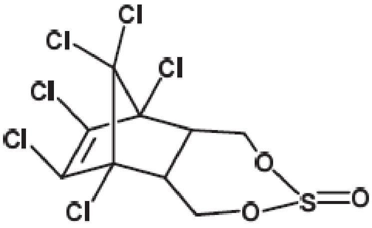
Organochlorines pesticides are organic compounds with five or more chlorine atoms. Among the synthetic organic pesticides, organochlorines were the first to be used in agriculture and in public health (Bajwa et al., 2016). Most of them were widely used as insecticides for the control of a wide range of insects in agriculture but have negative effects on human health and the environment (Nuapia et al., 2016; Rani et al., 2017). They have a long-term residual effect in the environment since they are resistant to most chemical and microbial degradations (Muzyed et al., 2017; Wang et al., 2017). Organochlorine insecticides act as nervous system disruptors leading to convulsions and paralysis of the insect and its eventual death (Thomas et al., 2017). Thus, long term exposure to organochlorines may trigger endocrine disruptions, neurotoxicity, cancer and other adverse health effects (Bonnineau et al., 2016). Some of the commonly used representative examples of organochlorine pesticides are DDT, lindane, endosulfan, aldrin, dieldrin and chlordane, and their chemical structures are shown in Fig. 1.



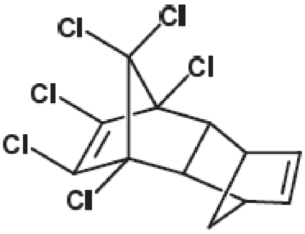
DDT



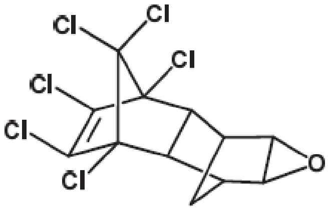
Lindane



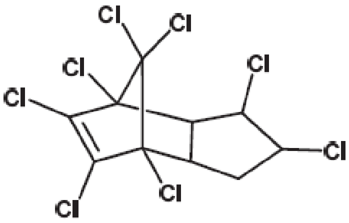
Endosulfan



Aldrin



Dieldrin



Chlordane

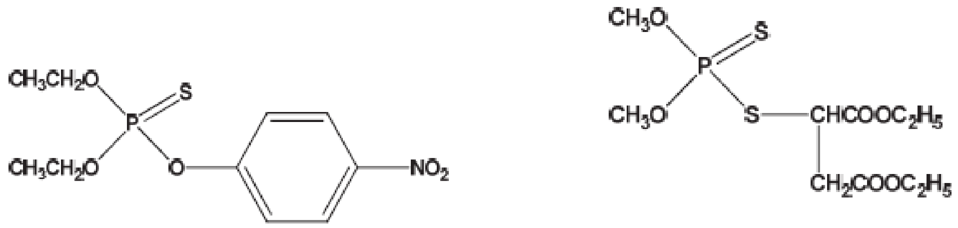
**Fig.1:** Structures of common organochlorines

### *2.1.1.2. Organophosphates*

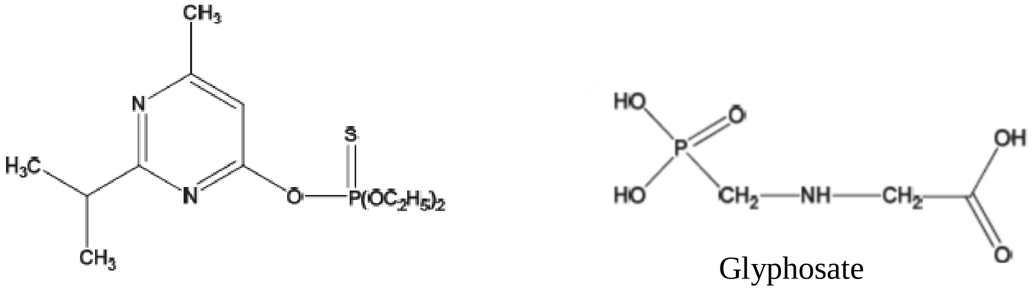
Organophosphates were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932 (Ko et al., 2014). They are synthetic esters, amides or thiol derivatives of phosphoric, phosphonic, phosphonothionic and phosphorothionic acids, which are extensively used to control agricultural, or household pests (Kumar et al., 2014). Some are very poisonous such that they were used in World War II as nerve agents. They are generally highly lipid soluble, and may be classified as direct or indirect acetylcholinesterase (AchE) inhibitors. However, they usually do not persist in the environment (Cheng et al., 2017).

The organophosphates, because of their widespread use and frequently high acute toxicity, are involved in more pesticide poisonings than any other class of pesticides (Sapahin et al., 2014). The organophosphates interfere with the activity of cholinesterase (Seebunrueng et al., 2014). When the cholinesterase enzyme cannot perform its normal function, the nerves in the body send “messages” to the muscles continuously leading to muscle twitching and weakness. If the poisoning is severe, the victim may have “fits” or convulsions, and may even die (Ren and Xia, 2016).

Organophosphates are irreversible cholinesterase inhibitors (Pirsaheb et al., 2013). Without medical treatment, the level of enzyme activity will return to normal only after several days, weeks or even months. Additive effects of small repeated doses over time, such as in a spraying season, may finally cause poisoning. The effects of mild poisoning in humans include fatigue, headache, and dizziness (Zhao et al., 2007). Moderate poisoning leads to inability to walk, weakness and chest discomfort. In severe cases, there will be unconsciousness, severe constriction of pupils and muscle twitching ultimately resulting in death (Sapahin et al., 2014). Some of the widely used organophosphorous insecticides include parathion, malathion, diaznon and glyphosate (Fig. 2).



Parathion Malathion

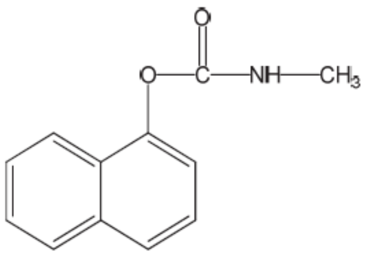


Diaznon Glyphosate

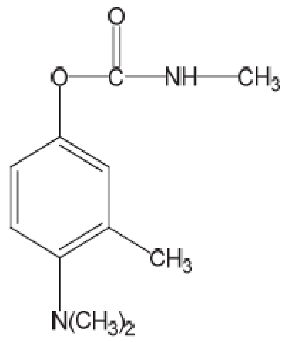
**Fig. 2:** Structures of common organophosphates

### *2.1.1.3. Carbamates*

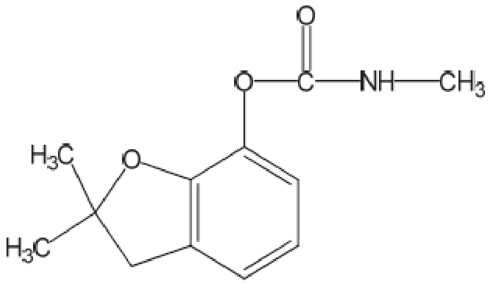
The carbamates were introduced as insecticides in 1951, and are derivatives of carbamic acid ($H_2NCOOH$) (Nantia et al., 2017). The effects of carbamates and organophosphates are similar because they both inhibit cholinesterase, affecting nerve impulse transmission (Zhou et al., 2018). They are widely used in homes, gardens, and agriculture. The carbamates are short-lived in the environment since they undergo hydrolysis reaction and decompose to simple non-toxic products (Rahman et al., 2017). Thus, while carbamates can cause severe poisoning, they do not normally produce long-term, cumulative poisoning. They can produce severe cholinergic toxicity following cutaneous exposure, inhalation, or ingestion, causing some adverse effects in human such as headaches, vomiting, abdominal cramps, uncontrolled urination or defecation, and even a comatose state (Nantia et al., 2017). Fig. 3 shows the structures of the common carbamates.



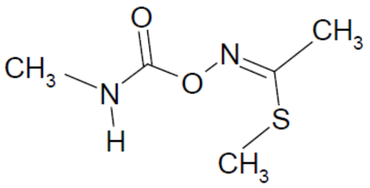
Carbaryl



Aminocarb



Carbofuran



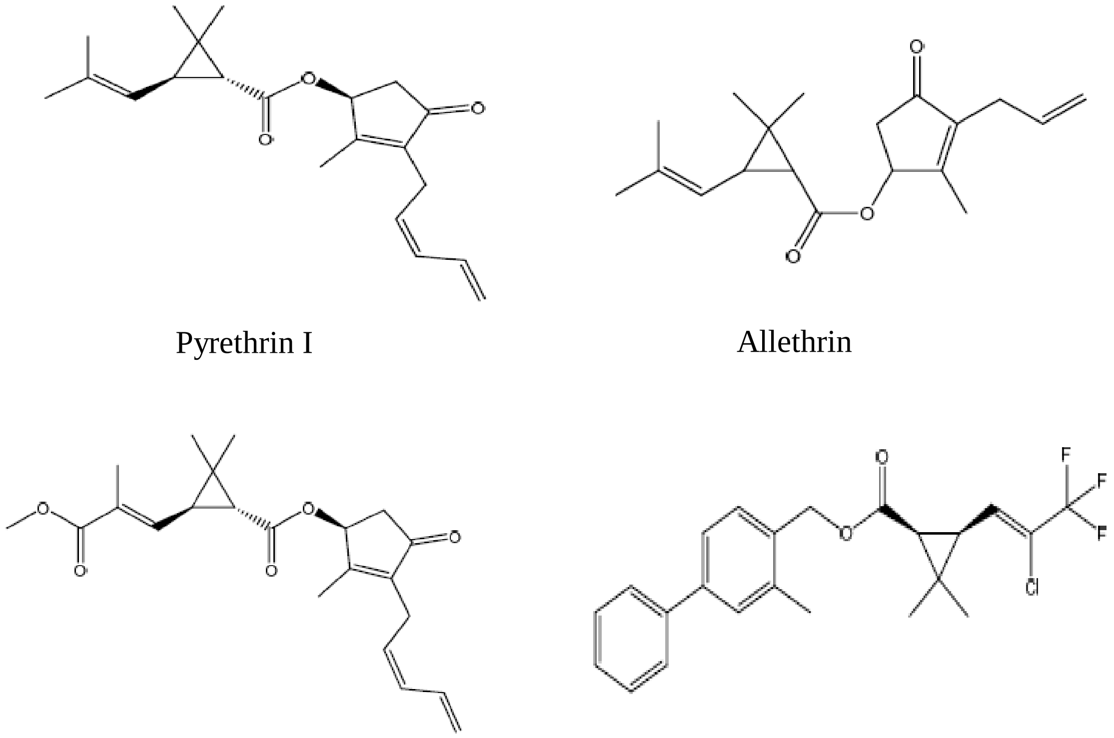
Methomyl

**Fig. 3:** Structures of common carbamates

### *2.1.1.4.Pyrethroids*

Pyrethroids originate from natural insecticide derived from chrysanthemum flowers, known as pyrethrins (Fig. 4) (Farajzadeh et al., 2014). To meet the increasing demand of pesticides in the agricultural industry, pyrethroids are now being obtained synthetically (Noori et al., 2017). Due to their high insecticidal activity, rapid degradation in the environment and relatively low toxicity to mammals, pyrethroids are widely used in agriculture, public health, veterinary medicine, forestry and horticulture (Hu et al., 2015; Zhang et al., 2014). The pyrethroids kill the insects through contact or ingestion. They act on the central nervous system causing changes in the dynamics of the sodium ion channels in the membrane of the nerve cell, causing it to increase its opening time, prolonging sodium current across the membrane in both insects and vertebrates (Ccanccapa-Cartagena et al., 2017) leading to neuronal hyper-excitation (Yue et al., 2018).

**Pyrethrins** **Pyrethroids**



Pyrethrin I

Allethrin

Pyrethrin II

Bifenthrin

**Fig. 4:** Structures of common pyrethroids

## 2.2. Sample pre-treatment techniques for pesticides

Residual pesticides at trace levels are difficult to extract completely due to the existence of complex components and interfering substances in certain matrices. Any interference in the sample treatment process may bring the uncertainty for determination of pesticide residues (Hou et al., 2014). Therefore, sample treatment is usually a critical step in the process of a comprehensive analysis, which directly affects accuracy and repeatability of the detection and analysis (Günter et al., 2016). Different techniques with varying figures of merits have been introduced. These are outlined hereunder.

### *2.2.1. Solid phase extraction*

Solid-phase extraction (SPE) is commonly used for pesticide residue analysis in agricultural samples (Pelajić et al., 2016; Shamsipur et al., 2016) and water (Zhang et al., 2016). SPE technology is simple, reproducible and easy to be coupled with detection instrument. It has become a universal sample treatment method (Zhao et al., 2016). In SPE procedure, the option of suitable adsorbent is a key factor to obtain high enrichment and good recovery. Based on the property of adsorbents, the sorbents are classified as normal phase (e.g., diatomite, silica gel and alumina), reversed phase (e.g., $C_8$ and $C_{18}$), polymeric sorbent, and mixed-mode sorbent (Shamsipur et al., 2016).

The first stage during the SPE procedure is the equilibration of the cartridge using a buffer of the same composition as the sample (Zhao et al., 2016). The sample is then loaded to the conditioned cartridge, and it elutes through the stationary SPE sorbent. As the sample passes through the SPE sorbent, analytes of interest are adsorbed on the sorbent resulting in their retention. The impregnated SPE sorbent is then washed to remove the impurities and finally, the analytes are eluted with appropriate buffer (Tokalıoğlu et al., 2016; Zhang et al., 2016).

Table 1 gives a summary of the reports where SPE was used in the analysis of pesticides. The commonly studied pesticides using SPE are the organochlorines. This can be attributed to their long half-lives and, therefore, their persistent nature in the environment. For instance, Zhao et al. (2016) used SPE to pre-concentrate organochlorine pesticides in red wine . In the same vein, Zhang et al. (2016) pre-concentrated organochlorines in water samples using SPE. Usually, after application of SPE, low LODs were obtained (Table 1) and this serves to show that SPE is a very effective and sensitive technique for pre-concentrating pesticides. Many different sorbents can be used when pre-concentrating pesticides using SPE which include florisil, $C_8$ and $C_{18}$. The $C_{18}$ sorbent, however, is very popular when pre-concentrating pesticides using SPE. Its popularity, probably, stems from the fact that $C_{18}$ has a high degree of hydrophilicity and as a result can effectively retain non-polar pesticides during SPE. GC is commonly used for the analysis of the pesticides after their pre-concentration with SPE. This could be attributed to the fact that most pesticides are volatile and volatility of the analyte is one of the basic requirement during GC analysis. The MS detector is very popular during trace analysis of the pesticides due to its high sensitivity and ability to identify them.

**Table 1**

Application of SPE in the analysis of pesticides in food samples

| Pesticide class | matrix | Sorbents | Instrument | LOD | Ref |
|---|---|---|---|---|---|
| Organochlorines | Surface water | $C_{18}$ | GC-MS | ng | Günter et al., 2016 |
| Multi-class pesticides | Red wine | Oasis HLB | GC-MS | 0.01-250 μg $L^{-1}$ | Pelajić et al., 2016 |
| Organochlorines | Alpina oxyphylla | Florisil | GC-ECD | 0.1-0.2 μg $kg^{-1}$ | Zhao et al., 2016 |
| Organochlorines | Antarctic waters | $C_{18}$ /silica gel | GC-MS | ng | Zhang et al., 2016 |
| Neonicotinoid | Sunflower seeds | $CH_3NH$-G | UPLC-MS/MS | 6 ng $kg^{-1}$ | Shi et al., 2017 |
| Multi-class pesticides | Water, milk, honey and fruit juice | $C_{18}$ | GC-MS | 0.5-1.0 ng $kg^{-1}$ | Shamsipur et al., 2016 |

**Note:** ng- not given

### *2.2.2. Solid phase micro-extraction*

One of the most significant developments in sample preparation has been, without doubt, solid phase micro-extraction (SPME), a technique first described by Arthur and Pawliszyn in 1990. SPME is an advanced sample pre-treatment technique integrating sampling, extraction, concentration and sample introduction into a single solvent-free step (Wang et al., 2013; Zhang et al., 2017).

In this approach, micro-quantities of the solid sorbent or liquid polymer in appropriate format are exposed to the sample. Quantification is based on the amount of analyte extracted at appropriate conditions (Xu et al., 2016). SPME is an equilibrium based extraction method. The extraction efficiency in SPME is determined by the distribution of the analytes between the sample matrix and the fibre coating (Liang et al., 2017). Therefore, the fibre coating material plays the most important role in SPME. Due to its convenience and speed, researchers worldwide in food and drugs analysis, clinical chemistry, biochemistry, forensic medicine and other fields implemented SPME (Jafari et al., 2016; Wang et al., 2016; Wu et al., 2016). Applications of SPME during the analysis of pesticides in food samples are summarised in Table 2.

SPME can be applied on a wide range of pesticides including organochlorines, organophosphates and carbamates. Most of these pesticides are volatile and can be easily pre-concentrated using HS-SPME. Thus, the HS-SPME mode is very popular when pre-concentrating pesticides using SPME (Table 2). Recently, many researchers are studying organophosphates using SPME as the pre-concentration technique (Sang et al., 2013; Wang et al., 2013; Zhang et al., 2017). Organophosphates have gained the attention of many researchers probably because they are widely used in agriculture ince they are biodegrade and non-persistent. However, their human and animal toxicity makes them a societal health and environmental concern. Hence, they are being widely studied using the HS-SPME mode for pre-concentration since they are volatile. The use of SPME to pre-concentrate pesticides is usually accompanied by low LODs (Table 2). Thus, the SPME is a very effective technique for the pre-concentration of pesticides. During SPME appropriate fibres should be used to adsorb the analyte during pre-concentration. Many fibres are used during the pre-concentration of pesticides using SPME (Table 2) and, among them, PDMS is widely used in conjunction with HS-SPME. The attractive properties of PDMS, which make it widely used, are its chemical inertness, non-toxicity and non-flammability. The common analytical technique used to analyse the pesticides after pre-concentration with SPME is gas chromatography. Most pesticides in general, and organophosphates in particular, can be analysed by gas chromatography due to their volatile nature. In Table 2, most of the pesticides analysed are organophosphates and as a result, the GC technique was used in conjunction with the nitrogen-phosphorus detector (NPD). NPD is a device that is highly sensitive and selective to nitrogen and phosphorus containing compounds. Thus, this detector is suitable for the detection of organophosphates since they contain phosphorus. With organochlorines, the electron capture detector can be used in conjunction with GC. The ECD is highly sensitive for compounds that contain electronegative atoms, which can easily attract electrons. Thus, organochlorines, due to the presence of chlorine atoms that are electronegative, can be easily detected by ECD in the gaseous phase. The MS, however, is a universal detector with sensitivity that surpasses both the ECD and NPD, can also be used during GC analysis of pesticides.

**Table 2**

Application of SPME for the analysis of pesticides in food samples

| Pesticide class | Food matrix | Fiber type | Mode of application type | Chromatographic technique | LOD | References |
|---|---|---|---|---|---|---|
| Organochlorines | Cocoa powder | Polydimethylsiloxane | HS-SPME | GC-MS | ng | Abdulra'uf et al., 2014 |
| Organochlorines | Water convolvulus | MOF-199/GO fibres | HS-SPME | GC-ECD | 2.3 – 6.9 ng $L^{-1}$ | Zhang et al., 2013 |
| Organophosphates | Cucumbers, green pepper, chinese cabbage, eggplant and lettuce | Sol-gel molecularly imprinted polymer | HS-SPME | GC-NPD | 0.017-0.77 µg $kg^{-1}$ | Wang et al., 2013 |
| Carbamates | Apple | Carbon nanotubes-reinforced hollow fibre | DI-SPME | HPLC-DAD | 0.09- 6.00 ng $g^{-1}$ | Song et al., 2013 |
| Organophosphates | Cucumber and lettuce | Polypyrrole/sol-gel composite | DI-SPME | GC-NPD | 1..5 – 10 ng $L^{-1}$ | Zhang et al., 2017 |
| Organophosphates | Turnips, green cabbage, French beans | PDMS | HS-SPME | GC-MS | 0.01- 2.5 µg $L^{-1}$ | Sang et al., 2013 |
| Organochlorines | Asparagus africanus | PDMS | QuEChERS/ HS-SPME | GC-ECD<br>GC-TOF-MF | 0.102-1.693 µg $L^{-1}$ | Obuseng et al., 2013 |
| Organophosphates | Pakchoi | OH-TSO/DVB | HS-SPME | GC-NPD | 0.007-0.07 ng $g^{-1}$ | Wang et al., 2008 |

**Note**: ng – not given

### *2.2.3. Micro-solid phase extraction*

Porous membrane protected micro-solid-phase extraction (μ-SPE) was first introduced by Basheer et al. (2006) as an alternative to multi-step SPE. In μ-SPE, few milligrams of sorbent are packed inside a sheet of porous polymer membrane and edges of this sheet are heat sealed to convert it into a small bag (Sajid, 2017). This sorbent containing bag, which can be represented as a μ-SPE device, is then cleaned and conditioned in a suitable solvent using ultra-sonication and stored in the same solvent until further use.

For extraction, the μ-SPE device is taken out from the conditioning solvent and is air-dried. After that, this device is added into the sample solution and the sample is rotated or stirred at a specified rate for an optimum period (Wang et al., 2013). After the extraction, the device is taken out from the sample solution and is rinsed with ultra-pure water and dried with lint-free tissue. Then this device is transferred to small glass or plastic vial and analytes are desorbed by adding a small volume of solvent. This desorption is assisted by ultra-sonication. The extract is then injected to GC or LC. Table 3 shows application of μ-SPE in the analysis of pesticides in food samples.

The μ-SPE technique has been widely used for the pre-concentration of organophosphates and organochlorines (Table 3). These classes are composed of non-polar pesticides that can easily adsorb on non-polar sorbents such as $C_{18}$ through the formation of hydrophobic interactions. After pre-concentration, the common analytical technique is also GC. The popularity of GC during pesticide analysis is largely based on their volatility. Detection, after pre-concentration using μ-SPE, is usually done using mass spectrometry, so as to take advantage of its high sensitivity. From Table 3, it can be seen that the use of μ-SPE in conjunction with GC-MS resulted in very low limits of detection. Thus, this technique is sensitive for the pre-concentration and analysis of organochlorines and organophosphates.

**Table 3**

Application of µ-SPE in the analysis of pesticides in food samples

| Pesticide class | Matrix | Sorbents | µ-SPE technique | Instrument | LOD | References |
|---|---|---|---|---|---|---|
| Organochlorines | Ovarian cancer tissue | Haye-Sepa/$C_{18}$ | MA-µ-SPE | GC-MS | 0.002-0.009 ng $g^{-1}$ | Basheer et al., 2007 |
| Organophosphates | Pakchoi, tomato, apple and grapes | Activated carbon | MA-µ-SPE | GC-MS | 006- 0.23 µg $kg^{-1}$ | Wang et al., 2013 |
| Organochlorines | Urine samples | LDH/G hybrid | SB-µ-SPE | GC-MS | 0.22 – 1.38 ng $mL^{-1}$ | Sajid, 2017 |
| Organochlorines | River water samples | MIL-101 | µ-SPE | GC-MS | 0.0025 -0.016 ng $mL^{-1}$ | Huang et al., 2015 |
| Organophosphates | Sewage sludge | MWCNTs | µ-SPE | GC-MS | 1-7 pg $g^{-1}$ | Basheer et al., 2006 |
| Organochlorines | Milk samples | ZnO incorporated carbon foam | µ-SPE | GC-MS | 0.19 – 1.64 ng $mL^{-1}$ | Sajid et al., 2016 |

### *2.2.4. The QuEChERS Method*

Anastassiades et al. (2003) developed a pre-treatment method of pesticide residues, which combined the extraction and clean-up of pesticides from the food matrix (Table 4). This method is named as QuEChERS because of the characteristics of being Quick, Easy, Cheap, Effective, Rugged and Safe. The original approach consists of extracting with acetonitrile, partitioning between the aqueous and acetonitrile phase through adding magnesium sulphate and sodium chloride, and a dispersive solid phase extraction (d-SPE) clean-up step by primary secondary amine (PSA) and anhydrous magnesium sulphate (Han et al., 2016). The QuEChERS technique is described in detail in Paper II of this dersertation.

The QuEChERS method has undergone several modifications and has become well-established for multi-residue analyses of pesticides in food and agricultural samples (Bernardi et al., 2016). Some proposed modifications to the QuEChERS method include buffering (Andraščíková et al., 2013), use of different sorbents such as graphitized carbon black (GCB) (Paz et al., 2014), octadecyl ($C_{18}$), Florisil (magnesium silicate) and alumina (Cerqueira et al., 2014) and the use of low temperature precipitation for fatty matrices (Sobahnzadeh et al., 2012). These modifications allowed the extraction of a large number of pesticides from different classes and matrices. Most part of the modifications was focused in the clean-up step and different sorbents have been used besides primary and secondary amine (PSA) and $C_{18}$ (Arias et al., 2014). Alumina or aluminum oxide ($Al_2O_3$), commonly applied in chromatographic separation of lipophilic compounds, has been used as d-SPE sorbent for pesticides determination (Koesukwiwat et al., 2008). Choi et al. (2015) used GCB for the determination of pesticides in different matrices.

Among other beneficial features, the QuEChERS procedure uses acetonitrile, which permits extraction of polar analytes and has an elevated degree of selectivity and detectability and direct compatibility with both gas and liquid chromatography coupled with mass spectrometry (MS) (Lehotay et al., 2010). The QuEChERS method, when compared with other techniques mentioned above, minimizes the number of sample preparation steps since it only involves two steps, first extraction with acetonitrile and a mixture of salts by partition and then clean-up steps by dispersive solid phase extraction (d-SPE) using a sorbent comprising of primary and secondary amines (PSA). Other advantages of the QuEChERS method compared with other techniques are their excellent recoveries, less time for sample preparation and less solvent consumption (Zhang et al., 2014).

The QuEChERS technique has found widespread applications in the pre-concentration of pesticides (Table 4). For instance, Wang et al. (2017) pre-concentrated organochlorines in fish using QuEChERS. Tette et al. (2016) used the QuEChERS technique to pre-concentrate multi-class pesticides in honey samples. The QuEChERS technique can also be used to pre-concentrate pesticides in plant samples (Table 4). Lee et al. (2016), for instance, successfully pre-concentrated herbicides in brown rice using QuEChERS. Dankyi et al. (2015) also used the QuEChERS technique to pre-concentrate neonicotinoids in cucumber samples.

The common extraction solvent used during QuEChERS is acetonitrile (Table 4). The choice of acetonitrile as an extraction solvent is based on its selectivity during the pre-concentration of pesticides using QuEChERS. Thus, when acetonitrile is used, only a few co-extractives from the matrix are extracted but covering a broad scope of pesticides. Another advantage of acetonitrile is its compatibity with both GC and LC. Moreover, acetonitrile is easily separated from water upon the addition of the partition salts during QuEChERS as compared to other solvents like acetone and ethyl acetate. Extraction of alkaline-sensitive pesticides is enhanced by the acidification of acetonitrile using acetic acid during QuEChERS.

**Table 4**

Pesticides analysed using QuEChERS technique

| Analyte | Pre-treatment technique | Extraction solvent | Clean-up process | Matrix | LOD | References |
|---|---|---|---|---|---|---|
| Organochlorines | QuEChERS-DLLME-SFO | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | Fish | 0.0065–0.00158 mg $kg^{-1}$ | Wang et al., 2017 |
| Multiclass pesticides | QuEChERS combined with freeze out | 1% acetic acid in acetonitrile | d-SPE using $MgSO_4$, $C_{18}$ and PSA sorbents | Coconuts | 10 µg $kg^{-1}$ | Ferreira et al., 2016 |
| Multiclass pesticides | Conventional QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, $C_{18}$, PSA and GCB | Tobacco | 25 -75 µg $kg^{-1}$ | Bernardi et al, 2016 |
| Organochlorines | Conventional QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | Beans, carbages, beef and fish | ng | Nuapia et al., 2016 |
| Multiclass pesticides | QuEChERS | 1% acetic acid in 10 mL of acetonitrile : ethyl acetate (70:30 v/v) | d-SPE using $MgSO_4$ , florisil and PSA sorbents | Honey | 0.005 mg $kg^{-1}$ | Tette et al., 2016 |
| Herbicides | EN-QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | Brown rice | 0.003- 0.015 mg $kg^{-1}$ | Lee et al., 2016 |
| Neonicotinoids | Conventional QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | Cucumber | 0.08 – 6.06 mg $kg^{-1}$ | Dankyi et al., 2015 |
| Multiclass pesticides | Conventional QuEChERS | Acetonitrile | r- DSPE using multi-walled carbon nanotubes | Cowpea | 0.01-0.03 mg $kg^{-1}$ | Han et al., 2016 |

**Note**: ng – not given

### *2.2.5. Molecular imprinting technology*

Molecular imprinting technology (MIT) has emerged as a versatile technique widely used for the synthesis of “tailor-made” polymeric materials affording the production of highly cross-linked materials, as stable recognition matrices for a wide range of analytes, mimicking the recognition mechanism of antigens and antibodies (Yan et al., 2014). These materials are considered to be artificial receptors possessing highly specific sites towards the target molecule presenting additionally improved properties, namely higher physical robustness, strength, resistance to temperature and pressure as well as stability in acid and basic media (Martins et al., 2015).

During the imprinting process, an *in-situ* formation of the monomer template complex is considered as a key step followed by the use of a cross-linker entity that allows the preservation of the structure of the monomer template complex (Bakas et al., 2014). Thus, the creation of an artificially generated three-dimensional polymer network which possesses binding sites with structural and functional groups complementary to the template molecule. After the polymerization process, the template molecule is removed from the polymer leaving specific recognition sites complementary in shape, size and chemical functionality to the template molecule, allowing the molecularly imprinted polymer (MIP) be able to recognize and bind selectively to only the template molecule (Martins et al., 2015). Moreover, the less expensive synthesis and the higher storage stability – keeping their recognition ability for several years at room temperature –and reusability, constitute the major advantages of these imprinting materials (Garcia et al., 2011). MIPs have been applied to pre-concentrate pesticides in different matrices (Table 5).

The common functional monomers used during MIP are acrylamide and metacrylic acid (Table 5). The functional monomer should have the ability to assemble around the template and subsequently get cross-linked to each other. The resultant is the polymerisation of the functional monomers causing the formation of a complimentary shape to that of the template. Thus, a functional monomer should be able to form interactions with functional groups on both the template molecules and the monomers. When acrylamide is used as a functional monomer in MIP, it interacts with the template through formation of either hydrogen bonds or dipole-dipole interactions. But between the acrylamide molecules, multiple amide bonds are formed resulting in the formation of polyacrylamide.

**Table 5**

Application of MIPS during pesticides analysis

| Analyte | Type of Polymerisation | Pre-polymerisation reagents | | | Matrix | LOD | References |
|---|---|---|---|---|---|---|---|
| | | Functional monomers | Cross-linker | Template | | | |
| Triazine herbicides | Precipitation | Metacrylic acid | EGDMA | Atrazine | Radix Paeoniae Alba | 0.09 – 0.39 ng $g^{-1}$ | Li et al., 2016 |
| Dicofol (DCF) | Precipitation | Acrylamide | Divinylbenzene (DVB) | $\alpha$-chloro DDT | Celery sample | 0.06 ng $g^{-1}$ | Yan et al., 2014 |
| Fenthion | Precipitation | Acrylamide | EGDMA | Fenthion | Olive oil | 5 $\mu$g $L^{-1}$ | Bakas et al., 2014 |
| Deltamethrin | Precipitation | Acrylamide | EGDMA | Deltamethrin | Olive oil | ng | Martins et al., 2015 |

**Note**: ng – not given

### *2.2.6. Dispersive liquid-liquid micro-extraction*

Dispersive liquid–liquid micro-extraction (DLLME), introduced by Rezaee et al. (2006) is a simple and fast micro-extraction technique based on the use of an appropriate extractant and dispersive solvent (Table 6). When the mixture of extractant phase and disperser is rapidly injected into the sample, high turbulence is produced (Hou et al., 2014). This turbulent mixture gives rise to the formation of small droplets, which are dispersed throughout the aqueous sample. Emulsified droplets have large interfacial area (Zhang et al., 2015). The nature of the emulsifier (disperser solvent) can also have an influence on droplet size distribution, the mean droplet size, and on emulsion viscosity (Zhang et al., 2014). Liquid–liquid dispersions play an essential role in separation processes and reaction systems. This is because the large interfacial area due to dispersion facilitates mass transfer and reaction rate (Wang et al., 2017).

DLLME is a well-established method for extraction/concentration of pesticides in aqueous samples (Chen et al., 2016), but it is difficult to use DLLME alone for food analysis due to the complex matrix (Table 6). Consequently, several hyphenated DLLME techniques have been developed to improve extraction and pre-concentration efficiency in complex matrix. Such hyphenated pre-treatment techniques used during pesticide analysis include QuEChERS-DLLME (Bresin et al., 2015; Zhang et al., 2014) and QuEChERS-DLLME-SFO (Wang et al., 2017).

Selection of an appropriate extraction solvent is the major parameter during the DLLME process. Halogenated hydrocarbons and ionic liquids are the common extraction solvents used during the pre-concentration of pesticides using DLLME (Table 6). Extraction solvents are selected based on their higher affinity of the pesticides, good chromatographic behaviour and having a density that is remarkably different to that of water. Thus, halogenated hydrocarbons are used as extraction solvents during DLLME because they have densities that are higher than that of water. On the other hand, some organic solvents, like dodecanol, are used for extraction purposes because they have lower densities than water.

Dispersive solvents play an equally important role during the DLLME process. Alcohols, such as ethanol and methanol, are in common use as dispersive solvents during the pre-concentration of pesticides using DLLME (Table 6). Miscibility in both extraction solvent and aqueous phase is essential during the selection of a dispersive solvent. Alcohols can interact with the extraction solvents, usually halogenated hydrocarbons, and the aqueous phase through dipole-dipole forces and hydrogen bonds respectively. Thus, alcohols are suitable to be used as dispersive solvents since they are miscible with both extraction solvents and water. However, other solvents such as acetone and acetonitrile can also be used as dispersive solvents (Table 6). Researchers who pre-concentrated pesticides using DLLME have reported Low LODs and this is an indication that the technique is very effective and sensitive.

**Table 6**

Pesticides analysed using DLLME techniques

| Pesticide class | DLLME technique | Extraction solvent | Dispersive solvent | Matrix | LOD | References |
|---|---|---|---|---|---|---|
| Organophosphates | VLDS-SD-DLLME | 1-dodecanol | Acetonitrile | Water | 0.25- 1.0 ng mL$^{-1}$ | Selbanruang, et al., 2014 |
| Pyrethroids | LLE-DLLME | 1,1,2-trichloroethane | Dimethylformamide | Vegetable oils | 0.02- 0.16 mg kg$^{-1}$ | Farajzadeh et al., 2014 |
| Benzoylurea | TCIL-DLLME | 1-hexyl-3-methylimidazolium hexafluorophosphate | Methanol | Wastewater | ng | Vázquez et al., 2014 |
| Benzoylurea | US-IL-DLLME | 1-octyl-3-methylimidazolium hexafluorophosphate | Methanol | Wastewater | 0.5- 1.0 ng L$^{-1}$ | Vázquez et al., 2014 |
| Pyrethroids | QuEChERS-DLLME | Carbon tetrachloride | Acetone | Fruit juices | 0.2 – 2 µg L$^{-1}$ | Zhang et al., 2014 |
| Pyrethroids | UA- DLLME-SFO | 1-dodecanol | Ethanol | Tea | ng | Hou et al., 2014 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sulfonylurea | VA-IL-DLLME | 1-hexyl-3-methylimidazolium hexafluorophosphate | Methanol | Wine | 3.2- 6.6 µg kg$^{-1}$ | Gure et al., 2015 |
| Herbicides | DLLME | Chloroform | Acetonitrile | Wine | 0.025- 0.88 µg L$^{-1}$ | Chen et al., 2016 |
| Fungicides | UDSA-DLLME | 1-octanol | ng | Wine | 0.007- 0.025 µg L$^{-1}$ | Chu et al., 2015 |
| Pyrethroids | SSIL-DLLME | Tributyldodecylphosphonium hexafluorophosphate | ng | Water | 0.71- 1.54 µg L$^{-1}$ | Zhang et al., 2015 |
| Mixture of pesticides | CCSHLIE-DLLME | 1,1,2,2-tetrachloroethane | Acetonitrile | Fruit juices | 2.0 – 12 µg L$^{-1}$ | Farajzadeh et al., 2016 |
| Benzoylurea | IL-DLLME | Tetrabutyl ammonium hexafluorophosphate | ng | Honey | 2.1 – 4.2 µg kg$^{-1}$ | Wang et al., 2016 |
| Benzoylurea | In-syringe SIL-DLLME | Tricaprymethyl ammonium hexafluorophosphate | ng | Tea beverages | 0.29 – 0.59 µg L$^{-1}$ | Wang et al., 2017 |

**Note**: ng – not given

## 2.3. Separation and detection techniques for pesticides

Besides the extraction and purification procedures, the choice of appropriate separation and detection techniques is a step of fundamental importance. Numerous analytical methods including gas chromatography (GC) (Bernardi et al., 2016; Farajzadeh et al., 2017; Wang et al., 2017), liquid chromatography (Bargańska et al., 2014; Jovanov et al., 2014), mass spectrometry (MS) (Wang et al., 2016; Zhang and Xu, 2014) and ultraviolet spectroscopy (Pirsaheb et al., 2015) have been developed for the detection and quantification of pesticides in contaminated samples. The analytical technique that found widespread application during the analysis of pesticides in different matrices is GC. This is because most pesticides are volatile substances and volatility of the analyte is one of the major requirements during DC analysis.

## 2.4. Heavy metals

Heavy metals are natural constituents of the earth's crust. They are spread into the environment through natural processes such as geological abrasion and metal excretion by living organisms (Mülayim and Balkıs, 2015). However, these natural processes discharge fewer metals into the environment than anthropological activities (Ardakani, 2018; Nazir et al., 2015). The spread of high amounts of these metals in the environment due to anthropological activities leads to their propagation in the food chain (Yan et al., 2012). Heavy metals such as Co, Cu, Mo, Zn, Cr, Cd, Hg, As and Pb are metallic elements that have a high density compared with water and are present in various matrices in trace amounts (Tchounwou et al., 2012). Their heaviness and toxicity are interrelated, as heavy metals are able to induce toxicity at trace levels.

Some heavy metals play crucial biochemical and physiological functions in humans, animals and plants (Cherfi et al., 2014). The nutritional requirements of these trace elements, such as cobalt, copper, chromium, iron, manganese, molybdenum, selenium and zinc are generally low and they are called microelements (Daşbaşi et al., 2018; Türkmen and Budur, 2018). They are present in various matrices, although with a different bioavailability, in trace concentrations. Essential trace elements are usually added as nutritional additives in animal feed and fertilizers to promote health, and to optimize production. However, excessive exposure with higher concentration of these elements has been linked with cellular or systemic disorders and could represent a source of pollution (Rossi et al., 2014; Tuzen et al., 2016). Other heavy metals (e.g. As, Cd, Pb and Hg) are xenobiotic. They have no established biological functions and are considered as contaminants and undesirable substances in animals and plants (Elik et al., 2017). Moreover, As, Cd, Cr, Pb and Hg present a high toxicity because they can induce organ damage, even at lower exposure levels (Mohmand et al., 2015).

The toxicity of heavy metals can be attributed to their physico-chemical properties. Heavy metals are not biodegradable and have long biological half-lives. Thus, they have the potential for accumulation in the different body organs leading to unwanted effects (Gebrekidan et al., 2013; Zhang et al., 2018). Most heavy metals are extremely toxic, and because of their solubility in water, contamination may readily reach toxic levels. Food chain contamination is one of the most important pathways for the entry of these toxic pollutants into the human body (Doroudi and Niazi, 2018).

### *2.4.1. Toxicity of heavy metals*

Toxic heavy metal species can bind to proteins and thereby affect the biological functions of the target molecule (Hossain et al., 2012). In addition, toxic metal may interact strongly with thiols and disulphides and subsequently cause disruption of the biological activity of certain proteins that contain sensitive sulphur groups (Lal, 2010). These reactions regularly produce reactive oxygen species (ROS), which are natural by-products of the normal metabolism. The destruction of sensitive thiol groups due to metal exposure may eventually impair the protein folding or binding of apoenzymes by cofactors, and thus the normal biological activity of the proteins is disrupted.

Certain heavy metals can participate in catalytic reactions, known as Fenton-type reactions that produce ROS (Kim et al., 2015). Collectively, these reactions can set the cell under an oxidative stress and thereby ROS levels significantly increased, which may result in DNA damage, destructions of lipids and proteins through a wide range of biochemical route (Mohamed et al., 2016).

Toxic metal species may also get into cells through various transporters, or penetrate the cellular membrane and subsequently bind to lipophilic carrier (González-Guerrero et al., 2016). The transporter-mediated uptake of toxic metals interferes with the normal transport of essential substrates, and thus results in competitive inhibition of the transport process. Moreover, this transport process acquires energy from the proton motive force or ATP pool. Some metal oxy-anions are reduced by the oxidoreductases that are able to draw electrons from the bacterial transport chain through the quinone pool (Yadav et al., 2016). Particular toxic metals can cause starving of microbial cells indirectly by siphoning electrons from respiratory chain. The ROS are also produced during normal metabolic processes and results in DNA damage, destructions of proteins and lipids; however, this production is promoted during metal pollution and subsequently may lead to additional cellular damages (Marchetti, 2013).

### *2.4.2. Sources and toxicity of selected heavy metals*

#### *2.4.2.1. Chromium*

Chromium is an element that can be found naturally in the environment (Onchoke and Sasu, 2016). It exists in many oxidation states, with Cr (III) and Cr (VI) being the primary existing ones in the environment (Bu et al., 2018). Anthropogenic activities involving various industrial processes usually increase the amount of chromium in the environment. Industrial processes involving the use of chromium include steel production, paint and dye production, leather tanning and wood preservation (Cui et al., 2017). Chromium enters the environment through deposition from air emissions and leaching at contaminated sites, mainly in the Cr (III) and Cr (VI) forms. Once introduced to a natural system, its fate depends upon the form at which it enters (Altunay et al., 2018). In aquatic systems, Cr (VI) tends to be very soluble, not readily sorbed to particulate matter. However, as anaerobic conditions prevail, Cr (VI) reduces to Cr (III), a state that can strongly bind onto organic particulates (Huang and Wei, 2018).

Exposure to chromium contamination occurs through inhalation, ingestion, or dermal contact. Inhalation of high levels of Cr (VI) has been shown to cause nasal irritations such as nosebleeds or ulcers (Yousefi and Shemirani, 2013). Ingestion of similar levels can cause stomach, liver or kidney damage, which may result in death (Arain et al., 2018). Dermal exposure to high levels of Cr (VI) may result into skin ulcers. Individuals with severe allergies may experience swelling and redness to exposed areas. Studies have shown Cr (VI) compounds can increase the risk of lung cancer, and the several health organizations have labelled Cr (VI) in various forms as a human carcinogen (Baranik et al., 2018; Munonde et al., 2017). Due to this, the World Health Organization (WHO) has set guidelines of the maximum permissible limits of chromium in different environmental matrices (Table 7).

#### *2.4.2.2. Cadmium*

Cadmium is a non-essential, toxic trace element that may accumulate in soils from various anthropogenic activities (Terán-Baamonde et al., 2018). Natural sources of cadmium include the weathering of rocks as well as forest fires and volcanoes. Most cadmium is used in Ni-Cd batteries as well as in pigments, coatings and as stabilizer for plastics (Moitra et al., 2013;

Sharma et al., 2015). It is also used particularly to electroplate steel in order to prevent corrosion. Cadmium has the ability to absorb neutrons, so it is used as a barrier to control nuclear fission (Bernhoft, 2013).

Compounds of cadmium as well as its elemental forms are extremely toxic at all levels and tend to bio-accumulate in organisms and ecosystems (Nazir et al., 2015). Therefore, it is one of the priority pollutants of the environment (Zhang et al., 2018). Cadmium derives its toxicological properties from its chemical similarity to zinc (Yang and Shu, 2015). Cadmium and zinc appear together in nature. In animals, they are biologically antagonistic to each other as they compete for binding sites on various carrier proteins (Husain, 2016). Cadmium is bio-persistent and, once absorbed by an organism, remains resident for a long period, although it is eventually excreted. In humans, long-term exposure is associated with renal dysfunction. High exposure can lead to obstructive lung disease and has been linked to lung cancer (Tuzen et al., 2016). Cadmium may also produce bone defects (e.g., osteoporosis) in humans and animals (Zhang et al., 2011). Thus, the concentration of cadmium should monitored and its maximum allowable limits are given in Table 7.

Cadmium is strongly adsorbed to organic matter in soils. When cadmium is present in soils, it can be extremely dangerous, as the uptake through food will increase (Khan et al., 2017). Soils that are acidified enhance the mobility of cadmium and therefore increase its uptake by plants. Human uptake of cadmium takes place mainly through food. Foodstuffs that are rich in cadmium (liver, mushrooms, shellfish and dried seaweed) can greatly increase the cadmium concentration in human body. Tobacco smoke transports cadmium into the lungs (Khan et al., 2014). Blood will transport it to the rest of the body. Other high exposures can occur with people who live near hazardous waste sites or factories that release cadmium into the air as well as people that work in the metal refining industry. When people breathe in cadmium, it can severely damage the lungs (Moitra et al., 2013). This may even cause death.

When cadmium is transported to the liver, it binds with proteins to form complexes that are transported to the kidneys where it is likely to damage the filtering mechanism (Rani et al., 2014). This causes the excretion of essential proteins and sugars from the body, damaging the kidney further (Yang and Shu, 2015). The highest concentration of cadmium is found in liver and kidney through its strong binding with cystine residue of metallothionin, with somewhat lower concentration in pancreas and spleen (Meng et al., 2017). Other health effects that can be caused by cadmium are: diarrhoea, stomach pains and severe vomiting, bone fracture, reproductive failure and possibly even infertility, damage to the central nervous system, damage to the immune system, psychological disorders, and - possibly DNA damage (Sharma et al., 2015).

### *2.4.2.3. Lead*

Lead is a naturally occurring bluish-grey metal present in small amounts in the earth's crust (Zhang et al., 2015). Although lead occurs naturally in the environment, anthropogenic activities such as fossil fuels burning, mining, and manufacturing contribute to the release of high concentrations (Emurotu and Onianwa, 2017; Rahnama and Ghadiri, 2015; Tareen et al., 2014). Lead has many different industrial, agricultural and domestic applications. It is currently used in the production of lead-acid batteries, ammunitions, metal products (solder and pipes), and devices to shield X-rays (Gillis et al., 2012; Jan et al., 2015; Zhang et al., 2015).

Exposure to lead occurs mainly via inhalation of lead-contaminated dust particles or aerosols, and ingestion of lead-contaminated food, water, and paints (Tiwari et al., 2013). Lead absorption is influenced by factors such as age and physiological status. In the human body, the greatest percentage of lead is taken into the kidney, followed by the liver and the other soft tissues such as heart and brain, however, the lead in the skeleton represents the major body fraction (Gillis et al., 2012; Yedjou et al., 2010). The nervous system is the most vulnerable target of lead poisoning. Headache, poor attention spam, irritability, loss of memory and dullness are the early symptoms of the effects of lead exposure on the central nervous system (Porova et al., 2014). Lead is the most systemic toxicant that affects several organs in the body including the kidneys, liver central nervous system, hematopoietic system, endocrine system, and reproductive system (Cherfi et al., 2014; Neal, 2015). The maximum allowable limits are given in Table 7.

Many published studies have documented the adverse effects of lead in children and the adult population (Carocci et al., 2016; Cherfi et al., 2014; Jan et al., 2015; Salahinejad and Aflaki, 2014). In children, these studies have shown an association between blood level poisoning and diminished intelligence, lower intelligence quotient, delayed or impaired neurobehavioral development, decreased hearing acuity, speech and language handicaps, growth retardation, poor attention span, and anti-social behaviour (Peter et al., 2012; Siraj and Kitte, 2013). In the adult population, reproductive effects, such as decreased sperm count in men and spontaneous abortions in women have been associated with high lead exposure (Mohmand et al., 2015). Acute exposure to lead induces brain damage, kidney damage, and gastrointestinal diseases, while chronic exposure may cause adverse effects on the blood, central nervous system, blood pressure, kidneys, and vitamin D metabolism (Gebrekidan et al., 2013; Tiwari et al., 2013).

One of the major mechanisms by which lead exerts its toxic effect is through biochemical processes that include lead's ability to inhibit or mimic the actions of calcium and to interact with proteins (Haleagrahara et al., 2011). Within the skeleton, lead is incorporated into the bones in place of calcium. Lead binds to biological molecules and thereby interfering with their function by a number of mechanisms (Haleagrahara et al., 2011; Zhang et al., 2015). Lead binds to sulfhydryl and amide groups of enzymes, altering their configuration and diminishing their activities. Lead may also compete with essential metallic cations for binding sites, inhibiting enzyme activity, or altering the transport of essential cations such as calcium (Pourrut et al., 2013).

### *2.4.2.4. Nickel*

Nickel is just one of a variety of ubiquitous trace metals emitted into the environment from both natural and anthropogenic sources (Haber et al., 2017). The primary sources of nickel emissions into the ambient air are combustion of coal and oil for heat or power generation, nickel mining, steel manufacture, and other miscellaneous sources, such as cement manufacture (Schaumlöffel, 2012).

Nickel particles in the air settle to the ground or are taken out of the air in rain. Much of the nickel in the environment is found in soil and sediments because nickel attaches to particles that contain iron or manganese, which are often present in soil and sediments. It is considered as a borderline element between hard and soft acid acceptors in chemical interactions towards donor atoms. This is reflected in its abundance in the earth’s crust as oxides, carbonates and silicates with iron and magnesium as well as sulphides, arsenides and telurides (Das et al., 2018). Nickel salts are soluble and can occur as a leachate from nickel bearing rocks. The effects of nickel exposure vary from skin irritation to damage to lungs, the nervous system, and mucous membranes. It is also a known carcinogen. Recently, Macomber and Hausinger (2011) have suggested many mechanisms of nickel toxicity that involves the replacement of essential metal by metalloprotein and binding to a protein with a metal ion co-factor. Nickel can also bind to catalytic sites of non-metalloenzymes as wee as binding outside the catalytic site of an enzyme to inhibit its function.

### *2.4.2.5. Arsenic*

Arsenic is a ubiquitous priority pollutant found in the atmosphere, soils and rocks, natural waters and organisms (Sun et al., 2014). It is mobilized in the environment through a combination of natural processes such as weathering reactions, biological activity and volcanic emissions as well as through a range of anthropogenic activities (Mohamed et al., 2016). Significant exposure to arsenic occurs through both anthropogenic and natural sources. Occupational and community exposures to arsenic from the activities of humans occur through the smelting industry, the use of gallium arsenide in the microelectronics industry, and the use of arsenic in common products such as wood preservatives, pesticides, herbicides, fungicides, and paints (Mandal, 2017). Widespread dispersion of arsenic is a by-product of the combustion of fossil fuels in which arsenic is a common contaminant (Balazs et al., 2012).

The toxicity of an arsenic-containing compound depends on its valence state (zero-valent, trivalent, or pentavalent), its form (inorganic or organic), and factors that modify its absorption and elimination (Tyler and Allan, 2014). Inorganic arsenic is generally more toxic than organic arsenic, and trivalent arsenic is more toxic than pentavalent and zero-valent arsenic (Tangahu et al., 2011).

Once absorbed into the body, arsenic undergoes some accumulation in soft tissue organs such as the liver, spleen, kidneys, and lungs, but the major long-term storage site for arsenic is keratin-rich tissues, such as skin, hair, and nails (Mohamed et al., 2016; Sikdar and Kundu, 2018). Acute arsenic poisoning is infamous for its lethality, which stems from arsenic's destruction of the integrity of blood vessels and gastrointestinal tissue and its effect on the heart and brain (Ventura-Lima et al., 2011). Chronic exposure to lower levels of arsenic results in somewhat unusual patterns of skin hyperpigmentation, peripheral nerve damage manifesting as numbness, tingling, and weakness in the hands and feet, diabetes, and blood vessel damage resulting in a gangrenous condition affecting the extremities (Alissa and Ferns, 2011; Maji et al., 2016; Tyler and Allan, 2014). Chronic arsenic exposure also causes a markedly elevated risk for developing a number of cancers, most notably skin cancer, cancers of the liver (angiosarcoma), lung, bladder, and possibly the kidney and colon (Kim et al., 2015; Taylor et al., 2017).

### *2.4.2.6. Mercury*

Mercury is well known as a dangerous neurotoxin enriched in the environment by human activities (Batrakova et al., 2014). It is unique in that it exists or is found in nature in three forms (elemental, inorganic, and organic), with each having its own profile of toxicity (Bernhoft, 2012). At room temperature, elemental mercury exists as a liquid, which has a high vapour pressure and is released into the environment as mercury vapour. Mercury also exists as a cation with oxidation states of +1 (mercurous) or +2 (mercuric) (Rafati-Rahimzadeh et al., 2014). Methylmercury is the most frequently encountered compound of the organic form found in the environment, and is formed as a result of the methylation of inorganic (mercuric) forms of mercury by microorganisms found in soil and water (Alissa and Ferns, 2011).

Humans are exposed to all forms of mercury through accidents, environmental pollution, food contamination, dental care, preventive medical practices, industrial and agricultural operations, and occupational operations (Rice et al., 2014). The major sources of chronic, low-level mercury exposure are dental amalgams and fish consumption (Jain et al., 2013). Mercury enters water as a natural process of off-gassing from the earth’s crust and also through industrial pollution. Algae and bacteria methylate the mercury entering the waterways (Batrakova et al., 2014). Methyl mercury then makes its way through the food chain into fish, shellfish, and eventually into humans.

The molecular mechanisms of toxicity of mercury are based on its chemical activity and biological features that suggest that oxidative stress is involved in its toxicity. Through oxidative stress, mercury has shown mechanisms of sulfhydryl reactivity (Carvalho et al., 2008). Once in the cell both $Hg^{2+}$ and MeHg form covalent bonds with cysteine residues of proteins and deplete cellular antioxidants. Antioxidant enzymes serve as a line of cellular defense against mercury compounds (Batrakova et al., 2014). The interaction of mercury compounds suggests the production of oxidative damage through the accumulation of reactive oxygen species that would normally be eliminated by cellular antioxidants.

**Table 7**

Maximum allowable limits of heavy metals (WHO, 1996)

| Heavy metals | Maximum allowable limits | | |
|---|---|---|---|
| | Soil (mg kg$^{-1}$) | plants (mg kg$^{-1}$) | Water (mg L$^{-1}$) |
| Nickel | 80.0 | 10.0 | 0.20 |
| Cadmium | 3.00 | 0.02 | 0.01 |
| Chromium | 100 | 1.30 | 0.10 |
| Lead | 100 | 2.00 | 0.05 |

### *2.5. Chemistry of heavy metal pollution*

Mining activities and other geochemical processes often result in the generation of acid mine drainage, a phenomenon commonly associated with mining activities (Nagajyoti et al., 2010). It is generated when pyrite ($FeS_2$) and other sulphide minerals in the aquifer and present in former mining sites are exposed to air and water in the presence of oxidizing bacteria, such as *Thiobacillus ferrooxidans*, and oxidized to produce metal ions, sulphate and acidity.

$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4$ …………………………………… (1)

$2FeSO_4 + 2H_2SO_4 \rightarrow Fe_2(SO_4)_3 + SO_2 + 2H_2O$ ………………………………….(2)

$2Fe_2(SO_4)_3 + 4FeAsS + 9O_2 + 6H_2O \rightarrow 4H_3AsO_4 + 8FeSO_4 + 2S$ ……………...(3)

Thus, heavy metals (M) at mining sites are leached and carried by acidic water downstream. They can be acted upon by bacterial activity and methylated to yield organic forms, such as monomethyl mercury and dimethylcladium (Batrakova et al., 2014). This conversion is effected by bacteria in water, in the presence of organic matter, according to the following simplified equation:

$M + \text{organic matter} \xrightarrow{H_2O\ \text{bacteria}} -CH_3 \text{ and } (CH_3)_2M$ …………………………… (4)

In the non-biological conversions, the following reactions have been identified for mercury:

$$Hg^{2+} \xrightarrow{H_2S} \underset{\text{Less soluble}}{HgS} \xrightarrow{\text{aeration}} \underset{\text{More soluble}}{Hg_2SO_4} \text{ ………. (5)}$$

These organic forms of heavy metals have been reported to be very toxic and adversely affect water qualities by seepage to pollute under ground water source. For instance, methylmercury (MeHg) is a potent neurotoxic compound. It is biomagnified in the food webs of aquatic systems, reaching high concentrations in carnivorous fish, thus posing a risk to human health (Schaefer et al., 2011)

## *2.6. Remediation technologies for heavy metals*

Recent concerns regarding the environmental contamination with heavy metals have initiated the development of appropriate technologies to assess the presence and mobility of metals in soil, water and wastewater (Abdi and Kazemi, 2015; Ochonogor and Atagana, 2014). Presently, phytoremediation and biosorption have become effective and affordable technological solutions used to extract or remove inactive metals and metal pollutants from contaminated environments (García et al., 2018; Hima et al., 2007).

### *2.6.1. Phytoremediation of heavy metals*

Phytoremediation is the cheapest and environmentally most friendly technology for cleaning up soil (Abubakar et al., 2017). This takes advantage of the fact that a living plant can extract and concentrate elements from the environment. Phytoremediation employs the use of plants to degrade, remediate and stabilize various heavy metals in soil, water and air (Laghlimi et al., 2015). Phytoremediation includes various processes like phytoextraction, rhizofilteration, phytostabilization, phytovolatilization, phytoimmobilization and phytostimulation.

The most widespread and most profitable technique is phytoextraction, used mainly for removing heavy metals and radioactive elements from the soil. To enhance the accumulative potential of plants, chelates can be used (Kumar et al., 2017). These compounds substantially intensify the uptake and translocation of heavy metals in plants in that they release metals from the soil and form soluble complexes with them, which are then transported by the xylem vessels and deposited in the leaves.

### *2.6.2. Mechanisms of phytoremediation of heavy metals*

#### *2.6.2.1. Phytoextraction of heavy metals*

Phytoextraction technology involves the extraction of heavy metals by plant roots and the translocation thereof to shoots (Somaye and Ziarati, 2014). The roots and shoots are subsequently harvested to remove the heavy metals from the soil. Phytoextraction has environmental benefits because it is considered a low impact technology. Furthermore, during the phytoextraction procedure, plants cover the soil and erosion and leaching will thus be reduced (Ochonogor and Atagana, 2014). With successive cropping and harvesting, the levels of heavy metals in the soil can be reduced. Some applications of phytoextraction are given in Table 8. Phytoextraction technique is the most common mechanism used during phytoremediation. One of its major advantage is its applicability to a wide range of media: soil, sediments, water and sludge (Table 8). During phytoextraction, a particular medium can be remediated using a mixture of trees, shrubs and grass. This mixed culture approach, as used by Čudić et al. (2016) (Table 8), results in effective clean up of the contaminated environment since most of the plants used during phytoextraction are heavy metal hyper-accumulators. The use of herbaceous plants, especially grasses during phytoextraction, is advantageous since they have the characteristics of rapid growth, large amount of biomass, strong resistance, and ability to extract heavy metals from different types of soils. The large surface area of their fibrous roots facilitates rapid extraction of heavy metals from the contaminated media.

#### *2.6.2.2. Phytostabilization of heavy metals*

Phytostabilization, also referred to as in-place inactivation, is primarily used for the remediation of soil, sediment, and sludge contaminated with heavy metals. It involves the use of plant roots to limit heavy metal mobility and bioavailability in the soil (Abubakar et al., 2017). The primary purposes of the plants during phytostabilization of heavy metals are to decrease the amount of water percolating through the soil matrix, which may result in the formation of a hazardous leachate as well as acting as a barrier to prevent direct contact with the contaminated soil. The plants also prevent soil erosion and the distribution of the toxic metal to other areas (Galal et al., 2017). Phytostabilization of heavy metals can occur through the processes of sorption, precipitation, complexation, or metal valence reduction. Table 8 gives some of the applications of phytostabilization during remediation of different environmental matrices contaminated with heavy metals. Some of the plants used during phytostabilisation of heavy metals include *Vossia cuspidata, Brachiaria decumbens* and *Pteris vittata* (Table 8). These plants have the ability to develop extended and abundant root systems, which can prevent the mobilisation of heavy metals. Their well-developed root system also have the ability to retain the heavy metals in the rhizosphere thereby limiting their spreading through the food chain.

### *2.6.2.3. Rhizofiltration*

Rhizofiltration or phytofiltration is primarily used to remediate extracted groundwater, surface water, and wastewater with low heavy metal concentrations (García et al., 2018). It is defined as the use of plants, both terrestrial and aquatic, to absorb, concentrate, and precipitate heavy metals from polluted aqueous sources in their roots (Laghlimi et al., 2015). Rhizofiltration can be used for Pb, Cd, Cu, Ni, Zn and Cr, which are primarily retained within the roots (Table 8). Some of the plants used for rhizofiltration include *Eichhornia crassipes* and *Pistia stratiotes*. These are terrestrial plants. Terrestrial plants are preferred because they have a fibrous and much longer root system, increasing the amount of root area.

### *2.6.2.4. Phytovolatilization of heavy metals*

Phytovolatilization involves the use of plants to take up heavy metals from the soil, transforming them into volatile forms and transpiring them into the atmosphere (Laghlimi et al., 2015) (Table 8). Mercuric mercury is the primary metal contaminant that this process has been used for (Alkorta et al., 2004). The advantage of this method is that the contaminant, mercuric ion, maybe transformed into a less toxic substance (that is, elemental Hg). The disadvantage to this is that the mercury released into the atmosphere is likely to be re-cycled by precipitation and then redeposited back into lakes and oceans, repeating the production of methyl-mercury by anaerobic bacteria.

**Table 8**

Application of phytoremediation of heavy metals in different matrices

| Phytoremediation mechanism for heavy metals | Inorganic contaminants | Matrix | Plant used for phytoremediation of heavy metals | References |
|---|---|---|---|---|
| Phytoextraction | Cadmium, zinc, copper, nickel, chromium and arsenic | Soil | Poplar (*Populus ssp*.), ailanthus (*Ailanthus glandulosa L*.), false acacia (*Robinia pseudoacacia L*.), ragweed (*Artemisia artemisiifolia L*.) and mullein (*Verbascum thapsus L*) | Čudić et al., 2016 |
| Phytoextraction | Iron and chromium | Soil | *Psoralea pinnata* | Ochonogor and Atagana, 2014 |
| Phytoextraction | Cadmium, lead and zinc | Bore-hole water, | Water hyacinth | Aisien et al., 2010 |
| Phytoextraction | Lead, copper, cadmium and nickel, | Soil | *Amaranthus sp* | Somaye and Ziarati, 2014 |
| Phytoextraction | Nickel and zinc | Soil | Wheat (*Triticum aesticum L*.), clover (*Trifolium resupinarum* L.) and rapeseed (*Brassica napus L*.) | Eskandari and Amraie, 2016 |
| Phytostabilization | Chromium, copper, lead, aluminium. Cadmium and zinc | Water | *Vossia cuspidata* | Galal et al., 2017 |

| | | | | |
|---|---|---|---|---|
| Phytostabilization and phytoextraction | copper | Soil | *Brachiaria decumbens* | Andreazza et al., 2013 |
| Phytostabilization | Zinc andcadmium | Industrial residue | *Brachiaria decumbens* | Santos et al., 2007 |
| Phytostabilization | Cadmium, manganese, lead and zinc | Manganese mine tailings | *Alternanthera philoxeroides, Artemisia princeps, Bidens frondosa, Bidens pilosa, Cynodon dactylon, Digitaria sanguinalis, Erigeron Canadensis* and *Setaria plicata* | Yang et al., 2014 |
| Phytovolatization | Arsenic | soil | *Pteris vittata* | Sakakibara et al., 2007 |
| Phytoextraction | Copper, zinc, lead and chromium | Water | *Phragmites australis* | Huang et al., 2017 |
| Rhizofiltration | Chromium, lead and nickel | Eutrophic water | *Pistia stratiotes* | Abubakar et al., 2014 |
| Rhizofiltration | Cadmium, lead and zinc | Fly ashes | *Eichhornia crassipes* | Yadav et al., 2015 |

*2.6.3. Biosorption*

Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physico-chemical pathways of uptake (García et al., 2016). Thus, biosorption is a term that describes the removal of heavy metals by the passive binding to non-living biomass from an aqueous solution. Algae, bacteria, fungi, and yeasts have proved to be potential metal biosorbents (Table 9). The major advantages of biosorption over conventional treatment methods include low cost, high efficiency, minimisation of chemical and biological sludge, no additional nutrient requirement, regeneration of biosorbent, possibility of metal recovery and bioprocess technologies are usually environmentally benign (no secondary pollution) (Sofiane and Sofia, 2015; Utomo et al., 2016).

The biosorption process involves a solid phase (sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be adsorbed (sorbate, metal ions) (Mustapha and Halimoon, 2015). Due to higher affinity of the sorbent for the sorbate species, the latter is attracted and bound there by different mechanisms. The process continues until equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity for the sorbate determines its distribution between the solid and liquid phases (Abbas et al., 2014).

**Table 9**

Application of biosorption for heavy metal remediation

| Matrix | Biosorbent | Heavy metals | References |
|---|---|---|---|
| Wastewater | *Bacillus SP* bacteria | Cadmium, chromium, lead and manganese | García et al., 2016 |
| Wastewater | Marine and freshwater algae | Copper, lead, cadmium and zinc | Utomo et al., 2016 |
| Wastewater | *Bacillus circulans*, *Pseudomonas aeruginosa*, *Staphylococcus xylosus*, *Streptomyces rimosus* and *Saccharomyces sp.* | Zinc, copper and manganese | Nwidi and Agunwamba, 2015 |
| Industrial and municipal wastewater | Chitin and chitosan | Zinc, cadmium and copper | Sofiane and Sofia, 2015 |
| Aqueous solutions | Coconut fibre | Cadmium | Kramer et al., 2014 |
| Industrial wastewater | Modified bark | Chromium | Kumar et al., 2012 |
| Wastewater | *Aspergillus flavus* and *Aspergillus niger* | Copper and lead | Iram and Abrar, 2015 |
| Polluted aqueous environments | Dried *Chlorella vulgaris* and *Spirulina platensis* | Lead, cadmium and copper | Kőnig-Péter et al., 2015 |

### *2.7. Sample preparation for heavy metals*

Analytical methods involving heavy metals consist of several steps including; sampling, sample preparation, analysis, calculations and statistical evaluation of the results. Each step has a direct impact on accuracy, precision and sensitivity of the method. Among these steps, sample preparation during analysis of heavy metals is the most time consuming step. Sample preparation follows two main aims; sample clean up and pre-concentration. Sample clean up is carried out for isolating the heavy metals from matrix components that interfere on determination and pre-concentration is done for enrichment of the heavy metals in sample because despite advances in analytical instrumentation, sensitivities are limited.

#### *2.7.1. Liquid-liquid extraction of heavy metals*

Liquid-liquid Extraction (LLE) is a versatile classical sample preparation technique (Rajendran et al., 2011) which can be used for both organic and inorganic analytes such as heavy metals (Table 10). LLE is based on establishment of distribution equilibrium of the heavy metals between two immiscible phases, an aqueous and an organic phase. If distribution equilibrium constant is large enough, a quantitative extraction of the heavy metals can occur in a few steps.

Solvent extraction is convenient for both group and selective pre-concentration of heavy metals. The pre-concentration procedure usually involves the reaction of the heavy metals with chelating agents such as 8-hydroxyquinoline, dithizone, ammonium pyrrolidin dithiocarbamate and diethyldithiocarbamate, followed by extraction with an appropriate organic solvent (Bouabdallah et al., 2006; Fetouhi et al., 2016). The major apparatus for LLE is the separating funnel. Thus, LLE has the advantage of not requiring sophisticated apparatus and is easy to perform. This technique has, besides its advantages, certain limitations such as lack of selectivity and the use of large volumes of toxic and volatile organic solvents (Pirkwieser et al., 2018)

Chelation of heavy metals prior to pre-concentration with LLE are a common feature in Table 10. The reaction of the heavy metals with chelating agents is done in order to reduce the polarity of the heavy metals and convert them into a non-polar form. This is an essential step as it facilitates the separation of the heavy metals from the aqueous medium into the non-polar organic solvent during LLE. Extraction solvents that have gained popularity during the pre-concentration of heavy metals with LLE are the ionic liquids (ILs) (Table 10). The ILs are ionic, non-molecular solvents with melting points below 100$^{o}$C. The most notable properties include their negligible vapour pressure at room temperature, high thermal stability, and variable viscosity. Their miscibility in water and organic solvents can becontrolled by selecting the cation/anion combination or by incorporating certain functional groups in the IL molecule. In addition, they possess a multitude of tunable physico-chemical properties. Thus, after chelation of the heavy metals, the non-polar complexes can be extracted during LLE using neutral ILs. After pre-concentration of the trace heavy metals, they will be ready for instrumental analysis. The common analytical technique used in conjunction with LLE, from Table 10, is atomic absorption spectroscopy. This is a sensitive technique for elemental analysis.

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**Table 10**

Application of liquid-liquid extraction to pre-concentrate heavy metals

| Matrix | Heavy metals | Chelating agent | Extraction solvent | Analytical instrument | References |
|---|---|---|---|---|---|
| Aqeuous solutions | Lead, nickel and cobalt | N-salicylideneaniline | 1- butyl-3-methylimidazolium hexafluorophosphate | Flame atomic absorption spectroscopy | Fetouhi et al., 2016 |
| Water | Mercury and cadmium | Dithizone | 1-butyl-3-methylimidazolium hexafluorophosphate | Uv-vis spectrometry | |
| Drinking water , pure water, saline water | Silver, cadmium, cobalt, copper, manganese, nickel and lead | 3-hydroxy-2-naphthoic acid | Trihexyltetradecylphosphoniu, methyltrioctylphosphonium and methyltrioctylammonium 3-hydroxy-2-naphthoate | Atomic absorption spectroscopy | Pirkwieser et al., 2018 |
| Water | Copper, cadmium and lead | N-donor pyrazole ligands | Methylene chloride | Atomic absorption spectroscopy | Bouabdallah et al., 2006 |
| Aqueous solutions | Zinc, cadmium, iron, copper | ng | Methyltrioctylammonium chloride and 1-methyl-3-octylimidazolium tetrafluoroborate | Atomic absorption spectroscopy | Hernández-Fernández et al., 2010 |

**Note**: ng- not given

### *2.7.2. Solid phase extraction of heavy metals*

One of the alternative methods for LLE, which can be used to pre-concentrate heavy metals, is solid phase extraction (SPE). SPE process is based on distribution of heavy metals between solid sorbent packed in a cartridge and liquid sample which moves through the solid phase (Zhang et al., 2016). Solid phase usually consists of small porous particles of silica with or without bonded organic phase, organic polymers and ion exchangers (Shamsipur et al., 2016). Mechanisms of extractions are based on adsorption, partitioning or ion exchange according to kind of solid phase. SPE has been used to pre-concentrate heavy metals in different environmental matrices (Babazadeh et al., 2015; Gouda and Zordok, 2018; Hossein et al., 2010) (Table 11).

SPE has many attractive features in comparison with classical liquid-liquid extraction during pre-concentration of heavy metals. However, it has its limitations. Some of the main limitations of SPE include clogging the pores of the solid phase by large biomolecules, oily materials and fine solids in the sample. It also uses large volumes of toxic organic solvents as well as being a time consuming method due to several steps of operation including; conditioning, sample loading and elution (Huo et al., 2016; Pelajić et al., 2016).

The effectiveness of the SPE pre-concentration of heavy metals hinges on the ability of the sorbents to adsorb the analyte. Many strategies are used in order to improve the ability of the sorbents to interact with heavy metals during their SPE pre-concentration. These include impregnating the sorbent with a suitable chelating agent, inclusion of cation exchange resin in the sorbent as well as inclusion of magnetic nanoparticles (Table 11). The presence of chelating agents in the sorbent will cause the retention of the heavy metals in the sorbent through complexation. The cation exchange resin has embedded negatively charged functional groups, which retain the heavy metal cations through electrostatic interactions while magnetic nanoparticles cause retention of the heavy metals on the sorbent by exerting a magnetic attractive force on them. The retained heavy metals are then eluted from the sorbent using an appropriate solvent and will be ready for instrumental analysis. From Table 11, the common analytical techniques used include flame atomic absorption spectroscopy and inductively coupled plasma atomic emission spectroscopy. These are effective and sensitive analytical techniques for elemental analysis.

**Table 11**

Application of SPE to pre-concentrate heavy metals

| matrix | Heavy metals | Sorbent | Analytical instrument | References |
|---|---|---|---|---|
| Street dust | Fe (II), Mn(II), Zn(II), Cu(II), Pb(II), Co(II) | Dowex marathon C, cation exchange resin | Flame atomic absorption spectrometry | Al-Rashdi et al., 2017 |
| Agricultural samples | Cd(II), Pb(II), Zn(II) and Cr(III) ions | $Fe_3O_4$–ethylenediamine (magnetic metal-organic framework) | Flame atomic absorption spectrometry | Babazadeh et al., 2015 |
| Industrial water | Cd(II) | Amberlite XAD-16 modified with α-benzoin oxime chelating resin | Flame atomic absorption spectrometry | Tiwari et al., 2016 |
| Water | Cu(II), Ni(II) and Co(II) | Indane-1,2,3-trione 1,2-dioxime immobilized on sodium dodecyl sulfate (SDS) coated alumina | Flame atomic absorption spectrometry | Hossein et al., 2010 |
| Water and food samples | Cd(II) and Pb(II) | Multiwalled carbon nanotubes in combination with 5-benzyl-4-[-chlororbenzylidene amine]-4H-1,2,4-triazole-3-thiol | Flame atomic absorption spectrometry | Gouda and Zordok, 2018 |
| Skin whitening cosmetics creams | Arsenic, bismuth, cadmium, mercury, lead and titanium | Multiwalled carbon nanotubes | Inductively coupled plasma atomic emission spectrometry | Alqadami et al., 2013 |
| Factory wastewater | Lead and copper | $SnO_2$ nanowires | Flame atomic absorption spectrometry | Alizadeh et al., 2018 |

### *2.7.3. Solid phase micro-extraction of heavy metals*

Solid phase micro-extraction (SPME) as a solvent free alternative method for conventional sample preparation methods (Souza-Silva et al., 2015). In SPME, a small volume of extraction phase coated on fused silica support is mounted in a modified Hamilton 7000 series syringe. Extraction phase could be a high molecular weight polymeric liquid or a solid porous sorbent with high surface area. The extraction process of fiber SPME can be conducted in three common ways: direct or immersion extraction (DI), headspace (HS) and membrane protection extraction (Merkle et al., 2015). In DI-SPME the fibre is directly immersed in a gaseous or liquid sample whereas in HS-SPME the fibre is exposed to the vapour phase above the liquid or solid sample. The third SPME technique is the membrane protection extraction. The extraction, in this case, is conducted using a membrane that is selectively permeable for analytes of interest (Wang et al., 2016).

Heavy metals cannot be extracted directly and efficiently by SPME due to their physicochemical properties. Thus, in certain cases derivatization is used to modify the heavy metals to make them volatile and improve their extraction by SPME relative to the matrix interferences (Table 12). Many researchers have used derivatization during the pre-concentration of some heavy metals using HS-SPME. Tyburska et al. (2011), for instance, pre-concentrated selenium and arsenic in water samples through derivatizing the heavy metals into volatile hydrides prior to HS-SPME. In the same vein, Lin et al. (2016) derivatized mercury in rice samples using sodium tetraphenylborate and then pre-concentrated it using HS-SPME. On the other hand, Zheng et al. (2018) volatized mercury and lead in rice and water samples using sodium tetraethylborate as a derivatizing agent prior to HS-SPME. Thus, derivatization to convert heavy metals into volatile forms is a pre-requisite for their analysis using SPME. From Table 12, the common SPME mode used during the pre-concentration is, therefore, HS-SPME since the analyte will be in a volatile form. The pre-concentration of the derivatized heavy metals using SPME is followed by direct injection of the analyte into the GC. Thus, GC is the common analytical technique that is used in conjunction with SPME.

**Table 12**

Application of SPME to pre-concentrate heavy metals

| Heavy metals | Matrix | Fibre type | Mode of application | Analytical instrument | LOD | References |
|---|---|---|---|---|---|---|
| Mercury and lead | Rice and water | PDMS/DVB | HS-SPME | Micro-plasma Optical Emission Spectrometry | 0.001µgL$^{-1}$ (Hg)<br>0.003µgL$^{-1}$ (Pb) | Zheng et al., 2018 |
| Mercury, methylmercury, ethylmercury | Rice | Porous carbon | HS-SPME | Gas chromatography | 0.5-1.0 µgkg$^{-1}$ | Lin et al., 2016 |
| Organomercury compounds and mercury | Sea water | Polydimethylsiloxane-coated fused-silica fibre | HS-SPME | Gas chromatography | ng | Carro et al., 2002 |
| Arsenic | Drinking and environmental waters | *N*-(β-aminoethyl)-γ-aminopropyltriethoxysilane | DI-SPME | Inductively coupled plasma mass spectrometry (ICP-MS) | 0.005 µg L$^{-1}$ | Li et al., 2014 |
| Arsenic and selenium | Water | Polydimethylsiloxane /Carboxen | HS-SPME | Optical emission spectrometry | 0.1ng mL$^{-1}$(As)<br>0.8 ng mL$^{-1}$(Se) | Tyburska et al., 2011 |

Note: ng – not given

### *2.7.4. Dispersive liquid-liquid micro-extraction of heavy metals*

Dispersive liquid-liquid micro-extraction (DLLME) is a versatile mode of liquid phase micro-extraction developed by Rezaee et al. (2006). DLLME is a ternary solvent system consisting of aqueous sample solution, extraction solvent and disperser solvent (Pourreza et al., 2015). Extraction solvent must be immiscible with aqueous sample solution and disperser solvent must soluble in both of the extraction solvent and aqueous sample solution. In DLLME, 5-10 mL of sample solution is placed in a centrifuge tube then optimized volumes of extraction solvent and disperser solvent are mixed. The mixture of these solvents is injected rapidly in the sample solution. After injection of solvents mixture, a stable cloudy suspension consisting of fine droplets of extraction solvent is formed which can be easily separated by centrifugation. After centrifugation, the aqueous phase is discarded and sedimented organic phase is transferred to an analytical instrument using a micro syringe.

Extraction of metal ions with organic solvents during DLLME requires the conversion of the metal ions to suitable forms that can interact with the organic solvents. Appropriate chelating agents must be selected and complexation conditions such as solution pH and concentration of ligand must be optimized (Gaubeur et al., 2015). Thus, application of DLLME technique to pre-concentrate metal ions is possible after converting the metals to organometallic compounds (Table 13).

A simple and powerful micro-extraction technique was used for determination of cadmium in water samples using DLLME followed by analysis using AAS (Rahmani et al., 2016). In this study, a mixture of methanol (disperser solvent) and carbon tetrachloride (extraction solvent) was used to extract a complex of cadmium and dithizone from water samples. In another study determination of trace levels of zinc and copper was possible with DLLME followed by AAS (Ghorbani et al., 2013). In the proposed approach, 8-hydroxyquinoline was used as a chelating agent, and chloroform and methanol were selected as extraction and dispersive solvents respectively. Table 13 gives more applications of DLLME during the pre-concentration of heavy metals in various environmental matrices. From Table 13, the commonly used extraction solvents during DLLME are halogenated hydrocarbons. The halogenated hydrocarbons used are denser than water and are non-polar organic solvents. Thus, they are able to extract effectively the metal complexes from the aqueous phase. Methanol, from Table 13, is the commonly used disperser solvent. It can interact with water through hydrogen bonding and is miscible with the halogenated extraction solvents. Thus, it is suitable for use as the disperser solvent.

**Table 13**

Application of DLLME to pre-concentrate heavy metals

| Heavy metals | matrix | Chelating agent | Pre-concentration technique | Extraction solvent | Dispersion solvent | LOD | References |
|---|---|---|---|---|---|---|---|
| Cadmium | Water | Dithizone | NB-DLLME | Carbon tetrachloride | Methanol | 6.3 µg $L^{-1}$ | Rahmani et al., 2016 |
| Lead and cadmium | Human teeth | DDTP | DLLME | Carbon tetrachloride | Methanol | 5.6 ng $L^{-1}$ (Cd)<br>45 ng $L^{-1}$(Pb) | Zhang et al., 2011 |
| Cadmium and lead | Water and fruit juices | Na-DDTC | EA-DLLME | Toluene | Tartaric acid, sodium bicarbonate and NaCl tablet | 0.43 µg $L^{-1}$(Pb)<br>0.05 µg $L^{-1}$(Cd) | Sorouraddin et al., 2018 |
| Copper and zinc | Natural water | 8-hydroxyquinoline | Conventional DLLME | Chloroform | Methanol | 0.025 µg $L^{-1}$ (Zn)<br>0.0033 µg $L^{-1}$(Cu) | Ghorbani et al., 2013 |
| Arsenic and cadmium | Sugar | APDC and DDTC | Conventional DLLME | Carbon tetrachloride | Acetone | ng | Seeger et al., 2017 |
| Cadmium | Water | Dithizone | Conventional DLLME | Chloroform | Methanol | 8.5 µg $L^{-1}$ | Pérez-Outeiral et al., 2014 |

**Note**: NB- narrow bore; DDTP- *O*,*O*-diethyldithiophosphate; Na-DDTC- sodium diethyldithiocarbamate; EA- effervescence assisted; APDC- ammonium pyrrolidinedithiocarbamate; DDTC- diethyldithiocarbamate

### *2.8. Synergistic effect of heavy metals and pesticides*

The living organisms in nature are frequently exposed to a mixture of xenobiotics (heavy metals, pesticides, and toxic gases) simultaneously. The xenobiotic substances have been reported to cause toxicity in animals as well as in key organs of the humans also (Omiecinski et al., 2011; Oesch et al., 2014). Therefore, the combined interactions between xenobiotic substances as well as xenobiotic and animal systems are very important and can lead to more serious health problems (Oesch et al., 2014). It was observed that combined oral exposure of rats to pesticides and heavy metals have effects which are different to when the rats are exposed to these xenobiotic chemicals separately. For instance, simultaneous exposure of rats to the dimethoate pesticide and cadmium was observed to cause general body weight gain and relative increase in the weight of the liver (Institóris et al., 2002). Thus, combined exposure to pesticides and heavy metals may result in the amplification of the toxic effects of these xenobiotic chemicals.

# Chapter 3

# Research objectives

This chapter gives the broad and specific objectives for the research as well as a brief outline of the general approaches for the DLLME and QuEChERS techniques during the pre-concentration of heavy metals and pesticides respectively.

## 3.1. Broad objective

- To develop methods for the pre-concentration of pesticides and heavy metals in complex matrices

## 3.2. Specific objectives

- To examine the recent developments and applications of DLLME during the pre-concentration of pesticides in food samples (**Paper I**).

- To outline the recent applications of QuEChERS to pre-concentrate pesticides in food samples in a review paper (**Paper II**).

- To review the green pre-concentration techniques applicable to food samples (**Paper III**).

- To apply the DLLME technique to pre-concentrate heavy metals in roadside dust using dithizone as the chelating agent (**Paper IV**).

- To speciate chromium in fruits using DLLME for pre-concentration and 1,5-diphenylcarbazide as the chelating agent (**Paper V**).

- To pre-concentrate malathion in fruits using Z-sep$^+$ based QuEChERS technique (**Paper VI**).

## 3.3. General research approach: DLLME technique

The DLLME technique was used to pre-concentrate heavy metals in roadside dust and fruit samples. The general approach during DLLME is given in Fig. 5.

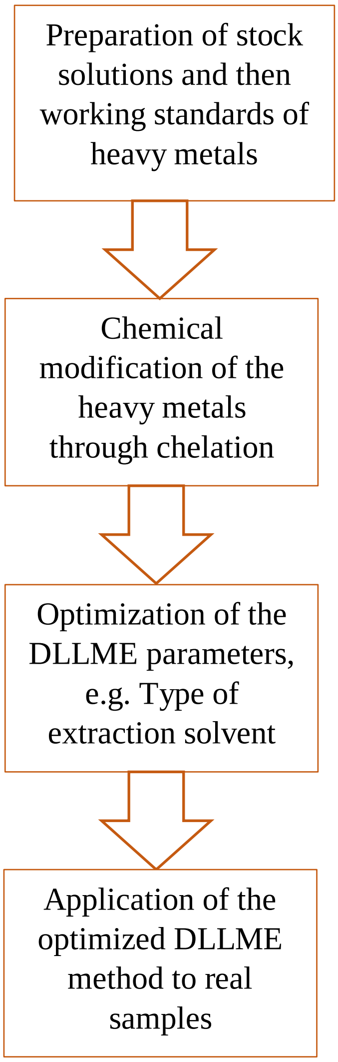

**Fig. 5.** Schematic diagram for the general research approach: DLLME

## 3.4. General research approach: QuEChERS technique

Analysis of pesticides was performed using the QuEChERS technique. Fig. 6 gives the general approach during the application of QuEChERS when analysing pesticides in various matrices.

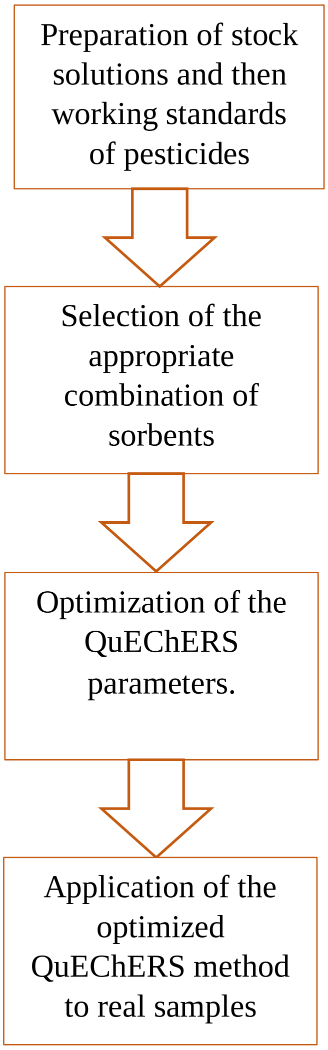

**Fig. 6.** Schematic diagram for the general research approach: QuEChERS

# Chapter 4

# List of publications

This chapter gives the publications that were done during the duration of MSc program.

## 4.1. Paper I

This paper "Recent developments and applications of dispersive liquid-liquid micro-extraction technique for the analysis of pesticides in food matrices" was submitted *to Journal of Food Control.* It describes the general principles of dispersive liquid-liquid micro-extraction as well as outlining its extensive application to analyse pesticides in food matrices

# Recent developments and applications of dispersive liquid-liquid micro-extraction technique for the analysis of pesticides in food matrices

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## Abstract

Recently, increasing interest on the use of dispersive liquid-liquid micro-extraction has been found in the field of pesticide analysis in food matrices. The major advantage, among others, of dispersive liquid-liquid micro-extraction (DLLME) is miniaturisation in which the acceptor-to-donor ratio is tremendously reduced compared to other sample preparation techniques such as liquid-liquid extraction and solid phase extraction. During DLLME, the dispersion of the extraction solvent presents a large surface area to volume ratio enabling faster extraction of the pesticides in the food samples. In the present work, the different complex matrices where the DLLME technique has been employed for the analysis of pesticides are reviewed. Analysis of solid samples in the reviewed papers was often done through offline coupling with limited usage of online coupling. In addition, the challenges of the DLLME techniques were reviewed.

*Keywords*

Sample pre-concentration, food matrices, optimization, dispersive liquid-liquid micro-extraction

## Contents

## 1. Introduction

Residual pesticides at trace levels are difficult to extract completely from food matrices due to the existence of complex components and interfering substances. Any interference in the sample treatment process may bring the uncertainty for determination of pesticide residues in food matrices (Amoli-Diva et al., 2017). Therefore, sample treatment is usually a critical step in the process of a comprehensive food analysis and is often the most work-intensive part of chemical analysis (Gure et al., 2015).

Many sample pre-concentration techniques have been reported during the trace analysis of residual pesticides in food matrices. These include sorbent-based techniques such as solid phase extraction (SPE) (Günter et al., 2016; Pelajić et al., 2016; L. Zhang et al., 2016), solid-phase micro-extraction (SPME) (Shamsipur et al., 2016; Souza et al., 2016; Souza-Silva et al., 2015; Wang et al., 2013; Zhang et al., 2013) and micro-solid phase extraction (μ-SPE) (Sajid, 2017; Sajid et al., 2016). Solvent-based pre-concentration techniques have also found widespread application during analysis of pesticides in food matrices. Such solvent-based techniques include hollow-fibre liquid-phase micro-extraction (HF-LPME) (Alsharif et al., 2017; Shi et al., 2014) and QuEChERS (Bernardi et al., 2016; Ferreira et al., 2015; Muhammad et al., 2017; Tette et al., 2016).

One of the solvent-based pre-concentration techniques that has generated enormous attention and interest among researchers during trace analysis of pesticides in food matrices is dispersive liquid-liquid micro-extraction (DLLME). The DLLME technique has been recently applied during trace analysis of pesticides in water and soil samples (Asghari et al., 2014; Cortada et al., 2014; Liang et al., 2017; Pastor-Belda et al., 2015; Toledo-Neira and Álvarez-Lueje, 2015). Recently, pesticides in food samples have also been pre-concentrated using the DLLME technique (Bresin et al., 2015; Chen et al., 2016; Farajzadeh et al., 2017; Mohammadi et al., 2013; Quigley et al., 2016; Wang et al., 2016; Zhang et al., 2014). Rezaee et al. (2006) introduced DLLME and is currently one of the high-performance pre-concentration technique during trace analysis of pesticides in food matrices.

The increasing use of pesticides in agriculture to promote growth of crops in a pest-free environment and meet the food demands of world, has resulted in the exposure of the consumers to food with a high content of residual pesticides. This has also prompted the rapid proliferation of researches that involve the use of DLLME technique during trace analysis of pesticides in food matrices. However, there are very few review papers on the application of the DLLME pre-concentration technique during analysis of pesticide residues in food matrices and this review seeks to close that research gap. In this paper, the recent developments and applications of various DLLME modes to pre-concentrate pesticides on food samples were reviewed as well as the challenges of DLLME configurations.

### *1.1.Basic principles of the conventional DLLME during pesticide analysis*

The conventional DLLME technique (Fig. 1), for trace analysis of pesticides in food matrices, is based on ternary solvent system including an aqueous food sample, a water immiscible solvent (extraction solvent) and a water miscible solvent (dispersion solvent) (Liang et al., 2017; Wang et al., 2016). The mixture of extraction solvent and dispersive solvent is injected rapidly into aqueous food sample solution. Then, a stable emulsion is formed containing fine droplets of extraction solvent dispersed in an aqueous food solution resulting in a large increase in contact area between the two phases (Chen et al., 2016; Farajzadeh et al., 2016). As a result, the pesticides are easily transferred into the extraction phase. After extraction, the emulsion is separated into two phases by centrifugation, which causes the extraction solvent, impregnated with pesticides, to sediment at the bottom of centrifugation tube (Zhang et al., 2014). If the extraction solvent is not compatible with the analytical instrument, the sedimented phase is dried followed by re-constitution before analysis.

High enrichment factor and clean-up efficiency, short extraction time and easy performance are the main advantages of the DLLME method (Kocúrová et al., 2012). However, there are still some drawbacks to conventional DLLME. Firstly, the extraction solvent must have low solubility in water, a density higher than that of the aqueous phase, and suitable chromatographic behaviour. Based on these requirements, halogenated solvents, which are toxic and environmentally hazardous such as chlorobenzene, carbon tetrachloride, and tetrachloroethylene, are often utilized in the conventional DLLME technique (Kocúrová et al., 2012). Secondly, conventional DLLME requires a third component that is miscible in both water and the extraction solvent as the dispersive solvent (Trujillo-Rodríguez et al., 2013). Toxic organic solvents, such as methanol, acetone, and acetonitrile, are often used as dispersion solvents. Finally, to facilitate the separation of the phases, a centrifugation step is necessary for conventional DLLME, and the extraction phase, which sediments at the bottom is removed with a micro-syringe before instrumental analysis (Trujillo-Rodríguez et al., 2013). Centrifugation, however, is a time-consuming process (Li et al., 2014). Therefore, modifications of the original DLLME process, for instance, by substituting toxic organic extraction solvents with eco-friendly solvents, eliminating or reducing the use of a dispersion solvent, and separating the phases rapidly without using the tedious centrifugation step, are welcome interventions.

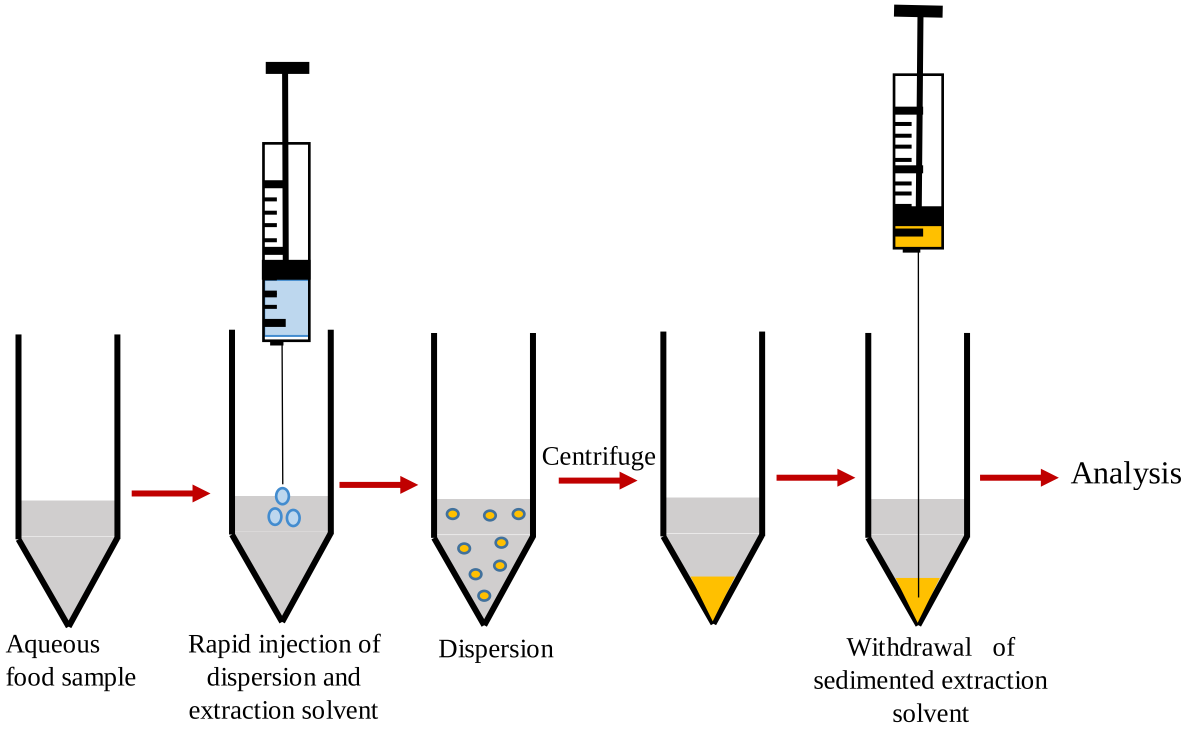

**Fig. 1:** Steps during analysis of pesticides from food matrices using conventional DLLME

### *1.2.Modifications of the original DLLME procedure*

### *1.2.1. Modified DLLME using ionic liquids*

Ionic liquids (ILs) are non-organic solvents that do not emit harmful vapours into the environment. They fulfil the requirements of “green solvents” (Trujillo-Rodríguez and Anderson, 2017). The ILs have emerged as very attractive alternatives to chlorinated organic solvents, usually employed as extraction solvents in conventional DLLME assays during trace analysis of pesticides in food matrices. This is due to their unique physico-chemical properties that depend on the nature and size of their cationic and anionic constituents (Wang et al., 2017). These valuable physico-chemical properties include negligible vapour pressure, high thermal stability, tunable viscosity and hydrophobicity (Yu et al., 2013). The DLLME mode that uses ionic liquids as extraction solvents is called ionic liquid dispersive liquid-liquid micro-extraction (IL-DLLME). Zhou et al., (2008) developed the first report on utilization of IL-DLLME and since then, it has been widely used during trace analysis of pesticides in food matrices (Asghari et al., 2014; Han et al., 2014; Liang et al., 2017; Trujillo-Rodríguez and Anderson, 2017; Wang et al., 2013; Wu et al., 2016; Yilmaz and Soylak, 2016; Yu et al., 2013).

IL-DLLME bears some resemblance to conventional DLLME during trace analysis of pesticides in food samples, but the extraction solvent is an IL. As in conventional DLLME the extraction solvent in IL-DLLME should have a higher density than water, have a high affinity to the pesticides and be immiscible in water (Trujillo-Rodríguez et al., 2013). The advantages of IL-DLLME during pre-concentration of pesticides are the environmentally benign extraction solvents, insolubility in water, low volatility and non-flammability of the ILs (Wang et al., 2017). ILs are, however, more costly than the organic solvents used in conventional DLLME during trace analysis of pesticides in food samples.

The commonly used IL-DLLME configurations include temperature-controlled (TC-IL-DLLME) (Rajabi et al., 2014) and ultrasound-assisted (US-IL-DLLME) (Quigley et al., 2016). TC-IL-DLLME utilizes an increase in temperature to fully dissolve the IL in the food sample solution followed by cooling and centrifugation to recover the IL phase. For instance, Zhang et al., (2011) injected a blend of acetonitrile and 1-octyl-3-methylimidazolium hexafluorophosphate ([$C_8$MIM][$PF_6$]) to a heated water sample and a homogeneous solution was formed. After a suitable time the sample was immersed into an ice bath inducing a cloudy solution. The sample was thereafter centrifuged followed by separation of the pesticide-impregnated extraction solvent and good recoveries were obtained on its analysis. On the other hand, in US-IL-DLLME, ultrasound is applied to disperse the IL phase, instead of the initial heating step, and then a cooling process is needed to obtain the cloudy solution (Zhang et al., 2011).

Some researchers pre-concentrated pesticides in food matrices by applying IL-DLLME. For instance, Wang et al. (2015) successfully used matrix solid-phase dispersion coupled with IL-DLLME for the pre-concentration of triazine herbicides in oilseeds. Gure et al. (2015), on the other hand, used vortex-assisted IL-DLLME during trace analysis of sulfoylurea herbicides in wine samples.

### *1.2.2. Modified DLLME with low density extraction solvents*

Recently, low-density solvents have been used as extraction solvents in DLLME in order to increase the range of extraction solvents compatible with the method. This mode is called low-density solvent-based DLLME (LDS-DLLME) (Kocúrová et al., 2012). The low-density extraction solvent, such as toluene, xylene, hexane, and heptane, floats on the surface of the aqueous phase after phase separation during LDS-DLLME. The solvent is then recovered using a fine needle, and this process is simplified when specialist glassware or other vessels are used to trap the floating solvent in a narrow restriction in the vessel (Quigley et al., 2016). An advantage of LDS-DLLME is that, after centrifugation, any matrix components will be sedimented at the bottom of the extraction vessel while the extraction solvent will be floating on top. This will lead to a cleaner extract and potentially cleaner chromatography (Loh et al., 2017). A typical example of an LDS-DLLME mode is DLLME based on the solidification of floating organic drop (DLLME-SFO) (Fig.2).

The DLLME-SFO pre-concentration technique of pesticides in food samples involves the use of an appropriate extraction solvent with lower density than water, low toxicity and a melting point near room temperature. The solvent is mixed with a water-miscible dispersive solvent and is then rapidly injected into an aqueous sample by syringe (Hu et al., 2016; Vera-Avila et al., 2013; Wu et al., 2012). A cloudy solution containing fine droplets of extraction solvent, dispersed entirely in the aqueous food sample phase, is formed and the mixture is centrifuged. After centrifugation, the extractant droplet floating on top of the centrifuge tube, can be easily solidified on an ice bath and then taken out from the aqueous food sample. It is then melted immediately at room temperature and the melted organic solvent is then analysed to determine pesticides in the food sample (Wang et al., 2017). The advantages of the method are rapid speed, simple operation, small consumption of organic solvent, simple extraction apparatus and high enrichment factor (Hou et al., 2014). This modified DLLME technique increases the scope of organic solvents that can be used during chemical analysis involving DLLME.

The DLLME-SFO technique have been used by researchers during the pre-concentration of pesticides in food samples. For instance, Wang et al. 2017) used QuEChERS coupled with DLLME-SFO during trace analysis of organochlorine pesticides in fish samples. Hou et al. (2014), on the other hand, successfully pre-concentrated pyrethroid pesticides in tea samples using ultrasound-assisted DLLME-SFO.

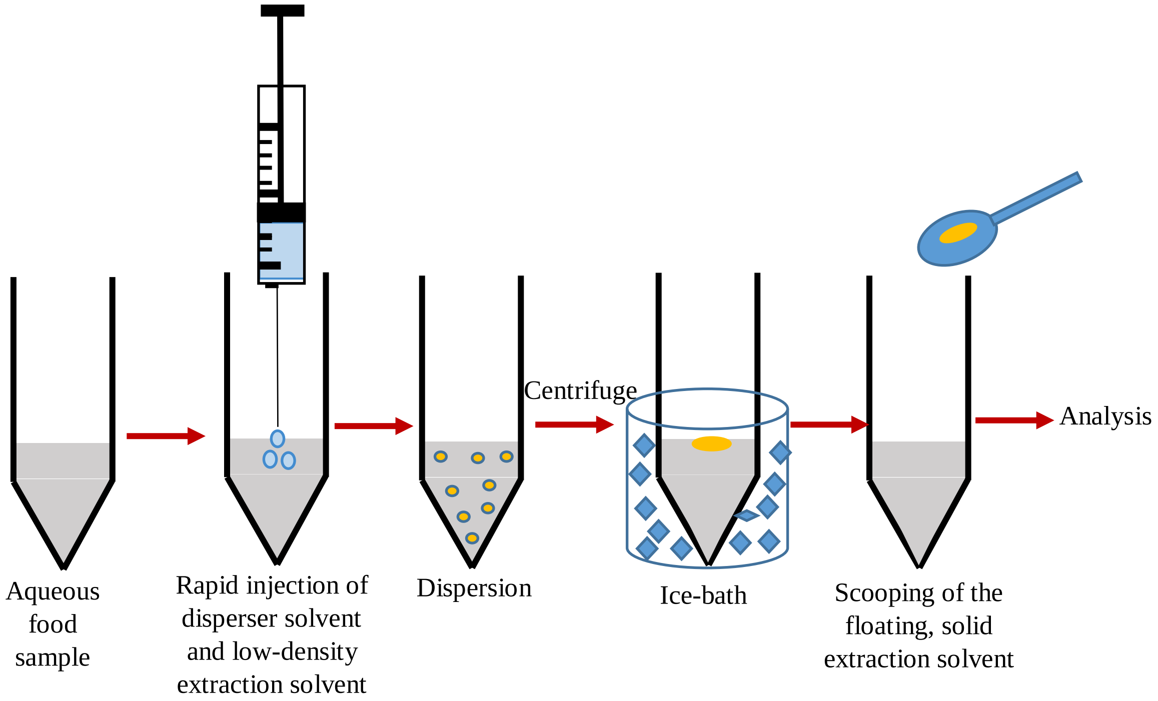

**Fig. 2.** Steps during analysis of pesticides from food matrices using DLLME-SFO

### *1.2.3. Modified DLLME without dispersive solvent*

In traditional DLLME, a dispersion solvent is required to dissipate the extraction solvent in an aqueous solution. However, the dispersion solvents may simultaneously increase the solubility of the pesticides in aqueous samples, thereby decreasing the recovery (Rajabi et al., 2014). Thus, some modifications of the original DLLME were developed to eliminate the need for a dispersion solvent or to reduce the amount of dispersion solvent used. Such modified DLLME procedures include effervescence- assisted dispersive liquid-liquid micro-extraction (EA-DLLME) (Lasarte et al., 2014), air-assisted dispersive liquid-liquid micro-extraction (AA-DLLME) (Farajzadeh and Mogaddam, 2012), ultrasound- assisted dispersive liquid-liquid micro-extraction (UA-DLLME) (Pirsaheb et al., 2013) and vortex-assisted dispersive liquid-liquid micro-extraction (VA-DLLME) (Seebunrueng et al., 2014).

EA-DLLME involves the in situ generation of bubbles of a gas, for instance $CO_2$, to assist the dispersion of the extraction solvent, removing the need for the dispersive solvent. The $CO_2$ is produced by adding a mixture of sodium carbonate and a weak acid (citric acid), usually in the form of a pressed tablet. Lasarte et al. (2014) used EA-DLLME to pre-concentrate herbicides in water samples. The technique used a mixture of 1-octanol (extraction solvent) and bare $Fe_3O_4$ magnetic nanoparticles (for phase separation). This mixture was injected into aqueous samples that were previously impregnated with a carbonate and effervescence of $CO_2$ bubbles that ensued caused dispersion of the extraction solvent. Hu et al. (2016) also employed EA-DLLME, with solidification of ionic liquid (IL) (extraction solvent), to determine benzoylurea insecticides in water samples. In this technique, the extraction solvent was also successfully dispersed using effervescence, eliminating the use of the dispersion solvent.

AA-DLLME also removes the need for a dispersive solvent by repeatedly aspirating the aqueous phase and the extraction solvent into a glass syringe until a cloudy solution is formed. To reduce the use of devices in DLLME， Farajzadeh and Mogaddam (2012) developed the air-assisted method to pre-concentrate phthalate esters in aqueous samples, in which the homogenous emulsion was obtained just by repeatedly aspirating and injecting the extraction mixture with a syringe. With only 15 µL of extraction solvent consumed and no use of any dispersion solvent, complete dispersion was achieved, and no other devices such as vortex, up-and-down-shaker and magnetic stirrer were required. Guo et al. (2016) performed similar work, however, the operation of aspiration and injection of sample mixture was performed by the auto-sampler of the instrument.

Vortex–assisted DLLME (VA-DLLME) (Fig.3) is yet another modification of the original DLLME, where vortex agitation is used to facilitate dispersion. In the vortex-assisted method, the dispersion of the extraction solvent is aided by vortex-mixing resulting in the use of a reduced volume of the dispersion solvent or no dispersion solvent at all. Seebunrueng et al. (2014) used vortex method to enhance the dispersion efficiency of low-density extraction solvent during pre-concentration of organophosphorus pesticides in water samples. Gure et al. (2015) used VA-IL-DLLME as a pre-concentration technique during trace analysis of sulfonylurea herbicides in wine samples and a well-dispersed emulsion was obtained in only 30 s due to vortex agitation.

Ultra-sound energy can be used during the DLLME technique to promote dispersion and to accelerate the formation of fine droplets of the extraction solvent in the aqueous food sample (Hou et al., 2014). The use of ultra-sound therefore eliminates the need for a dispersion solvent during DLLME procedures. It also reduces the amount required in situations where the dispersion solvent is used. Some researchers have used US-DLLME to pre-concentrate pesticides in food matrices. For instances, Pirsaheb et al. (2013) successfully used US-DLLME-SFO to pre-concentrate pesticides in summer crops (cucumber, watermelon, melon, and ribbed melon). Hou et al. (2014) used the same technique during the pre-concentration of pesticides in tea samples. Gao et al. (2012), on the other hand, used US-IL-DLLME during the trace analysis of sulphonamides in milk samples.

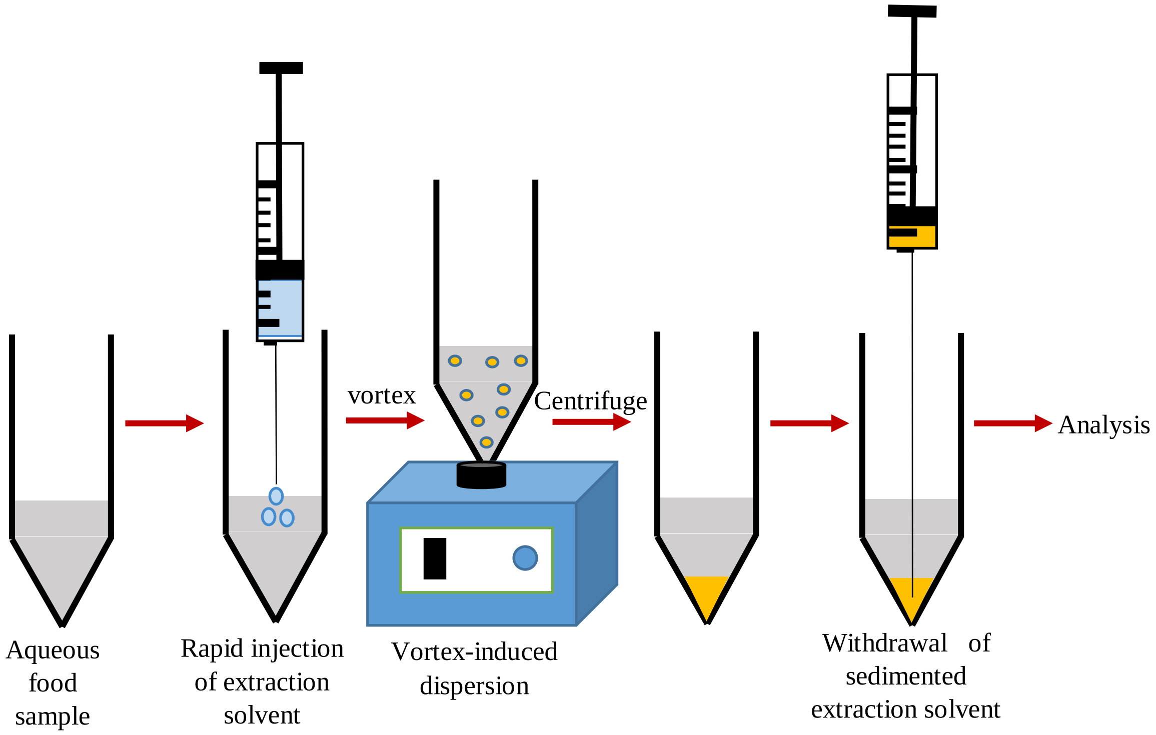

**Fig. 3.** Steps during analysis of pesticides in food matrices using VA-DLLME

### *1.2.4. Modified DLLME which by-passes the centrifugation step*

Modifications of the conventional DLLME technique, so as to by-pass the centrifugation step during pre-concentration of pesticides in food samples, have also been developed. The centrifugation step is the most time-consuming step during the original DLLME procedure (Li et al., 2014). Thus, its absence will cause a reduction in the extraction time as well as causing simplification of the DLLME process since it reduces the number of devices used during the extraction process. Such modifications involve the use of hydrophobic magnetic nanoparticles (Seebunrueng et al., 2014), the use of de-emulsification solvent (Caldas et al., 2016) and causing phase separation by cooling (Farajzadeh et al., 2014).

Phase separation, during the DLLME procedure, can be effected by adding hydrophobic magnetic nanoparticles to the sample and then subjecting it to a magnetic field. The hydrophobic magnetic nanoparticles interact with the extraction phase and can be sedimented by applying a magnet; this eliminates the centrifugation step (Seebunrueng et al., 2014). The de-emulsification solvent, on the other hand, causes phase separation of the emulsion on its addition (Behbahani et al., 2014; Caldas et al., 2016)

The solubility of the extraction solvent during DLLME is temperature-dependent. Thus, temperature adjustment may be used to cause phase separation and by-pass the centrifugation step (Farajzadeh et al., 2014; Zhao et al., 2011). The procedure used by Farajzadeh et al. (2014) had two stages: elevating temperature stage and the cooling stage. The tube containing sample solution was incubated in water bath at 75 °C for 4 min, and then the mixture of extraction solvent and dispersion solvent was rapidly injected. The extraction solvent could be dispersed completely by the aid of the elevated temperature and trace amount of dispersive solvent. The tube was then cooled by water flow for 3 min. As solubility of extraction solvent decreased with the decrease of temperature, fine droplets of extraction solvent were formed when the tube was cooled with tap water. To avoid considerable loss of organic solvents during this DLLME procedure, Farajzadeh et al. (2014) used organic solvents with high boiling points.

### *1.3.Important calculations in DLLME*

When performing the DLLME procedure, during pre-concentration of pesticides in food matrices, two important calculations are frequently taken into consideration. These are the calculations of enrichment factor (EF) and extraction recovery (ER). EF, during pre-concentration of pesticides in food matrices, is defined as the ratio of the concentration of the pesticides in the sedimented phase to the concentration of the pesticides in the food sample (Quigley et al., 2016; Rezaee et al., 2006). This ratio is expressed mathematically as follows:

$$EF = \frac{C_{sed}}{C_0} \quad \ldots\ldots (1)$$

where $C_{sed}$ is analyte concentration in the sedimented phase and $C_0$ is the initial analyte concentration in the sample.

During the pre-concentration of pesticides in food matrices, extraction recovery is the ratio of number of moles of pesticides in the sedimented phase to the number of moles of pesticides in the food sample:

$$ER = \frac{n_{sed}}{n_0} \text{ x } 100 = \frac{C_{sed}\ x\ V_{sed}}{C_0\ x\ V_0} \text{ x } 100 \quad \ldots\ldots (2)$$

where, $n_{sed}$ is the amount of the pesticides in the sedimented phase, $n_0$ is the initial pesticide amount in the sample, $V_{sed}$ is the volume of the sedimented phase and $V_0$ is the initial volume of the food sample. The equation for the calculation of ER is also written as:

$$ER = \frac{n_{sed}}{n_0} \text{ x EF x } 100 \ldots\ldots (3)$$

*1.4.Parameters that affect DLLME extraction efficiency of pesticides from food samples*

The extraction efficiency during the DLLME procedure depends on several factors and these include type and volume of the extraction solvent, type and volume of the disperser solvent, pH of the sample, effect of salt, extraction time, centrifugation time, and sample volume (Bordagaray et al., 2016; Farajzadeh et al., 2017; Wang et al., 2017; Yu et al., 2013). These parameters have to be optimized during trace analysis of pesticides in food matrices in order to achieve high extraction efficiency.

The success of the DLLME technique during trace analysis of pesticides in food matrices hinges on the ability of the researcher to select the appropriate extraction solvent. A suitable extraction solvent must meet the following requirements: (i) it must be immiscible with water and should form tiny droplets in it, (ii) it must have a higher affinity for the analyte and (iii) it must have a different density than water (Daneshfar and Babaee, 2016). The extraction ability for the solvent is dependent on its volume. Therefore, the volume of the extraction solvent should be carefully optimised during the DLLME procedure when pre-concentrating pesticides in food samples. In addition, its compatibility with the desired analytical instrument should be taken into consideration.

During the DLLME technique, a dispersion solvent is used. The extraction efficiency during pre-concentration of pesticides in food matrices is influenced greatly by the type and volume of the dispersion solvent (Yilmaz and Soylak, 2016). Generally, disperser solvents should be miscible with both the extraction solvent and the aqueous food sample solution (Ahmad et al., 2015). The function of the disperser solvent is to facilitate the partitioning of the extraction solvent uniformly in the aqueous food sample during pre-concentration of pesticides, in order to achieve good extraction efficiency (Guo et al., 2016). Thus, the ratio of volume of extracting solvent to disperser solvent should be carefully controlled. The volume of the sedimented phase is, significantly influenced by disperser type and volume. The dispersion solvents that are in common use during the DLLME procedures when pre-concentrating pesticides in food matrices are acetone, methanol and acetonitrile (Farajzadeh et al., 2017).

Centrifugation time has a great bearing on the extraction efficiency and should be optimised during trace analysis of pesticides in food samples using DLLME. The process of centrifugation is important for phase separation and is the most time-consuming stage during the DLLME procedure (Li et al., 2014). The injection of the mixture of the extraction and dispersive solvent into the sample solution during DLLME results in the formation of an emulsion. Because the formed emulsion is thermodynamically unfavourable, it can be terminated simply by centrifugation (Toledo-Neira and Álvarez-Lueje, 2015). It usually takes between 5-10 min. A long centrifugation time causes the sedimented phase to dissolve thereby having a negative impact on the extraction efficiency.

Selection of the appropriate salt concentration is of great importance in order to obtain satisfactory extraction performance of the pesticides from food matrices. The addition of salt to aqueous solutions generally causes decrease in the solubility of the organic compounds in water (Ren and Xia, 2016). Therefore, addition of salt has been widely used to enhance the extraction recovery of pesticides from food samples. When salt is added to the solution, the water molecules can form hydration spheres around the ionic salt molecules. These hydration spheres reduce the amount of water available to dissolve pesticide molecules, driving additional pesticides into the organic phase.

Mass transfer is a time dependent process and one of the salient factors in the DLLME procedure (Zhao et al., 2011) and has a direct effect on extraction time. In DLLME, the extraction time is defined as the time interval from injection of the mixture of extraction and dispersive solvents to starting of centrifuge (Ren and Xia, 2016). The formation of the cloudy emulsion, after the injection of a mixture of extraction and dispersive solvents, increases the migration of the pesticides from the aqueous food phase into the extraction phase resulting in the attainment of the equilibrium state. Thus, the optimum extraction time is dependent on the rate of equilibration. If the equilibrium state is reached quickly then the extraction time will be short. Thus, the extraction time should be optimised during trace analysis of pesticides in food matrices in order to increase extraction efficiency.

Optimization of the pH of the aqueous food sample solution is very important during trace analysis of pesticides using the DLLME procedure because it determines the existing state of pesticides, as well as their extraction efficiency (Rezaee et al., 2006). Thus, the partitioning of a pesticide from an aqueous food phase into a hydrophobic organic solvent is greater for a neutral molecule than for an ionized species. An appropriate pH should, therefore be selected so that the pesticides are extracted from the aqueous phase into the extraction solvent in their neutral form.

## 2. Analytical application of DLLME for pesticide analysis in different matrices

### *2.1. Analysis of pesticides in fruits and fruit juices using DLLME*

The DLLME technique has been used during the analysis of pesticide residues in fruit and fruit juices (Farajzadeh et al., 2016; Ghoraba et al., 2018; Jafari et al., 2016; Pastor-Belda et al., 2017; Pirsaheb et al., 2015; Shamsipur et al., 2016) (Table 1) which resulted generally in efficient extraction, high enrichment factors and low detection limits. The general procedure, used by some researchers (Farajzadeh et al., 2016; Shamsipur et al., 2016) involves the extraction of the pesticides from the complex fruit matrices followed by the application of different modes of the DLLME technique.

Farajzadeh et al. (2016), for instance, developed an extraction method based on counter current salting-out homogenous liquid–liquid extraction (CCSHLLE) followed by dispersive liquid–liquid micro-extraction for the pre-concentration of widely used pesticides in fruit juice samples prior to their analysis by gas chromatography–flame ionization detection (GC–FID). In this method, initially, sodium chloride as a separation reagent was filled into a small column and a mixture of fruit juice and acetonitrile was passed through the column. This resulted in the formation of fine droplets of acetonitrile due to salting-out effect. The produced droplets collected at the top of the mixture as a separated layer and was removed with a syringe followed by the addition of 1,1,2,2-tetrachloroethane (extraction solvent ). The extraction recoveries and enrichment factors ranged from 87% to 96% and 544 to 600, respectively. Limits of detection where between 2 and 12 mg $L^{-1}$.

Some researchers (Jafari et al., 2016; Melo et al., 2013; Shamsipur et al., 2016) through combinations of DLLME with other pre-concentration techniques achieved great enhancement of the quality of chromatograms and detection limits during analysis of pesticides in fruit matrices. DLLME and solid–phase micro-extraction (SPME), for instance, were successfully combined by Jafari et al. (2016) for the analysis of organophosphate pesticides (diazinon and parathion) in fruit juices. For the DLLME step, 1,1,2,2- tetrachloroethane and acetonitrile were used as extraction and disperser solvents, respectively. Halloysite nanotubes–titanium dioxide was used as the fiber coating in the SPME step. The limits of quantification and detection were obtained to be 0.015 and 0.005 g $L^{-1}$ for diazinon, and 0.020 and 0.007 g $L^{-1}$ for parathion. A good linearity range ($r^2 = 0.993$) was obtained in the range of 0.015–3.000 and 0.020–3.000 g

$L^{-1}$ for diazinon and parathion, respectively. This method showed high sensitivity with good recovery values (between 87 and 99%) for the extraction of pesticides in the fruit juice. Another effective offline coupling method for the extraction and determination of traces of multi-residue pesticides in fruits was developed by Shamsipur et al. (2016) using a combination of solid-phase extraction (SPE) and DLLME (SPE-DLLME). During the SPE-DLLME method, variables affecting the performance of both extraction steps such as type and volume of elution and extraction solvents, breakthrough volume, salt addition and extraction time were optimised. The SPE-DLLME method resulted in good linearities ($R^2 > 0.9915$), low limits of detection (LODs) in the range of 0.5–1.0 ng $kg^{-1}$ at S/N = 3, and precision of RSD% of 611.8. Under optimal conditions, the pre-concentration factors were obtained in the range of 2362–10,593 for 100 mL sample solutions. Thus, analysis of pesticide residues in fruit matrices using a combination of two pre-concentration techniques provides higher extraction efficiency and larger pre-concentration factor.

**Table 1**

Application of the DLLME technique to fruit samples

| Pesticides | Matrix | DLLME mode | Extraction solvent | Dispersive solvent | Enrichment factor | LOD | Ref. |
|---|---|---|---|---|---|---|---|
| Pyrethroids | Apple, pear, grape, peach, orange, lemon, kiwi and mango juices | Conventional DLLME | Tetrachloromethane | Acetone | 64-110 | 0.2- 2 µg $L^{-1}$ | Zhang et al., 2014 |
| Pyrethroids | Apple, pear, grape, peach, orange, lemon, kiwi and mango juices | QuEChERS-DLLME | Tetrachloromethane | 1mL of QuEChERS extract | 16 - 26 | 0.2- 2 µg $L^{-1}$ | Zhang et al., 2014 |
| Mixture of pesticides | Apple, sour cherry, peach and grape juices | CCSHLIE-DLLME | 1,1,2,2-tetrachloroethane | Acetonitrile | 544 - 600 | 2.0 – 12 µg $L^{-1}$ | Farajzadeh et al., 2016 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Organophosphates | Apple and grape juices | SPME-DLLME | 1,1,2,2 tetrachloroethane | Acetonitrile | 88-95 | 0.01-0.03 µg L$^{-1}$ | Jafari et al., 2016 |
| Multiclass pesticides | Orange juice | SPE-DLLME | Chlorobenzene | SPE extract | 2362-10593 | 0.5-1.0 ng kg$^{-1}$ | Shamsipur et al., 2016 |
| Organophosphates | Fruits and vegetables | Conventional DLLME | Tetrachloromethane | Acetonitrile | ng | 0.02- 0.23 ng L$^{-1}$ | Ho et al., 2013 |
| Multi-pesticides | Tomatoes | QuEChERS-DLLME | Trichloromethane | QuEChERS acetonitrile extract | 86 - 116 | 0.01-1.00 mg Kg$^{-1}$ | Melo et al., 2013 |
| Multi-pesticides | Celery | QuEChERS-DLLME | Trichloromethane | QuEChERS acetonitrile extract | 70.8 – 93.2 | 2.4-14.2 µg Kg$^{-1}$ | Wang et al., 2016 |
| Imidacloprid and diazinon | Apple and pear | SA-DLLME-SFO | 1-undecanol | Acetone | 58 - 67 | 1-2 µg Kg$^{-1}$ | Pirsaheb et al., 2015 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Triazole fungicides | Pear, apple and grapefruit | QuEChERS-IL-DLLME | 1-hexyl-3-methylimidazolium hexafluorophosphate | QuEChERS acetonitrile extract | 63.8 – 119.1 | 3.4-26.8 µg $Kg^{-1}$ | Zhang et al., 2016 |
| Triazole fungicides | Fruit juice | VA-IL-DLLME | 1-hexyl-3-methylimidazolium hexafluorophosphate | Acetonitrile | 71- 104.5 | 0.4- 6.7 µg $L^{-1}$ | Zhang et al., 2016 |
| Fungicides | Pear, watermelon, peach and grape | BS-DLLME | Mixture of chloroform and undecanol | Acetonitrile | 12 - 60 | ng | Pastor-Belda et al., 2017 |
| Bendiocarb (carbamate) and azinphos-ethyl (orgaophosphate) | Tomato and orange juice | UA-DLLME-IMS | Chloroform | ng | 6.9 – 67.6 | 1.04 ng $mL^{-1}$ (azinphos-ethyl)<br>1.31 ng $mL^{-1}$ (bendiocarb | Ghoraba et al., 2018 |

Note: ng – not given; SA- sonication assisted; BS- binary extraction solvents; IMS- Ion-mobility spectroscopy; UA- ultrasound assisted

### *2.2. Analysis of pesticides in beverages using DLLME*

Various DLLME modes have found application in the analysis of many different beverages such as wine, tea and coffee (Deng et al., 2019; Hrouzková et al., 2017; Lu et al., 2018; Timofeeva et al., 2017; Wang et al., 2017) (Table 2). Good recoveries, low limits of quantification and low limits of detection were obtained most of the time and this is a good indication for the suitability and versatility of the DLLME approach when analysing pesticides in beverage samples.

Analysis of pesticides in wine beverages have gained the attention of many researchers in recent years (Bresin et al., 2015; Chen et al., 2016; Chu et al., 2015; Gure et al., 2015; Hrouzková et al., 2017; Lu et al., 2018). Gure et al. (2015) developed a vortex-assisted ionic liquid dispersive liquid–liquid micro-extraction (VA-IL-DLLME) for the determination of sulfonylurea herbicides in wine samples. The ionic liquid (IL) 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$) was used as extraction solvent and was dispersed using methanol into the wine solution, assisted by a vortex mixer. Various parameters influencing the extraction efficiency, such as type and amount of IL, type and volume of disperser solvent, sample pH, salting-out effect, vortex and centrifugation time were studied and optimised. Under the optimum conditions, the limits of detection and quantification of the proposed method were in the ranges of 3.2–6.6 and 10.8–22.0 $\mu g\ kg^{-1}$, respectively. The proposed method was successfully applied to different wine samples and satisfactory recoveries were obtained. On the other hand, Chu et al. (2015) developed an up-and-down-shaker-assisted dispersive liquid–liquid micro-extraction (UDSA–DLLME) for the determination of fungicides in wine samples. The developed method used 1-octanol as an extraction solvent without the need for dispersive solvents. Chen et al. (2016) also successfully used the traditional DLLME technique for the determination of pesticides in wine samples.

Some researchers used the DLLME technique for the analysis of pesticides in tea and coffee samples (Bresin et al., 2015; Deng et al., 2019; Hou et al., 2014; Lu et al., 2018; Wang et al., 2017; Zhang and Xu, 2014). For instance, Hou et al. (2014) developed an ultrasound-assisted dispersive liquid–liquid micro-extraction based on solidification of floating organic droplet method (UA-DLLME-SFO) combined with gas chromatography for the determination of pyrethroid pesticides in tea samples. After ultrasound and centrifugation, 1-dodecanol and ethanol were used as the extraction and dispersive solvent, respectively. A series of parameters, including extraction solvent and volume, dispersive solvent and volume, extraction time, pH, and ultrasonic time influencing the micro-extraction efficiency were optimised. Under the optimal conditions, the enrichment factors were from 292 to 883 for the pyrethroid pesticides.

The clean-up process during analysis of pesticides in beverage samples by DLLME can be improved greatly by combining it with other pre-treatment techniques. For example, Bresin et al. (2015) minimized interference during analysis of coffee samples through the combination of QueChERS and DLLME resulting in good enrichment factors. Li et al. (2017), to analyse multiclass pesticides in tea samples, used a similar coupling technique. Zhang and Xu (2014), on the other hand, combined dispersive solid phase extraction (DSPE) with DLLME method for the determination of triazole fungicide residues in tea samples. DSPE with $C_{18}$, primary secondary amine, and florisil as sorbents was used to clean up and minimize matrix interference from tea samples. This was then followed by the enrichment of target pesticides using the DLLME procedure and detection with liquid chromatography–tandem mass spectrometry (LCMS/ MS). Good linearity and low limits of detection were achieved using this technique for the analysis of pesticides in tea beverages.

**Table 2**

Application of the DLLME technique to beverage samples

| Analytes | Matrix | DLLME mode | Extraction solvent | Dispersion solvent | Enrichment factor | LOD | Ref. |
|---|---|---|---|---|---|---|---|
| sulfonylurea herbicides | wine | VA-IL-DLLME | 1-hexyl-3-methylimidazolium Hexafluorophosphate | methanol | ng | 3.2–6.6 µg kg$^{-1}$ | Gure et al., 2015 |
| fungicides | wine | UDSA–DLLME) | 1-octanol | ng | ng | 0.007–0.025 µg L$^{-1}$ | Chu et al., 2015 |
| Multi-pesticides | wine | Conventional DLLME | chloroform | acetonitrile | 73.8 -121.7 | 0.025-0.88 µg L$^{-1}$ | Chen et al., 2016 |
| pyrethroid pesticides | Tea | UA-DLLME-SFO | 1-dodecanol | Ethanol | 292 - 883 | 0.08- 0.2 $\mu$g kg$^{-1}$ | Hou et al., 2014 |
| organochlorine | coffee | QueChERS-DLLME | carbon disulfide | Acetonitrile QuEChERS extract | 17.6-98.6 | ng | Bresin et al., 2015 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| organophosphates | White and red wine | EVA-DLLME | Hexanol | ng<br>(Dichloromethane used as volatile solvent | 92- 103 | $3\text{x}10^{-10}$ – $3\text{x}10^{-7}$ g $L^{-1}$ | Timofeeva et al., 2017 |
| Multiclass pesticides | Green tea, oolong tea, black tea and puer tea | QuEChERS-DLLME | Tetrachloromethane | QuEChERS extract | 70-120 | ng | Li et al., 2017 |
| Pyrethroids | Green tea, oolong tea and black tea | DES-based DLLME | Hydrophobic DESs | Acetone | 85-109.4 | 0.06 – 0.17 ng $mL^{-1}$ | Deng et al., 2019 |
| Triazole fungicides | Different types of green tea | DSPE-DLLME | Chlorobenzene | DSPE acetonitrile extract | 91-118 | ng | Zhang and Xu, 2014 |
| Multiclass pesticides | Plum brandy with ethanol content of 40% | VA-DLLME | tetrachloroethane | ng | 70-120 | 0.02-1.4 µg $L^{-1}$ | Hrouzková et al., 2017 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Benzoylurea insecticides | Green tea, oolong tea, black tea, | In-syringe SIL-DLLME | Hydrophobic ionic liquid $[N_{8881}][PF_6]$ | ng | 85.93- 90.52 | 0.29-0.59 $\mu g\ L^{-1}$ | Wang et al., 2017 |
| Pyrethroids | Two types of tea | MSPE-DLLME-SFO | 1-dodecanol | MSPE methanol extract | 78.3-103.6 | 0.008-0.015 $ng\ mL^{-1}$ | Lu et al., 2018 |

Note: ng- not given; DES- deep eutectic solvent; EVA- evaporation- assisted; UA- ultrasound-assisted; SIL- solidification of ionic liquid

*2.3. Analysis of pesticides in animal products using DLLME*

Animal products, such as honey and fish, have also been analysed to determine the presence of trace amounts of pesticides using the DLLME technique (Asati et al., 2018; Mousavi et al., 2016; Petrarca et al., 2017; Wang et al., 2017) (Table 3). The application of the various modes of DLLME on animal products is generally characterised by good linearity of calibration curves, low limits of quantification as well as low limits of detection (Jovanov et al., 2013; Zacharis et al., 2012). This, therefore, points to the suitability and versatility of the DLLME technique for analysis of animal products.

The determination of pesticides in animal products is a formidable challenge mainly because of small quantities of the pesticides and large quantities of interfering substances which may adversely affect the results of the analysis (Shirani et al., 2016; Wang et al., 2017). Such interfering substances include lipids and proteins. These interfering substances must be eliminated since they can act like surfactants and disrupt the interfacial tension at the droplet surface, hindering phase separation during the DLLME technique (Quigley et al., 2016). Researchers, in recent years, circumvented this problem during analysis of animal products through combinations of DLLME with other extraction techniques (Shirani et al., 2016; Wang et al., 2017). Combination of two extraction techniques helps to obtain low detection limits, acceptable linear range and low RSD% (Shirani et al., 2016) as well as causing massive reduction of the matrix effect and formation of high quality chromatograms.

Shirani et al. (2016) combined solid-phase extraction with dispersive liquid–liquid micro-extraction for the simultaneous determination of trace amounts of deltamethrin and permethrin in honey by gas chromatography–mass spectrometry. These pesticides were initially extracted from polluted honey samples by ultrasound-assisted liquid–liquid extraction followed by solid-phase extraction (SPE). Then, the pesticides were eluted from the SPE cartridge. The elution solvent containing trace amounts of the pesticides was used in the DLLME step as disperser solvent for further purification and pre-concentration of deltamethrin and permethrin. Under the optimal conditions, good linearity in the range of 0.2–800 ng $g^{-1}$ with the coefficient of determination ($r^2$) of 0.9986 and 0.9990, low limits of detection (LODs) of 0.02 and 0.04 ng $g^{-1}$ and relative standard deviations (RSD%) of 1.8 and 2 % were obtained for permethrin and deltamethrin, respectively. Wang et al. (2017), on the other hand, coupled QuEChERS and DLLME-SFO for the determination of organochlorine pesticides in fish tissue through gas chromatography with electron capture detection. QuEChERS, using acetonitrile as the extraction solvent, was initially used to extract the pesticides in fish tissue. The QuEChERS extract was then pre-concentrated through DLLME–SFO. Dispersive solid phase extraction (d-SPE) during QuEChERS was not a separate step but integrated into DLLME-SFO procedure. Using the optimised method, the limits of quantification and the limits of detection for the organochlorines ranged from $1.94\times10^{-3}$ to $4.93\times10^{-3}$ mg kg$^{-1}$ and $6.50\times10^{-4}$ to $1.58\times10^{-3}$ mg kg$^{-1}$, respectively. The organochlorine pesticides in fish tissue were successfully determined Recently, there is an increase in the number of DLLME-based researches, which do not include the dispersion solvent when analysing pesticides in animal products (Asati et al., 2018; Petrarca et al., 2017; Wu et al., 2017). This can be attributed to the conscious attempt by researchers to improve the greenness of researches involving DLLME. For instance, Asati et al. (2018) successfully determined organochlorine pesticides in fish liver using US-DLLME-SFO without the use of a dispersion solvent. The DLLME technique can also be made to be more environmentally friendly through the replacement of the halogenated extraction solvents with eco-friendly solvents during analysis of pesticides in animal products. Wu et al. (2017), for instance, used ionic liquid-based $TiO_2$ nanofluids (extraction solvent) during the determination of acaricide pesticides in honey and tea samples by EA-DLLME. Deng et al. (2019), on the other hand, used a deep eutectic solvent as an extraction solvent during the determination of pyrethroids in tea beverages and fruit juices by DLLME.

**Table 3**

Application of the DLLME technique to animal products

| Pesticides | Matrix | DLLME mode | Extraction solvent | Dispersion solvent | Enrichment factor | LOD | Ref. |
|---|---|---|---|---|---|---|---|
| Organochlorines | Honey | Conventional DLLME | dibromoethane | Acetonitrile | 171-199 | 0.004-0.07 ng $g^{-1}$ | Mousavi et al., 2016 |
| Pyrethroids | Baby food | UA-DLLME | Chloroform | ng | 75-120 | 0.2-1.7 µg $Kg^{-1}$ | Petrarca et al., 2017 |
| Organochlorines | Fish liver | UA-DLLME-SFO | 1-undecanol | ng | 85.3- 112 | 1.06-3.94 ng $g^{-1}$ | Asati et al., 2018 |
| Neonicotinoid insecticides | Honey liqueur | Conventional DLLME | Dichloromethane | Acetonitrile | 69.2-113.4 | 0.5-1.5 µg $L^{-1}$ | Jovanov et al., 2013 |
| Organochlorines | Honey | Conventional DLLME | Chloroform | Acetonitrile | 36-114 | 0.02-0.15 µg $L^{-1}$ | Zacharis et al., 2012 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Organochlorines | Fish tissues | QuEChERS-DLLME-SFO | 1-undecanol | QuEChERS extract | 88.1 – 121.2 | 6.50x10$^{-4}$-1.58x10$^{-3}$ mg Kg$^{-1}$ | Wang et al., 2017 |
| Pyrethroids (permethrin, deltamethrin) | Honey | SPE-DLLME | tetrachloromethane | SPE extract | 4955 (permethrin)<br>4925 (deltamethrin) | 0.02 ng g$^{-1}$ (permethrin)<br>0.04 ng g$^{-1}$ (deltamethrin) | Shirani et al., 2016 |
| Acaricide pesticides | Honey | EA-DLLME | [OMIM]NTF$_2$ - based nanofluids | ng | 74.12-87.86 | 0.04-0.18 µg L$^{-1}$ | Wu et al., 2017 |

**Note:** ng- not given

## 3. Challenges and future work

The DLLME technique is a miniaturised sample pre-concentration method that is based on a ternary solvent system: water, extraction solvent and dispersion solvent. The major challenge is that most of the DLLME configurations, currently being used by researchers, still use toxic organic solvents, either as extraction solvents or dispersion solvent. Therefore, currently, the use of the various DLLME modes reduces the release of toxic organic solvents into the environment but the negative impacts are not eliminated. Thus, future work should have, as its main premises, zero tolerance on pollution of the environment with organic solvents during the DLLME technique. This noble goal may be achieved when research efforts are focused on the use of eco-friendly solvents during the DLLME technique and a conscious attempt to make it organic solvent-free. In addition, this goal can be achieved if the organic solvents used during DLLME are recovered and re-cycled.

## 4. Conclusion

DLLME is a versatile and robust sample enrichment technique that can be applied to a variety of liquid samples. The DLLME technique has been extensively applied during the analysis of pesticides in different food matrices. From the reviewed literature, it can be seen that the DLLME technique is desirable for the analysis of residual pesticides in food matrices due to: (i) its ability to significantly enrich trace pesticides, (ii) its ability to in-cooperate pesticides into suitable solvents for analysis and (iii) its ability to remove interferences from the food samples. The scope of the application of DLLME has been tremendously broadened by the incorporation of low-density solvents during analysis of food samples.

There is a conscious drive among researchers, recently, to enhance the greenness of the DLLME technique. This is evidenced by the recent rapid proliferation of researches where the halogenated extraction solvents are being replaced by eco-friendly solvents such as ionic liquids (Wang et al., 2017), deep eutectic solvents (Deng et al., 2019) and nanofluids (Wu et al., 2017).

Modifications of the conventional DLLME have also made possible the application of DLLME to solid food samples. The procedure during the analysis of solid food samples usually involves the use of an appropriate technique to extract the pesticides from the solid sample followed by the DLLME procedure. Thus, DLLME has been combined with other sample pre-treatment techniques such as QuEChERS, SPE and solvent extraction during analysis of solid food samples. Most of the coupling techniques used, however, are offline in nature.

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## Conflict of interests

There is no conflict of interests

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## 4.2. Paper II

This paper “Recent developments and applications of QuEChERS based technique on food samples during pesticide analysis” was submitted *to Journal of Food Chemistry*. The basic principles of the QuEChERS technique are described in this paper as well as its extensive application to analyse pesticides in cereals, animal products, fruits and vegetables.

# Recent developments and applications of QuEChERS based technique on food samples during pesticide analysis

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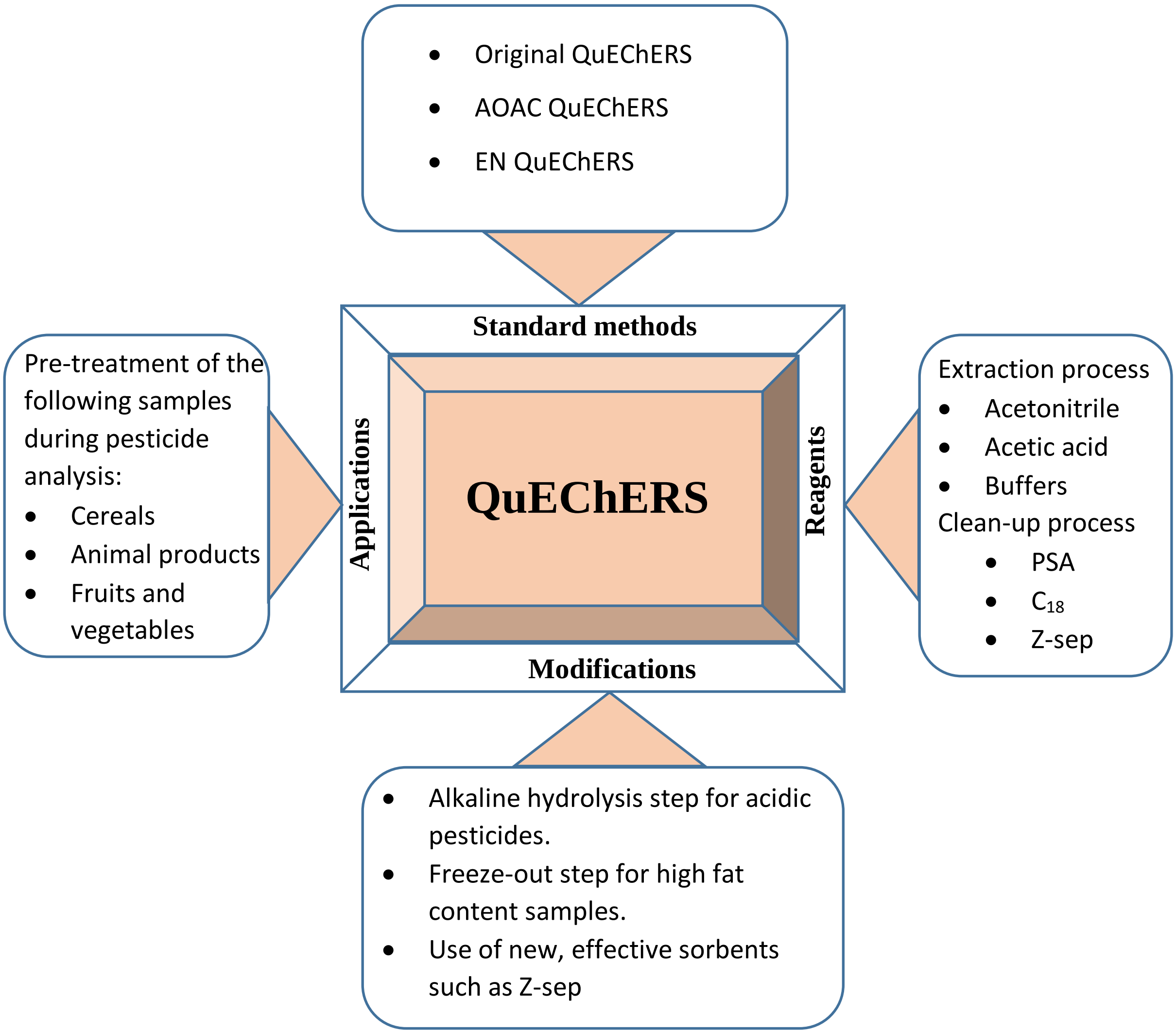
HIGHLIGHTS

- Standard QuEChERS techniques used for analysis of pesticides in food matrices.
- Reagents used during the extraction and clean-up processes when performing QuEChERS.
- Modifications of standard QuEChERS methods to accommodate problematic matrices and pesticides.
- Applications of the QuEChERS techniques to analyse pesticides in cereals, animal products, fruits and vegetables.

## GRAPHICAL ABSTRACT



## ABSTRACT

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is one of the pre-treatment techniques that has gained tremendous popularity among researchers during pesticide analysis of food and agricultural samples. Since its inception, this method is considered particularly suitable and effective in the extraction of a wide range of compounds. Consequently, most of the methods, developed using QuEChERS involve extracting pesticides and contaminants from various food samples, particularly fruits, vegetables, and other food products. The QuEChERS technique involves two steps: A liquid–liquid extraction and dispersive solid-phase extraction clean-up. The samples pre-treated using QuEChERS are clean enough to be analysed using gas or liquid chromatography. There are three common standard methods used during QuEChERS and these are the original QuEChERS, AOAC and the EN methods. In this review, recent developments in the QuEChERS technique are discussed as well as application of these techniques in the analysis of pesticides in food samples. Recently, many modifications of the QuEChERS technique have been effected during analysis of pesticides in food samples and are reviewed in this paper. Such modifications include the use of alkaline hydrolysis during analysis of acidic pesticides, inclusion of the freeze-out step when analysing food samples with high fat content as well as the use of new, effective sorbents such as Z-sep. The applications of the QuEChERS techniques to analyse pesticides in cereal, animal products as well as fruits and vegetables were also reviewed in this paper.

*Keywords*

Sample pre-treatment, food samples, miniaturization, pesticides, QuEChERS, optimisation

**Contents**

## 1. Introduction

Pesticides such as organochlorine, organophosphate, pyrethroids and carbamates are widely used in agricultural practice (Chen et al., 2018; Dubey et al., 2018; Lin et al., 2018). Many of these pesticides are resistant to chemical, physical and biological degradation, thus they are ubiquitously found in the environment and food products despite restrictions on their use (Du et al., 2017). Besides being effective in controlling pests, many of the pesticides are also harmful to human health when some threshold concentrations are exceeded. Thus, there is widespread concern about the potential health effects on non-target organisms, including human beings (Souza et al., 2016; Wittayanan et al., 2017). It is therefore imperative that pesticide residue analysis in different food matrices need to be prioritised.

Pesticide residue analysis is a difficult task, especially because of the complexity of some food matrices and due to the low concentrations that these compounds are usually present (Bordin et al., 2017; Muñoz et al., 2017; Ni et al., 2018; Rahman et al., 2018; Rong et al., 2018; Valverde et al., 2018). Despite the advances in the development of highly efficient analytical instrumentation, sample preparation remains an important step to obtaining accurate quantitative results (da Luz et al., 2017; Golge et al., 2018; Guo et al., 2018) through the reduction of matric effects. Extracts with high content of co-extractives can cause unreliable results when the pesticide concentration is too low, besides other problems related to matrix effect and analytical instrumentation damage, particularly when complex food samples are analysed (Parrilla Vázquez et al., 2016). In gas chromatography, matric effect can occur in different parts of the system such as sample injector, analytical column and detector. This occurs typically because of the interactions between pesticides and the active sites of the liner and in the column (Sousa et al., 2013). In liquid chromatography with mass spectrometry, matrix effect occurs during ionization, when interfering components co-elute with the pesticides causing signal suppression or enhancement.

Sample pre-treatment can go a long way to reduce matrix effect during the analysis of pesticides in food matrices. The sample pre-treatment methods that have been recently reported by other researchers for the extraction of pesticides in food matrices include dispersive liquid − liquid micro-extraction (DLLME) (Behbahani et al., 2014; Cacho et al., 2018; Chu et al., 2015; Dong et al., 2013; Farajzadeh et al., 2017; Gaubeur et al., 2015; Guo et al., 2016; Seebunrueng et al., 2014; Wang et al., 2017), solid-phase extraction (SPE) (Günter et al., 2016; Huo et al., 2016; Shamsipur et al., 2016; Zhang et al., 2016; Zhao et al., 2016), solid-phase micro-extraction (SPME) (Kenessov et al., 2016; Liang et al., 2017; Pelit et al., 2015; Souza-Silva et al., 2015; Wu et al., 2016), micro-solid phase extraction (µ-SPE) (Sajid et al., 2016) and molecularly imprinted technology (MIT) (Bakas et al., 2014; Martins et al., 2015; Yan et al., 2014).

A pre-treatment technique that has gained a lot of popularity among researchers recently during the analysis of pesticides in food matrices is QuEChERS (Bernardi et al., 2016; Ferreira et al., 2016; Golge et al., 2018; Lee et al., 2018; Nuapia et al., 2016; Parrilla Vázquez et al., 2016; Rahman et al., 2018; Tette et al., 2016; Wang et al., 2017). Anastassiades et al. (2003) developed the QuEChERS technique and was named so because of the characteristics of being Quick, Easy, Cheap, Effective, Rugged and Safe. The original approach (Fig. 1) consists of extracting with acetonitrile, partitioning between the aqueous and acetonitrile phase through adding magnesium sulphate and sodium chloride, and a d-SPE clean-up step by primary secondary amine (PSA) and anhydrous magnesium sulphate (Han et al., 2016).

Recently, the QuEChERS method has undergone several modifications. These modifications have made it to become well established for multi-residue analyses of pesticides in complex food and agricultural samples (He et al., 2015). Some proposed modifications to the QuEChERS method include buffering (Lee et al., 2016), use of different sorbents such as graphitized carbon black (GCB) (Bernardi et al., 2016), octadecylsilane ($C_{18}$) (Dankyi et al., 2015), Florisil (magnesium silicate) (Kemmerich et al., 2018) and the use of low temperature precipitation for fatty matrices (Ferreira et al., 2016). These modifications allowed the extraction of a large number of pesticides from different food classes and matrices. Most of the modifications have focused in the clean-up step and different sorbents have been used besides PSA and $C_{18}$ (Ferreira et al., 2016). For instance, zirconium-based sorbents have been used as d-SPE sorbent during pesticides determination in fish muscle and liver samples (Kaczyński et al., 2017). Lee et al. (2016), on the other hand, used GCB during the determination of pesticides in brown rice.

Among other beneficial features, the QuEChERS procedure uses acetonitrile, which permits extraction of polar analytes and has an elevated degree of selectivity and detectability and direct compatibility with both gas and liquid chromatography coupled with mass spectrometry (Lehotay et al., 2010). The QuEChERS method, when compared with other techniques mentioned above, minimizes the number of sample preparation steps since it only involves two steps. The first step is extraction with acetonitrile and a mixture of salts by partition. The second step is clean-up by dispersive solid phase extraction (d-SPE) using a sorbent comprising of primary and secondary amines (PSA) (Muhammad et al., 2017). Other advantages of the QuEChERS methods compared with other techniques are their excellent recoveries, less time for sample preparation and less solvent consumption (Rong et al., 2018; Zhang et al., 2014).

A lot of publications have been made on the analysis of pesticide residues in food matrices using the QuEChERS techniques for pre-concentration (Han et al., 2016; Lee et al., 2016; Parrilla Vázquez et al., 2016; Rahman et al., 2018; Tette et al., 2016; Valverde et al., 2018). However, very few reviews have been recently published on the application of the QuEChERS based techniques on food samples during pesticide analysis. Thus, this paper seeks to update readers on the current developments and applications of the QuEChERS technique for pesticide pre-concentration in different food matrices. The different strategies to tailor QuEChERS for selective pre-concentration of pesticides in food samples of different matrices were discussed and this included recent trends as well as the analytical merits and limitations of the technique.

### *1.1. QuEChERS standard methods*

There are three common standard procedures when performing the QuEChERS technique. These are the original (unbuffered) QuEChERS method, AOAC QuEChERS method and the buffered EN QuEChERS method (Fig. 1). These methods are referred to as standard methods because international regulatory bodies such as the European Committee and the AOAC in the United States accept them. Many researchers have used these QuEChERS standard procedures to analyse pesticides in food matrices. For instance, Golge et al. (2018**)** used the AOAC QuEChERS method to analyse pesticides in milk, cheese and cream samples while Zheng et al. (2018**)** analysed pesticides in honey samples using the EN QuEChERS.

The basic steps are the same for all the three standard methods: a liquid-liquid extraction (LLE) between an organic phase and water using salts for liquid–liquid partitioning. The sample is stirred, centrifuged, and an aliquot of the organic extract is removed and subjected to a d-SPE clean-up using a sorbent such as primary secondary amine (PSA). The sample can then be analysed using GC or LC. The LLE and d-SPE steps, where the actual chemistry occurs, are discussed in subsequent sections and differences between the three aforementioned methods are outlined.

### *1.1.1. Original QuEChERS method*

QuEChERS method has attracted the attention of residual pesticide analysis studies worldwide since Anastassiades et al. (2003) first published it. The original approach (Fig. 1.) consists of extracting with acetonitrile, partitioning between the aqueous and acetonitrile phase through adding magnesium sulphate and sodium chloride, and a d-SPE clean-up step by primary secondary amine (PSA) and anhydrous magnesium sulphate. Generally, PSA is used as the sorbent in the original method to remove polar pigments, polar organic acids, fatty acids, and some sugars (Suganthi et al., 2018)**.** The interfering matrix components are removed by PSA through an ion-exchange mechanism. However, the clean-up performance is not always satisfactory, especially for pigments (Han et al., 2016)**.** GCB is sometimes used in modified QuEChERS method and is effective at retaining neutral, acidic and basic co-extractives as well as pigments. The versatility of this sorbent is due to the fact that it can form van der Waals interactions with matrix components as well as being an anion-exchange sorbent. Its disadvantage, however, is that it can adsorb planar pesticides (Chen et al., 2018)**.** The original QuEChERS method has recently found a lot of application during the analysis of pesticides in complex matrices such as cowpea (Han et al., 2016)**,** honey samples (Tette et al., 2016)**,** tobacco samples (Bernardi et al., 2016) and cucumber samples (Dankyi et al., 2015)**.**

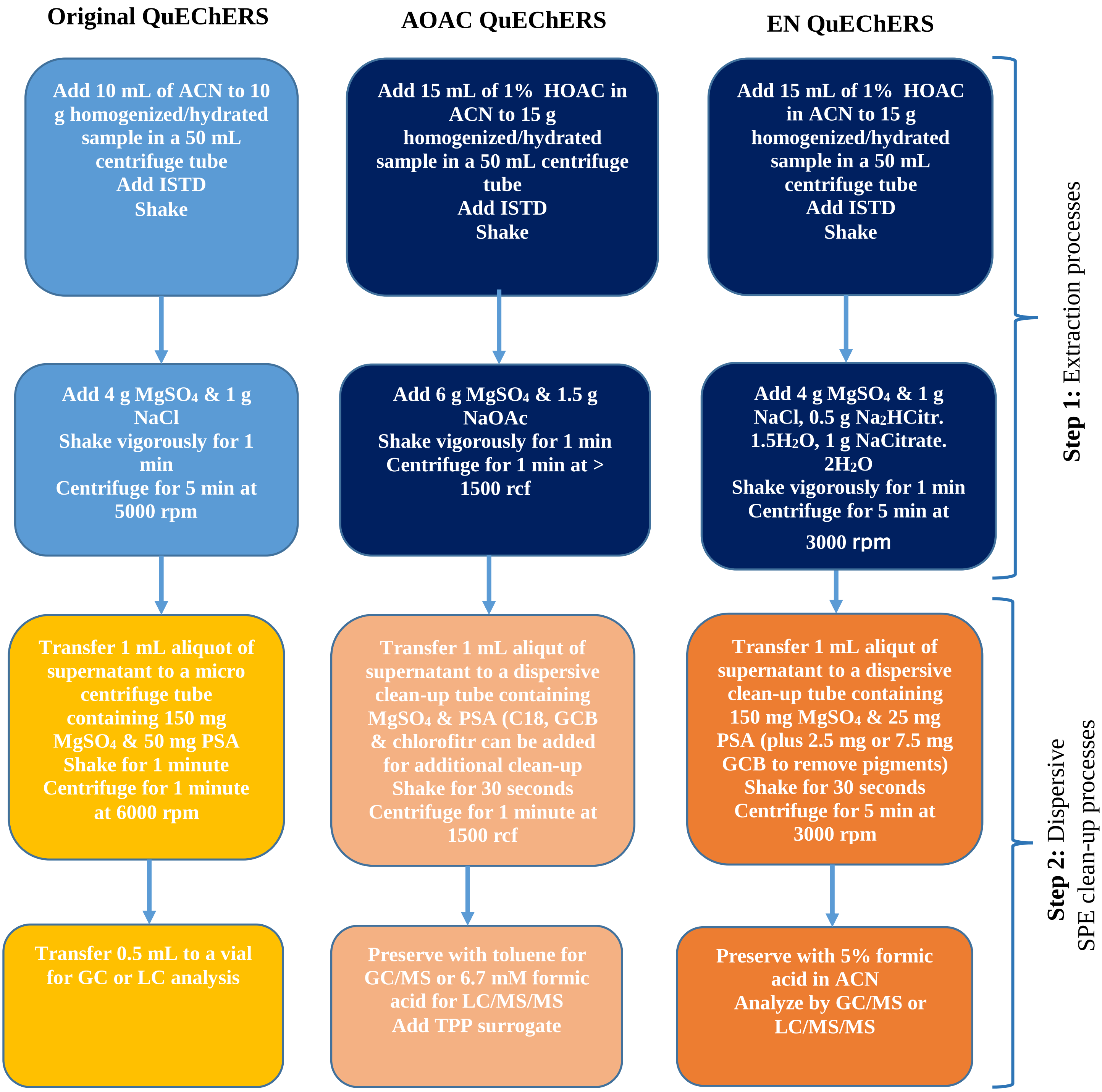


**Fig. 1.** Steps during the original, AOAC and EN QuEChERS methods (Rejczak and Tuzimski, 2015)

### *1.1.2. AOAC QuEChERS method*

One of the standard QuEChERS techniques is the AOAC method (Fig. 1). The extraction step during this method involves the use of 1% acetic acid in acetonitrile as the extraction solvent. Salting out of water from the sample is done using anhydrous $MgSO_4$ and this induces liquid-liquid partitioning (He et al., 2015). An aliquot of the organic layer, now containing the analyte, is then taken and subjected to a further clean-up step using d-SPE. During d-SPE, a combination of PSA and $C_{18}$ is used as sorbent while $MgSO_4$ is used to facilitate the partitioning process (Ferreira et al., 2016). The PSA removes fatty acids from the extract while the $MgSO_4$ reduces the amount of water in the extract. The mixture is then vigorously shaken, and centrifuged followed by analysis of the upper layer. Many researchers have used this technique to analyse pesticides in food matrices such as corn, wheat and rice (He et al., 2015), honey samples (Tette et al., 2016) and milk, cheese and cream samples (Golge et al., 2018). Fig. 1 summaries the steps during the AOAC QuEChERS technique.

### *1.1.3. European (EN) official QuEChERS method*

The EN method 15662: 2007 (Fig. 1.) is a European variation to the QuEChERS method. The method uses acetonitrile extraction, followed by the salting out of water from the sample using anhydrous $MgSO_4$ and NaCl (Lee et al., 2016). During the EN method, citrate salts are used as buffers in order to create suitable pH conditions to induce liquid-liquid partitioning. A dispersive solid phase extraction is conducted for clean up using a combination of PSA to remove fatty acids among other components, $C_{18}$ to remove lipids and anhydrous $MgSO_4$ to reduce the remaining water in the extract (Zheng et al., 2018). Sometimes, it is necessary to hydrate the food samples if they have low water content (Table 1). After mixing and centrifugation, the upper organic layer is ready for analysis. Many researchers have used the EN QuEChERS technique to analyse pesticides in food matrices. For instance, Lee et al. (2016) analysed herbicides in brown rice using the EN QuEChERS technique while Zheng et al. (2018) used the same technique to analyse pesticides in honey. Herrmann and Poulsen (2015) also successfully used the EN QuEChERS technique to analyse pesticides in oats and wheat.

The EN QuEChERS method is very similar to the AOAC QuEChERS method. However, the two techniques have some differences. One of the differences is that the extraction buffered system in the EN method uses disodium citrate sesquihidrate while sodium acetate is used in the AOAC extraction step. The other difference is that in the dispersive SPE step, the AOAC method uses 50 mg PSA per millilitre of extract instead of the 25 mg PSA per millilitre of extract used in the EN method.

### *1.2. Modifications of the standard QuEChERS techniques*

The standard QuEChERS techniques were modified to facilitate the analysis of problematic pesticides. Such pesticides include those with high fat content matrices, low water content and pH sensitive pesticides (Fig. 2.).

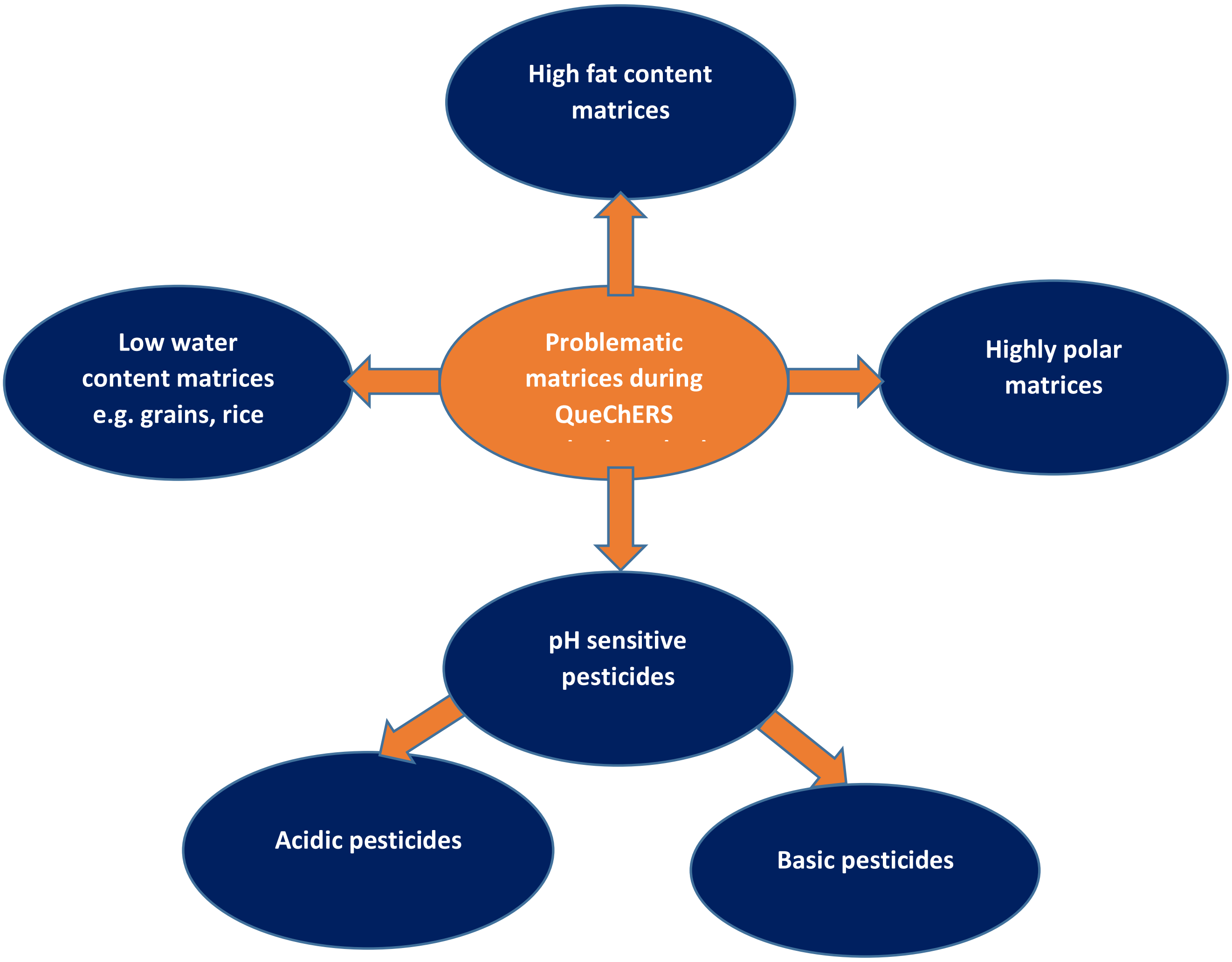


**Fig. 2**. Problematic matrices during pesticide analysis using the QuEChERS technique

The application of the QuEChERS technique on pH sensitive pesticides is problematic. Such pesticides can either be acidic or basic. Acidic pesticides have carboxyl groups and can be covalently bonded to the matrix components making their concentration to be underestimated during QuEChERS techniques (Anastassiades et al., 2010; Ghoniem et al., 2017)

Some acidic pesticides also form mesomeric structures (Fig. 3). Electrons that form double bonds in conjugated acidic pesticides can switch between atoms slightly changing the way the structure appears and behaves chemically. Thus, the pesticide molecule actually exist in a hybrid state between the different structures formed by variations in bonds and these different structures are called mesomeric structures. The formation of these mesomeric structures make chromatographic analysis of acidic pesticides complicated.

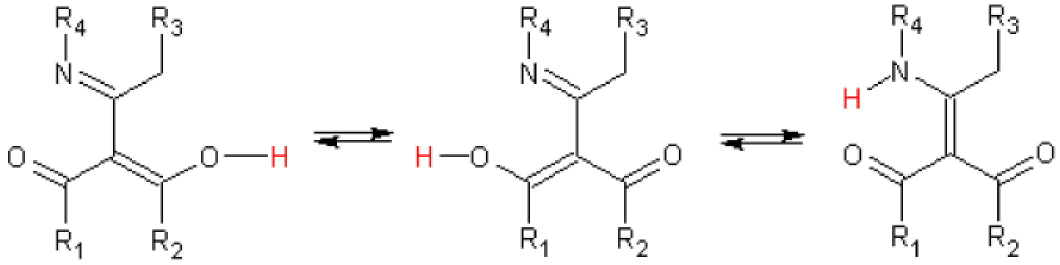


Fig. 3. Mesomeric structures of acidic pesticides (Kostelac and Anastassiades, 2007)

The acidic pesticides also ionizes in aqueous medium and these ionic forms are hydrophilic and difficult to extract using organic, non-polar solvents. Thus, the standard QuEChERS techniques should be modified to allow for the efficient analysis of acidic pesticides. PSA sorbents are basic and are not suitable for use during analysis of acidic pesticides since the pesticides would form acid-base interactions with the sorbent (Kostelac and Anastassiades, 2007). Thus, the use of PSA is avoided during the analysis of acidic pesticides to prevent their loss during the clean-up process.

Another important modification of the standard QuEChERS methods during analysis of acidic pesticides is the inclusion of an alkaline hydrolysis step (Fig. 4). Alkaline hydrolysis will break the covalent bounds formed between matrix components and acidic pesticides, thereby making the pesticides free during analysis. Steinborn et al. (2017) successfully applied alkaline hydrolysis during analysis of acidic pesticides (dichlorprop, fluazifop and haloxyfop) from different food matrices (peanut butter, sunflower seeds, cornbread, white bread and oranges) using the QuEChERS techniques.

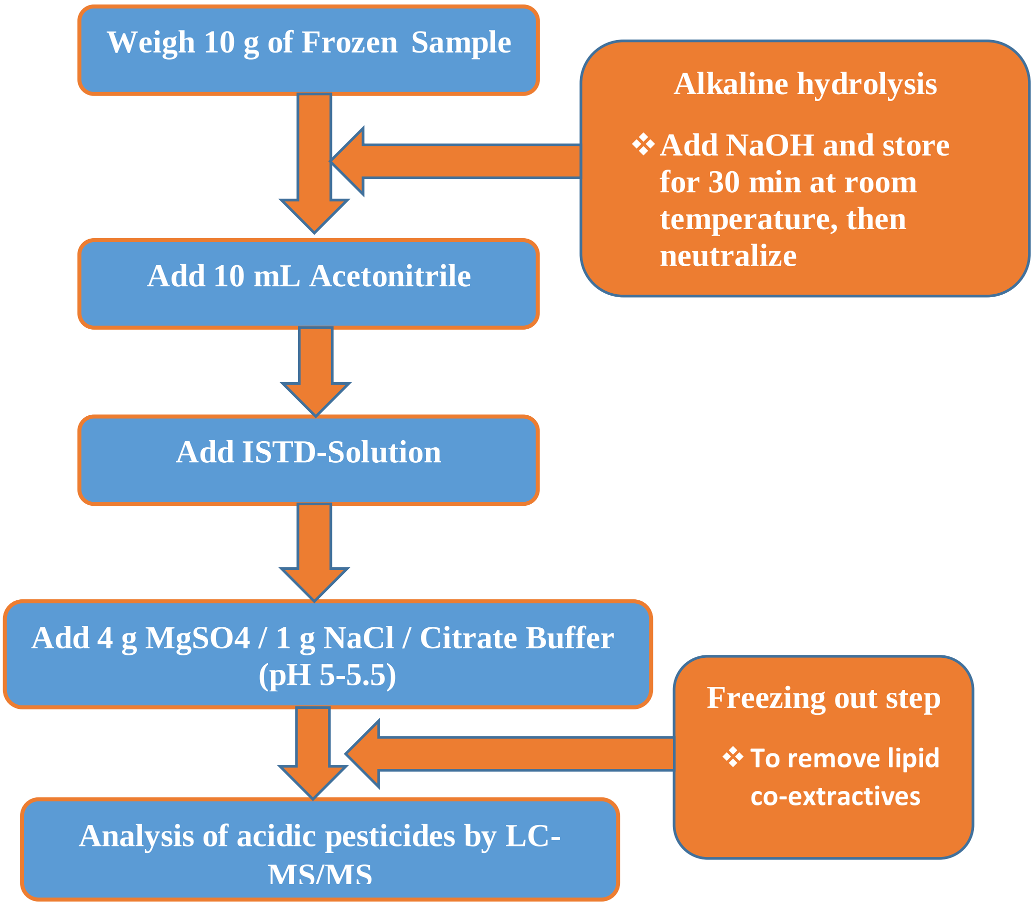


**Fig. 4**. Modified citrate-buffered QuEChERS with alkaline hydrolysis and freeze out step (Kostelac and Anastassiades, 2007)

The high lipid content matrices are among the problematic matrices during the QuEChERS technique. Some pesticides are lipophilic and they form hydrophobic interactions with lipids. They are, therefore, absorbed and trapped in the fatty layer causing the under-estimation of the pesticides in such medium (Parrilla Vázquez et al., 2016; Xu et al., 2016). On the other hand, some fats are also co-extracted during the QuEChERS technique and these lipids will be problematic during chromatographic analysis since they may cause the clogging of the analytical columns. Thus, the standard QuEChERS methods should be modified to accommodate the matrices with high fat content.

Lipid co-extractives can be greatly reduced by incorporating a liquid-liquid extraction step with hexane into the QuEChERS technique. For instance, Bargańska et al. (2014) successfully used n-hexane to eliminate beewax during the application of QuEChERS on honey samples. Fig. 5 shows the modified QuEChERS technique used by Bargańska et al. (2014) where n-hexxane was used remove lipids from sample matrix. Xu et al. (2016) used a similar technique to remove lipids from edible animal tissue during their analysis for pesticides using

QuEChERS. Also, during the analysis of high fat content matrices, a freeze-out step over night, for removal of co-extracted fats as well as other components with limited solubility in acetonitrile, could be performed (Fig. 4). Ferreira et al. (2016) successfully used the freezing-out technique for the analysis of multi-class pesticides in coconut samples

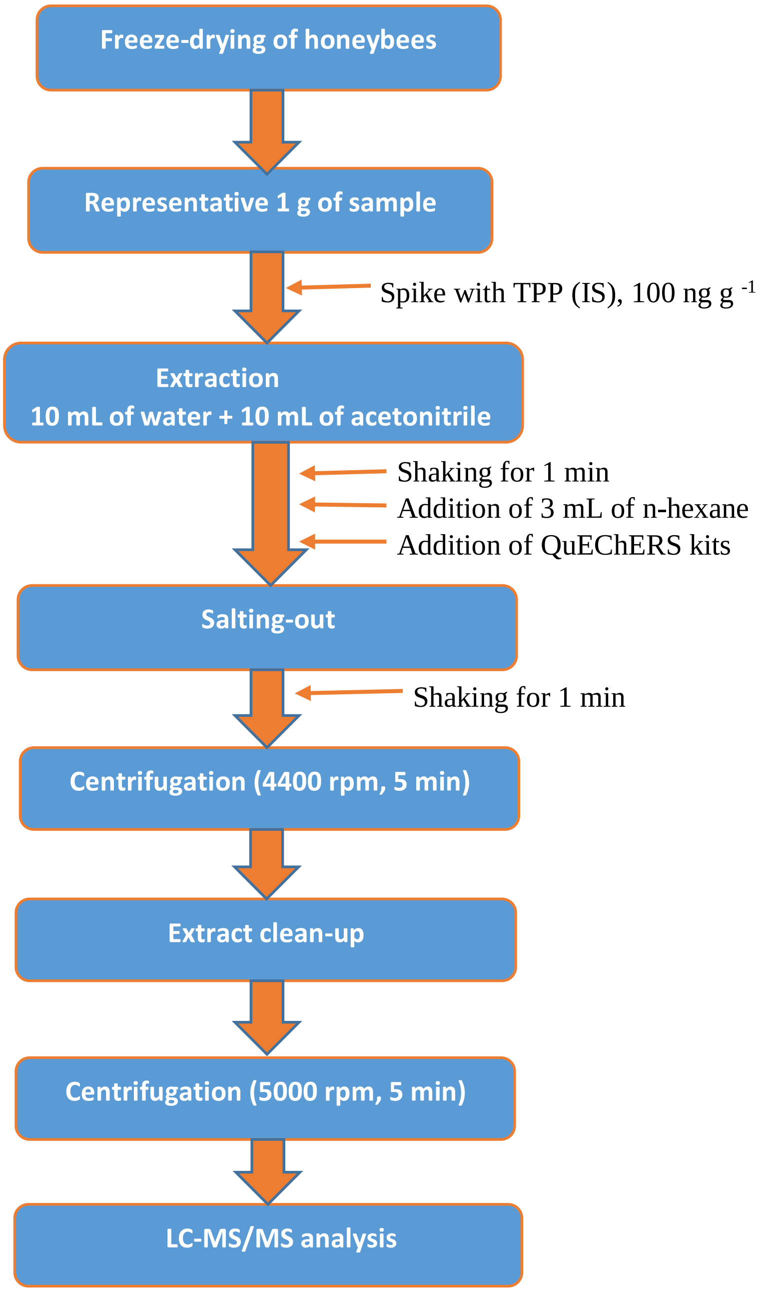


**Fig. 5**. Modified QuEChERS with liquid-liquid extraction step using n-hexane (Bargańska et al., 2014).

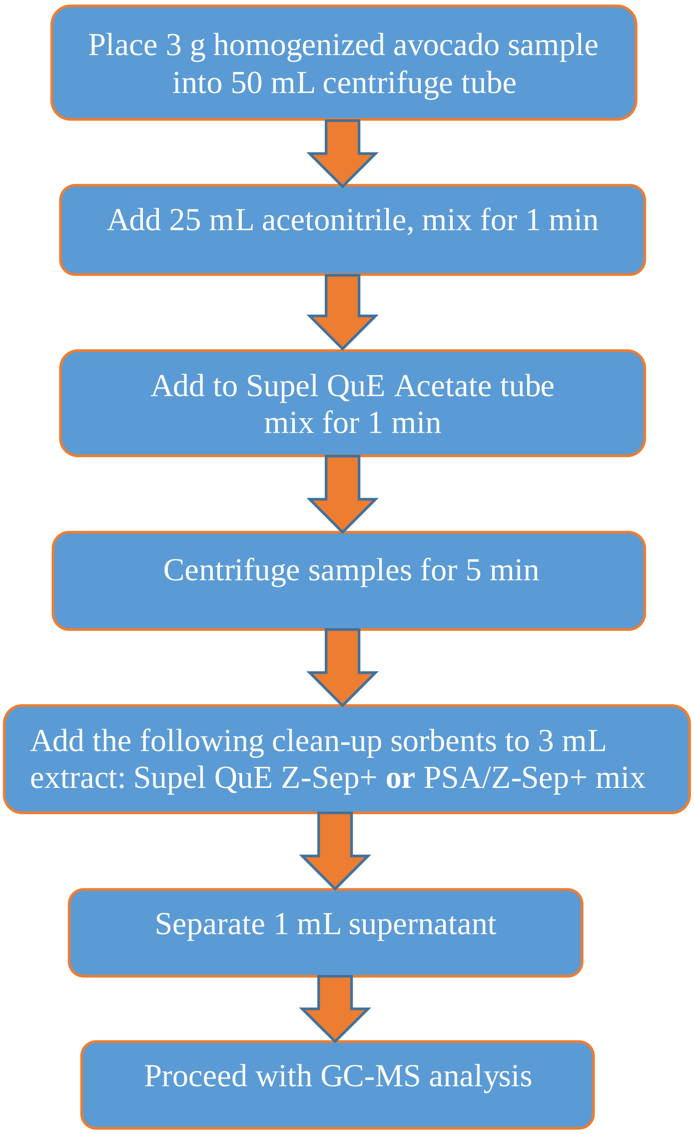


**Fig. 6.** Modified QuEChERS for fatty matrices using Z-sep+ sorbents (Dias et al., 2016)

The QuEChERS technique is most effective for the extraction of pesticides in samples with a high water content such as fruits and vegetables. For samples with a low water content, additional water is added to ensure optimum extraction. The EN method guidelines for the addition of water to samples with low water content are shown in Table 1. The technique of hydrating dry samples was successfully used by He et al. (2015) during analysis of cereal samples (ground rice, wheat flour and ground corn) using the QuEChERS methodology. During the QuEChERS procedure, 5 g of each cereal sample (rice, wheat flour and corn) was hydrated using 10 mL of purified water to ensure effective extraction of pesticides. Lee et al. (2016), when analysing rice samples using the QuEChERS technique, used a similar hydrating strategy. 10 mL of water was added to each 2 g of rice sample that was subjected to the QuEChERS methodology.

**Table 1**

EN method guidelines for the addition of water to samples with low water content (Rejczak and Tuzimski, 2015)

| Sample type | Sample weight (g) | Water added (g) | Note |
|---|---|---|---|
| Fruits and vegetables (> 80% water content) | 10 | 0 | No water added |
| Fruits and vegetables (25 - 80% water content) | 10 | X | X = 10 – water amount in 10 g sample |
| Cereals | 5 | 10 | Mass of water is double that of the sample |
| Dried fruits | 5 | 7.5 | Water can be added during comminution step |
| Honey | 5 | 10 | Mass of water added is double that of sample |
| Spices | 2 | 10 | Water added is 5 times mass of sample |

## *1.3. Reagents used during QuEChERSs*

### *1.3.1. Reagents used during the QuEChERS extraction and their functions*

During analysis of pesticides in food samples using the QuEChERS technique extraction reagents are used to ensure that as much pesticides as possible are removed from the base matrix. The success of the QuEChERS technique during pesticide analysis depends, among other factors, on the correct choice of extraction reagents. The extraction reagents should be carefully controlled in order to minimize co-extracting compounds and to enhance the pre-concentration of the pesticides of interest. The commonly used reagents by researchers during the extraction process of pesticides in food matrices include anhydrous magnesium sulphate, acetic acid, acetonitrile, buffers and sodium chloride (Guo et al., 2018; Muhammad et al., 2017; Rahman et al., 2018; Rong et al., 2018; Tette et al., 2016) (Table 2).

**Table 2**

Reagents used during QuEChERS extraction process and their functions

| Extraction reagent | Functions | Matrices | Analyte | Ref. |
|---|---|---|---|---|
| Anhydrous magnesium sulfate | Facilitates solvent partitioning and improves recovery of polar pesticides | Dry herbs | Multiclass pesticides | Abbas et al., 2017 |
| Acetic acid | Used to adjust pH | Honey | Multiclass pesticides | Tette et al., 2016 |
| Acetonitrile | Organic solvent providing the best characteristics for extracting the broadest range of pesticides with the least number of co-extractables. Amenable for both LC and GC analysis | Fish samples | Organochlorine | Wang et al., 2017a |
| Buffers | Prevents degradation of pH sensitive analytes by maintaining optimal pH | Honey | neonicotinoids | Valverde et al., 2018 |
| Sodium chloride | Reduces the amount of polar interferences | Rice, wheat, maize | Herbicides | Ni et al., 2018 |

### *1.3.2. Reagents used during the QuEChERS clean-up process and their functions*

Sample clean up is necessary during QuEChERS to reduce interferences. The presence of interferences can damage analytical instrumentation and complicate pesticide identification and quantification. Thus, a sorbent in the clean-up step is chosen to retain undesired, co-extracting compounds from the matrix and to allow the pesticide of interest to remain in the liquid phase. Sorbent (clean-up reagent), therefore, form an integral part of the QuEChERS technique during pesticide analysis in food matrices. Several sorbents have been effectively used by different researchers during pesticide analysis in food matrices using the QuEChERS technique and these include $C_{18}$ (Lee et al., 2016) graphitized carbon black (GCB) (Malhat et al., 2017) and primary Secondary Amine (PSA) (Tette et al., 2016). Table 3 shows the functions of these sorbents.

**Table 3**

QuEChERS clean-up reagents used during pesticide analysis and their functions

| Clean-up reagents | Functions | Matrices | Pesticides | Ref. |
|---|---|---|---|---|
| Aminopropyl | Removes sugars and fatty acids. Serves the same function as PSA, but is less likely to catalyze degradation of base sensitive analytes. Aminopropyl has a lower capacity for clean-up than PSA | strawberries | Multiclass pesticides | Oshita and Jardim, 2014 |
| ChloroFiltr® | Polymeric sorbent for selective removal of chlorophyll from acetonitrile extracts without loss of polar aromatic pesticides | Lupin, mustard, sorghum | Multiclass pesticides | Walorczyk et al., 2015 |
| $C_{18}$ | Removes long chain fatty compounds, sterols and other non-polar interferences | Brown rice | Herbicides | Lee et al., 2016 |
| Graphitized carbon black (GCB) | Strong sorbent for removing pigments, polyphenols, and other polar compounds: examples of planar (polar aromatic) pesticides which may be removed: chlorothalonil, coumaphos, hexachlorobenzene, thiabendazole, terbufos, and quintozene | Tobacco | Multiclass pesticides | Bernardi et al., 2016 |
| Anhydrous magnesium sulphate | Removes water from organic phase | Coconut | Multiclass pesticides | Ferreira et al., 2016 |

| | | | | |
|---|---|---|---|---|
| Primary Secondary Amine (PSA) | Used in the removal of sugars and fatty acids, organic acids, lipids and some pigments. When used in combination with $C_{18}$, additional lipids and sterols can be removed | Honey | Multiclass pesticides | Tette et al., 2016 |
| Z-sep | Used to remove lipids | Fish muscle and liver | Multiclass pesticides | Kaczyński et al., 2017 |

### *1.4. Optimization of QuEChERS parameters*

With the appropriate sorbents and extraction reagents at hand, one will be ready to perform the QuEChERS technique to analyse pesticides in food matrices. The first step, before analysing real food samples, is optimisation of the QuEChERS extraction parameters. Optimisation involves the investigation of most of the parameters that influence the QuEChERS extraction efficiency of pesticides from food matrices to pave way for the use of optimal conditions during analyses and to achieve the best possible extraction results (Muhammad et al., 2017). Thus, the importance of optimisation cannot be over-emphasised during pesticide analysis using the QuEChERS methodologies. The parameters to be optimised during pesticide analysis include type and volume of extraction solvent, quantity of sorbents, pH of the sample, centrifugation time and speed, and quantity of extraction salts (Lin et al., 2018; Rahman et al., 2018; Suganthi et al., 2018; Tripathy et al., 2017).

It should be noted that although the original publications for all the standard methods indicate specific amounts of reagents, it is important to optimize each step in QuEChERS for the compounds under investigation, as the optimal parameters may vary by analyte and matrix. The QuEChERS parameters should be optimised with the ultimate goal of increasing recovery and removal of matrix interfering peaks (Nantia et al., 2017).

## 2. Analysis of pesticides in food matrices using QuEChERS

The QuEChERS technique has found applications during pesticide analysis in many different food matrices. These matrices include fruits and vegetables (Dankyi et al., 2015; Prodhan et al., 2016; Tripathy et al., 2017), cereals (Grande-Martínez et al., 2016; Rebelo et al., 2014; Xia et al., 2017) and animal products (Mu et al., 2016; Rahman et al., 2018; Zheng et al., 2018). The subsequent sections give brief outlines of these applications of QuEChERS during pesticide analysis in different food matrices.

### *2.1. Fruits and vegetables*

The determination of pesticide residues in food matrices, such as fruits and vegetables, is a challenge especially because of the low concentration of pesticides and large amounts of interfering substances that can be co-extracted with pesticides and, in most of the cases, adversely affect the analysis results (Han et al., 2016). Several sample pre-concentration procedures have been proposed for pesticide residues determination in fruits and vegetables, including: solid phase extraction (SPE) (Shamsipur et al., 2016; Zhao et al., 2016), solid phase micro-extraction (SPME) (N. Liang et al., 2017), micro-solid phase extraction (μ-SPE) (Sajid et al., 2016) and microwave assisted extraction (MAE) (Fernandes et al., 2011). While these methods are highly efficient, they generally require considerable investment in instrumentation and allow a limited scope of pesticides that can be extracted under certain conditions. Since 2003, when Anastassiades et al. (2003) introduced the QuEChERS method, there has been a revolution in sample preparation step for pesticide residue analysis in fruits and vegetables.

Fruits and vegetables can be placed into different categories, which are summarised in Table 3. Selection of a suitable sorbent combinations during the QuEChERS technique is largely dependent on the category the fruits and vegetables are found. This is because different categories of fruits and vegetables have different matrix interfering components.

During pesticide residue analysis in fruits and vegetables, the widely used QuEChERS techniques are AOAC and EN official QuEChERS methods (buffered QuEChERS techniques) (Table 4). Extraction of pesticides in the fruit and vegetable samples is usually done using acetonitrile as the extraction solvent and the clean-up sorbents used depends on the nature of the fruit and vegetable samples.

**Table 3**

Fruit and vegetable categories and suitable sorbent combinations

| Fruit and vegetable category | Functions of sorbents | Sorbent combinations | Ref. |
|---|---|---|---|
| General fruits and vegetables | Removes polar organic acids, some sugars and lipids | PSA and $MgSO_4$ | Sivaperumal et al., 2017; Suganthi et al., 2018 |
| Highly pigmented fruits and vegetables | Removes polar organic acids, some sugars and lipids, plus high levels of carotenoids and chlorophyll. | PSA, GCB and $MgSO_4$ | Chen et al., 2018; Malhat et al., 2017 |
| Fruits and vegetables with pigments and fats | Removes polar organic acids, some sugars and lipids, plus carotenoids and chlorophyll. | PSA, GCB, $MgSO_4$ and $C_{18}$ | Abbas et al., 2017; Lin et al., 2018 |

**Table 4**

Analysis of pesticides in fruits and vegetables using different QuEChERS techniques

| Pesticides | Matrix | Pre-concentration technique | Extraction solvent | Clean-up process | LOQ (mg kg$^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| Neonicotinoids | Cucumber | QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | 0.01 | Dankyi et al., 2015 |
| Multiclass pesticides | Tobacco | QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and, $C_{18}$, PSA and GCB | ng | Bernardi et al., 2016 |
| 5 insecticides and 2 fungicides | Cabbage | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | 0.01 | Prodhan et al., 2016 |
| Pyrethroids | 20 fruits and 20 vegetables | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ , PSA and $C_{18}$ sorbents | 0.0003-0.0049* | Lin et al., 2018 |
| Multiclass pesticides | Pear | Modified acetate QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ , PSA, florisil and $C_{18}$ sorbents | 0.0025* | Kemmerich et al., 2018 |
| Multiclass pesticides | Medicinal herbs | Modified QuEChERS | 1% acetic acid in acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | 0.01-0.069 | Tripathy et al., 2017 |

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|---|---|---|---|---|---|---|
| Multiclass pesticides | Mango | Modified QuEChERS | Acetonitrile and ethyl acetate mixture, 25:75 (v/v) | d-SPE using $MgSO_4$ and PSA sorbents | 0.0005-0.007* | Sivaperumal et al., 2017 |
| Multiclass pesticides | Dry herbs | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ , PSA, GCB and $C_{18}$ sorbents | ng | Abbas et al., 2017 |
| Fenitrothion | Tomatoes | Modified QuEChERS | Ethyl acetate | d-SPE using $MgSO_4$ , PSA and GCB sorbents | 0.01 | Malhat et al., 2017 |
| Organochlorines and pyrethroids | | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | 0.01 (organochlorines)<br>0.05 (pyrethroids) | Dubey et al., 2018 |
| Multiclass pesticides | Watermelon, pakchoi, cowpea, pepper, peaches, grapes | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, $C_{18}$, carb and PSA sorbents | 0.00001-0.00667* | Xiu-ping et al., 2017 |

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|---|---|---|---|---|---|---|
| Thiamethoxam, fipronil, pyraclostrobin | Cucumber, apples, bananas, tomatoes | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, $C_{18}$, GCB and PSA sorbents | 0.010* | Rong et al., 2018 |
| Afidopyropen and metabolites | Tomato, watermelon, pepper, cucumber, pear, grape, cabbage | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, GCB and PSA sorbents | 0.001* | Chen et al., 2018 |
| Multiclass pesticides | Lettuce | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, GCB and PSA sorbents | 0.005* | Ribeiro Begnini Konatu et al., 2017 |
| Multiclass pesticides | Many types of fruits and vegetables from 29 markets | Modified QuEChERS | Acetonitrile | SPE using $MgSO_4$, GCB and PSA sorbents | $\leq 0.01$ | Ahammed Shabeer et al., 2018 |
| Neonicotinoids | Sugarcane | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | 0.002-0.005** | Suganthi et al., 2018 |
| Multiclass pesticides | Golden berry | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, $C_{18}$, GCB and PSA sorbents | 0.005 | Muñoz et al., 2017 |

**Note:** ng – not given; *- converted from μg $kg^{-1}$ to mg $kg^{-1}$; **- converted from μg $g^{-1}$ to mg $kg^{-1}$

### *2.2. Cereals*

Pesticides are used to improve cereal crop yields and to minimize degradation during storage and processing. However, the widespread use of pesticides and the potential for residues to remain on the final product is of concern to consumers and to governments whose responsibility it is to ensure a safe food supply (Zhang et al., 2013). Pesticides have adverse effects on human health even at trace levels. Thus, efficient sample pre-treatment techniques should be used to monitor pesticide levels in food samples such as cereals.

Due to the high complexity and diversity of the matrices, the analysis of pesticides in dried samples such as cereals is considered a difficult task (He et al., 2015). Compared to vegetables, cereals, such as corns, have high fat content that makes them even more difficult to extract and clean (He et al., 2015; Xia et al., 2017; Xu et al., 2015). The QuEChERS procedure was developed to determine pesticide residues in samples with more than 75% moisture and non-fatty matrices (da Luz et al., 2017; Grande-Martínez et al., 2016). Thus, the QuEChERS procedure is rarely used in pesticide residue analysis of cereals compared to its use in fruits and vegetables.

The other challenges in the application of the QuEChERS method to some cereal matrices are due to their highly pigmented nature (Xia et al., 2017) and presence of low water content. Thus, the standard QuEChERS methods should be modified in order to effectively extract pesticides from complex cereal matrices. The cereal samples should be hydrated in order to have the appropriate amount of water required during QuEChERS (Bordin et al., 2017; Ni et al., 2018; Xia et al., 2017; Xu et al., 2015). Effective sorbents such as $C_{18}$ and Z-sep should be used for the removal of lipids in some cereal samples (Ferreira et al., 2016; Lee et al., 2016) and sorbents such as GCB and chlorofiltr should be used if the cereal sample is highly pigmented. A freeze-out step may also be included to rid the cereal samples of lipid co-extractives (Ferreira et al., 2016). Table 5 has some of the researches on pesticides performed on cereal samples.

**Table 5**

Analysis of pesticides in cereals and grain products using QuEChERS

| Pesticides | Matrix | Pre-concentration technique | Extraction solvent | Clean-up process | LOQ (mg kg$^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| Multiclass pesticides | Coconuts | QuEChERS combined with freeze out using dry ice. | 1% acetic acid in acetonitrile | d-SPE using $MgSO_4$ and, $C_{18}$ and PSA sorbents | 0.010* | Ferreira et al., 2016 |
| Multiclass pesticides | Cowpea | QuEChERS | Acetonitrile | r-DSPE using multi-walled carbon nanotubes | 0.001-0.003 | Han et al., 2016 |
| Herbicides | Brown rice | EN-QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | 0.001-0.05 | Lee et al., 2016 |
| Multiclass pesticides | Rice, wheat flour | Modified QuEChERS | Ethyl acetate | d-SPE using $MgSO_4$ , PSA and $C_{18}$ sorbents | 0.0036 | Grande-Martínez et al., 2016 |
| Sulphonamide herbicides | Wheat, rice, corn | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | 0.005 | Xu et al., 2015 |
| Sulfonylurea herbicides | Rice, maize, wheat, soybeans | Modified QuEChERS | Acidified acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | 0.0005-0.001 | Ni et al., 2018 |
| Multiclass pesticides | Wheat, oats extracts | EN QuEChERS | Acidified acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | ng | Herrmann and Poulsen, 2015 |

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|---|---|---|---|---|---|---|
| Herbicides | Corn flour | Modified QuEChERS | Acetonitrile | d-SPE using $MgSO_4$, GCB and PSA sorbents | 0.004-0.048** | Xia et al., 2017 |
| Multiclass pesticides | Whole wheat flour | Modified QuEChERS without clean-up | Acidified acetonitrile | Ng | 0.010* | Bordin et al., 2017 |
| Multiclass pesticides | Corn, wheat flour, rice | AOAC QuEChERS | Acidified acetonitrile | d-SPE using $MgSO_4$ , PSA and $C_{18}$ sorbents | 0.005-0.05* | He et al., 2015 |
| Fungicides | Wheat grain | QuEChERS | Acidified acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | ng | da Luz et al., 2017 |
| Multiclass pesticides | rice | Modified QuEChERS | Acidified acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | 0.0004-0.0588** | Rebelo et al., 2014 |

**Note:** ng – not given; r-DSPE – reversed- dispersive solid phase extraction; * - converted from μg $kg^{-1}$ to mg $kg^{-1}$;  **- converted from ng $g^{-1}$ to mg $kg^{-1}$

### *2.3. Animal products*

Food of animal origin such as muscle, liver, and eggs are usually chemically complex (Golge et al., 2018; Zhao et al., 2014). Animal tissues are known to be rich in protein and lipid components (Jeong et al., 2012), which can bind to pesticides during the QuEChERS technique. The non-polar pesticides bind with the lipids through hydrophobic interactions while the zwitterionic forms of proteins and amino acids form ionic interactions with both basic and acidic pesticides. A zwitterion is a result of intra-molecular donation of a proton from the acidic carboxyl group to the basic amino group in an amino acid. Therefore, an appropriate sample treatment is essential to weaken and break the interactions between proteins and pesticides in order to pave way for obtaining reliable results in pesticide analyses from animal products.

Suitable analytical strategies should be employed to free polar pesticides that are bound to proteins during application of the QuEChERS technique on food matrices that are rich in proteins. One such strategy is protein precipitation. Organic solvents, such as acetonitrile, methanol, and ethanol, are commonly employed in the precipitation of proteins in biological matrices (Jeong et al., 2012) during QuEChERS. Acetonitrile is usually the extraction solvent of choice used by many researchers (Golge et al., 2018; Guo et al., 2018; Nuapia et al., 2016; Rahman et al., 2018; Tette et al., 2016; Valverde et al., 2018; Wang et al., 2017) when using QuEChERS for sample pre-treatment. The acetonitrile solvent typically provides high extraction efficiency and often minimizes co-extraction of lipids from animal tissues (Han et al., 2016).

Samples from animal products also contain a high amount of lipids and this presents a problematic matrix during the QuEChERS methodology. The major challenge when analysing pesticides from samples with a high lipid content is the co-extraction of lipids during QuEChERS (Han et al., 2016). Many strategies have been employed by researchers to avoid co-extraction of lipids during application of QuEChERS on animal products with the aim of determining the level of pesticides in such samples. These strategies include the use of effective sorbents such Z-sep (Kaczyński, 2017) and florisil (Seddik et al., 2017; Tette et al., 2016) that can effectively remove the lipids from the matrix; inclusion of a freeze-out step during the QuEChERS technique (Parrilla Vázquez et al., 2016) and coupling of QuEChERS with other pre-treatment techniques to improve the clean-up process. For instance, Wang et al. (2017) successfully extracted organochlorines from fish samples by using the QuEChERS technique coupled with DLLME-SFO. Table 6 shows some pesticides extracted using QuEChERS technique from food matrices of animal origin.

**Table 6**

Analysis of pesticides in animal products using QuEChERS

| Pesticides | Matrix | Pre-concentration technique | Extraction solvent | Clean-up process | LOQ (mg $kg^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| Organochlorines | Fish | QuEChERS-DLLME-SFO | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | $6.5x10^{-4}$ – $1.58x\ 10^{-3}$ | Wang et al., 2017 |
| Multiclass pesticides | Honey | AOAC QuEChERS | 1% acetic acid in 10ml of acetonitrile : ethyl acetate (70:30 v/v) | d-SPE using $MgSO_4$, Florisil and PSA sorbents | 0.005 | Tette et al., 2016 |
| Multiclass pesticides | Milk | AOAC QuEChERS | Acidified acetonitrile | d-SPE with $MgSO_4$, PSA and $C_{18}$ | 0.52 – 3.67 | Jeong et al., 2012 |
| Organochlorines | Beef and fish | Modified QuEChERS | Acetonitrile | d-SPE with $MgSO_4$ and PSA | 10.8 – 193.01 | Nuapia et al., 2016 |
| Multiclass pesticides | Chicken, fish, sheep, rabbit, pork and clam | Modified QuEChERS | Acetonitrile | d-SPE with $MgSO_4$, PSA, GCB and $C_{18}$ | 0.001-0.010* | Zhang et al., 2017 |
| Endrin | Chicken, pork, beef, egg and milk | Modified QuEChERS | Acidified acetonitrile | d-SPE with $MgSO_4$ and PSA | 0.01 | Rahman et al., 2018 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Carbamates | Porcine, shrimp, egg and whole milk | EN QuEChERS | Acidified acetonitrile | d-SPE with $MgSO_4$ and $C_{18}$ | 0.002* | Zheng et al., 2017 |
| Fipronil and metabolites | Chicken egg, chicken muscle | Modified QuEChERS | Acetonitrile | d-SPE with $MgSO_4$, PSA and $C_{18}$ | $2 \times 10^{-7}$ - $5 \times 10^{-7}$ ** | Guo et al., 2018 |
| Multiclass pesticides | Honey | EN QuEChERS | Acetonitrile | d-SPE with $MgSO_4$, PSA and $C_{18}$ | 0.001- 0.005 | Zheng et al., 2018 |
| neonicotinoid insecticides | Honey | Modified QuEChERS | Acetonitrile and ethyl acetate mixture | d-SPE with $MgSO_4$, PSA and $C_{18}$ | ng | Valverde et al., 2018 |
| Multiclass pesticides | Milk, cheese, cream | AOAC QuEChERS | Acidified acetonitrile | d-SPE with $MgSO_4$, PSA and $C_{18}$ | 0.0011 – 0.00131* | Golge et al., 2018 |
| Methiocarb and its metabolites | Chicken, pork, beef, egg and milk | Modified QuEChERS | Acetonitrile | d-SPE with $MgSO_4$ and $C_{18}$ | 0.005 | Rahman et al., 2017 |
| Fungicides | Chicken, egg, milk, pork, pork liver and pork kidney | Modified QuEChERS | Acetonitrile | d-SPE with $MgSO_4$, PSA and $C_{18}$ | 0.00013- 0.0042* | Mu et al., 2016 |

**Note:** ng - not given; *- converted from µg $kg^{-1}$ to mg $kg^{-1}$; **- converted from ng $kg^{-1}$ to mg $kg^{-1}$

## 3. Challenges and future work

Miniaturisation is an outstanding feature of the QuEChERS methodology during pesticide analysis of food samples. It results in a massive reduction of the volume of organic solvent used during the QuEChERS extraction process. Despite this, the challenge of exposing the environment to toxic organic solvent is still there during the QuEChERS methodologies. The level of pollution of the environment with organic solvents is reduced if comparing QuEChERS with other sample preparation techniques but it still contributes to pollution to a certain extend. Thus, it is imperative that researchers should explore the possibility of performing QuEChERS using non-organic solvents, such as ionic liquids and natural deep eutectic solvents, which are environmentally benign and make the technique relatively greener. In addition, recovering and re-cycling of the organic solvents used during QuEChERS can enhance the greenness of this technique.

The QuEChERS pre-treatment technique make the sample just clean enough to enable analysis of pesticides using GC or LC. A certain percentage of interfering components in the matrix remains in the sample after application of the QuEChERS technique. These interfering components may be co-extracted and may affect negatively on the chromatographic analysis. The cleanliness of the sample can be greatly improved through coupling of the QuEChERS technique with other sample preparation techniques during pesticide analysis in food matrices.

Another future perspective on QuEChERS approach could be its automation. There is limited use, if any, of automation when using the QuEChERS technique. Taking into account the fact that laboratories are now encountering large numbers of samples, automation would ensure rapid pre-concentration of large quantities of samples and would make the QuEChERS technique even faster. Thus, automation of the QuEChERS clean-up procedure helps save cost while improving productivity, throughput and reproducibility.

## 4. Conclusion

QuEChERS is a versatile pre-treatment technique that has been successfully applied during the analysis of pesticides in different complex matrices such as biological, environmental and agricultural samples. The versatility of the QuEChERS technique is derived from, among other things, the possibility of introducing modifications based on the application of different solvents, salts, buffers and sorbents. This allows the QuEChERS methodology to be implemented in the analysis of broad spectrum of analytes and matrices. The QuEChERS extraction method has the advantage of being a dynamic, simple and fast procedure, with few analytical steps, which minimize errors. In addition, it is cheap and environmentally benign due to the use of small volumes of organic solvents. However, there are very few researches where the QuEChERS technique is coupled with other pre-concentration methods. Coupling of QuEChERS is an aspect that can be the focal point of future researches with the aim of improving selectivity and extraction efficiency.

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## Conflict of interests

There is no conflict of interests

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# 4.3. Paper III

This paper “Green pre-concentration techniques during pesticide analysis in food samples: A review” was submitted *to Journal of Cleaner Production.* The eco-friendly pre-concentration techiques were described in this paper as well as their application for the analysis of pesticides in food samples.

# Green pre-concentration techniques during pesticide analysis in food samples: A review

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## ABSTRACT

The ever-increasing demand for determining pesticides at low concentration levels in food matrices requires a preliminary step of pre-concentration in order to employ a detection technique characterised by high sensitivity at low limits of quantification. Pesticide pre-concentration, therefore, is considered as crucial part of analytical procedures used during trace analysis of pesticides in food matrices. Environmental concerns should be taken into consideration when selecting the pre-concentration technique to use. Recently, the parameter of "greenness" during sample pre-concentration of pesticides in food matrices is as important as selectivity in order to avoid using harmful organic solvents during sample preparation. Developing new green pre-concentration techniques is one of the key subjects in green chemistry in order to minimize the release of large volume of toxic organic solvents into the environment. Thus, to reduce the impact on the environment during trace analysis of pesticides in food matrices, new developments in pesticide pre-concentration have gone in three separate directions: one is the search for more environmentally friendly solvents, the second one is miniaturization and the third one is the development of solvent-free pre-concentration techniques. Eco-friendly solvents such as supercritical fluids, ionic liquids and natural deep eutectic solvents have been developed for use as extraction solvents during pre-concentration of pesticides in food matrices. Also miniaturized pre-concentration techniques such as QuEChERS, dispersive liquid-liquid micro-extraction and hollow-fibre liquid phase micro-extraction have been used during trace analysis of pesticides in food samples as well as solvent-free techniques such as solid phase micro-extraction and stir bar sorptive extraction. All these developments are geared to ensure that pesticide pre-concentration in food matrices is green.

*Keywords*

Green chemistry, food samples, miniaturization, pesticides, pre-concentration

## Contents

## 1. Introduction

There is general trend recently towards pre-concentration techniques that are environmentally benign during pesticide analysis in food samples. Green pre-concentration techniques and methodologies should be, among other things, capable of reducing or eliminating chemicals hazardous to human health or environment (Tranchida et al., 2015). During pesticide analysis in food matrices, more often than not, hazardous chemicals are released into the environment at the sample preparation step since it is the most solvent-consuming stage (Kong et al., 2016; Pinto et al., 2010). Thus, sample preparation should be carefully managed if researchers are to move towards the goal of green chemistry.

The importance of sample preparation during pesticide analysis in food matrices cannot be over-emphasized. Despite the significant advances in analytical instruments and approaches that have been made in recent years, sample pretreatment procedures must still be considered prior to an instrumental determination of pesticides in food samples since they have a large amount of potential interferences (Cheng et al., 2010) and the pesticides are usually found in trace amounts. Thus, the pre-treatment step generally plays the combined roles of extraction, cleanup and pre-concentration as well as rendering the pesticides into a form that is compatible with the analytical instrumentation (Ahmad et al., 2015; Yilmaz and Soylak, 2016).

There is a variety of traditional methods that can be used for pre-concentration during trace analysis of pesticides in food matrices. Such methods include liquid-liquid extraction (LLE) and solid phase extraction (SPE) (Hou et al., 2014; Sajid et al., 2017; Shamsipur et al., 2016; Zhao et al., 2016). The operation of LLE is based on the equilibrium distribution/partition coefficient between two immiscible liquids. On the other hand, SPE operation principle is based on the selective distribution of analytes between the solid packing material and liquid mobile phase (Bordagaray et al., 2016). While these methods perform the above tasks adequately, they also suffer a number of drawbacks. Both LLE and SPE are environmentally unfriendly due to the large amounts of organic solvents used, they are slow, and labour intensive (Quigley et al., 2016). These traditional methods do not conform to the dictates of green chemistry. Organic solvents are recognized as problematic for several reasons. Most of them are toxic to living organisms and harmful to the environment, therefore special care has to taken for their proper disposal. Moreover, they should be of high-grade purity to avoid the contamination of extracts in trace analysis, and this alone significantly contributes to the high cost of analysis. The use of an SPE method also requires the purchase of solid phase extraction cartridges (Prosen, 2014).

In order to overcome the limitations of the traditional pre-concentration techniques mentioned above, many green methods based on principles of green analytical chemistry have been developed in recent years for the pre-concentration of pesticides in food matrices. For instance, new protocols of liquid-phase micro-extraction (LPME) methods such as single-drop micro-extraction (SDME), hollow-fibre LPME (HF-LPME), and dispersive liquid- liquid micro-extraction (DLLME) have found recently widespread application during pre-concentration of pesticides in food samples (Farajzadeh et al., 2014). These miniaturized pre-concentration techniques are cheap, quick and very effective during trace analysis of pesticides in food matrices. The volume of solvent used during pre-concentration of pesticides is on the microliter scale and therefore there is limited interaction with the solvent during execution of these micro-extraction techniques. Moreover, most of them combine separation, pre-concentration, and sample introduction in a single step. The most significant advantage of these methods is that almost all of the microliter volumes of the organic extraction phase used can be introduced into the detection systems while only a small amount of the large volume of concentrated solvent used in traditional pre-concentration techniques is introduced into the analytical instrument (Yilmaz and Soylak, 2016). Thus, the miniaturized, modern pre-concentration techniques are green and have an edge over their traditional counterparts.

The main focal point of the present paper is to review the green pre-concentration techniques that are used during trace analysis of pesticides in food samples that are environmentally friendly. The green direction in pre-concentration methodologies is achieved mainly through miniaturization and the use of environmentally friendly solvents during pesticide analysis. Many green, miniaturized pre-concentration techniques have been reported but there are very few review papers that solely and clearly outline the green methodologies that pertain to pesticide trace analysis in food matrices. Thus, this paper seeks to put together the recent green micro-extraction techniques as well as outlining their basic principles. The analytical merits and challenges, when using these techniques during analysis of pesticides in food samples, were discussed in this review.

### *1.1.Strategies to make pesticide pre-concentration green*

Researchers, in a bid to reduce the negative environmental impacts of organic solvents during trace analysis of pesticides, have come up with strategies that make the pre-concentration process greener. These green strategies generally take three dimensions: miniaturisation, use of environmentally friendly solvents and the use of solvent-free pre-concentration methods (Fig. 1).

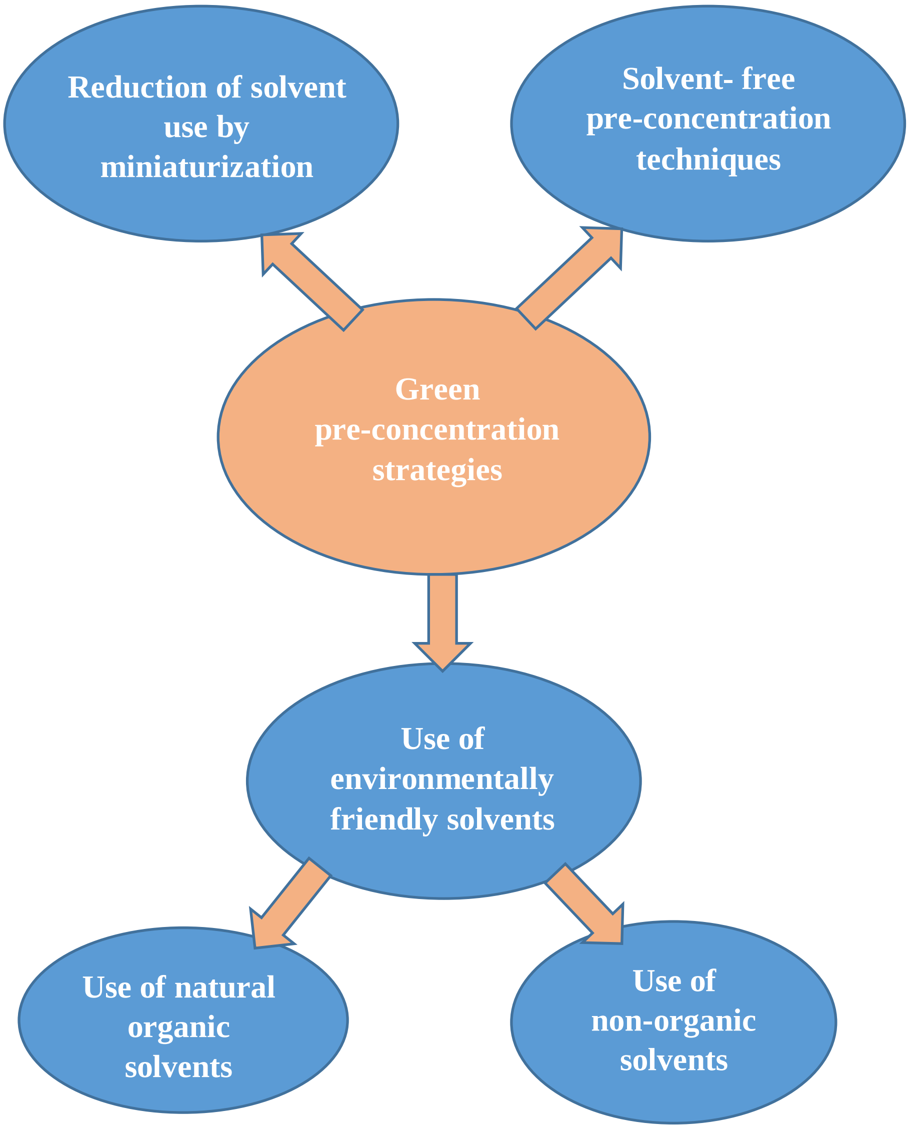

**Fig. 1.** Strategies to make pesticide pre-concentration in food matrices green

### *1.1.1. Miniaturization*

Environmental concerns and economical standpoints, recently, are the most important issues in considering the analytical process to use during pesticide analysis in food matrices (Kabir et al., 2017). In order to achieve ecological, environmentally friendly and economic analyses we have to think about the miniaturization of the sample pre-concentration step and analytical instrumentation used during pesticide analysis (Huat and Bola, 2012; Rutkowska et al., 2014).

Microscale pre-concentration of pesticides in food matrices can result in numerous analytical merits (Saito et al., 2002). Miniaturized pre-concentration can cause high extraction efficiency of pesticides as well as high speed during pesticide analysis. The operational cost of the pre-concentration process is greatly reduced in a miniaturized procedure due to low or no solvent consumption (Manousi et al., 2017). Above all, miniaturisation results in greener pesticide pre-concentration processes due to massive reduction in organic solvent consumption during trace analysis of pesticides (Yilmaz and Soylak, 2016) (Table 1). A lot of green, miniaturized techniques have been used by researchers to pre-concentrate pesticides in food samples and these include QuEChERS, DLLME, SPME and HF-LPME.

### *1.1.2. Environmentally friendly solvents*

Developing a cost effective and environmentally benign solvent system is of the utmost importance during the green pre-concentration of pesticides in food matrices (Liu et al., 2015). One dimension to ensure green pre-concentration of pesticides in food samples is the replacement of conventional hazardous volatile organic solvents by non-volatile alternatives that are environmentally friendly (Chen et al., 2016). The parameter of "greenness" is as important as selectivity in order to reduce the intensity of anthropogenic activities related to analytical laboratories (Płotka-Wasylka et al., 2017) during pre-concentration of pesticides in food matrices. Recently, some green solvents have appeared that can be used as possible replacements of the toxic organic solvents during trace analysis of pesticides in food matrices. Such solvents include supercritical fluids, ionic liquids, surface-active agents (surfactants) and natural deep eutectic solvents.

Supercritical fluid extraction has become one of the green pre-concentration techniques used in the field of pesticide analysis in food (Poustka et al., 2003). Its main advantages are the replacement of organic solvents with environmentally acceptable supercritical fluids (such as carbon dioxide), high recovery, short extraction time, and selectivity in the extraction process (Berglof et al., 1999). The SFE technique has been exploited for the quantitative extraction of organic compounds, such as pesticides from a variety of environmental and food matrices. For instance, Pearce et al. (1997) pre-concentrated multi-class pesticides in strawberries using supercritical extraction with carbon dioxide at 4000 psi and 50°C as the supercritical fluid. Poustka et al. (2003) also used supercritical extraction to pre-concentrate several groups of pesticides in cereals, cereal products, fruits and vegetables. The widespread use of supercritical fluid extraction during pesticide pre-concentration in food matrices, however, is hampered by high operating costs and complex method optimization.

Ionic liquids (ILs) are gaining widespread recognition as greener solvents during trace analysis of pesticides in food matrices (Wang et al., 2016). ILs generally consist of bulky, non-symmetrical organic cations such as imidazolium, pyrrolidinium, pyridinium, ammonium or phosphonium combined with numerous different inorganic or organic anions such as tetrafluoroborate and bromide anions (Han and Row, 2010). Their structure gives them unique properties such as a negligible vapour pressure, good thermal stability, tuneable viscosity and miscibility with water and organic solvents, as well as good extractability for various organic compounds and metal ions (Toledo-Neira and Álvarez-Lueje, 2015). The ILs are suitable replacements for toxic organic solvents during pre-concentration of pesticides in food matrices. The non-flammable, non-volatile nature of ILs makes them an excellent choice for the development of greener pre-concentration techniques. Two important aspects make ILs interesting materials. First, small modifications to the IL structure are accompanied by a dramatic change in a number of IL physical and chemical properties such as hydrophobicity, viscosity, water solubility and viscosity. Furthermore, the incorporation of different moieties within the IL structure can promote different interactions with analytes, resulting in impressive solvation capabilities for different compounds (Trujillo-Rodríguez and Anderson, 2017). ILs, therefore, are regarded as “designer solvents” because of this tuneable nature, which increases their potential applications (Han and Row, 2010) during pre-concentration of pesticides in food matrices. The ILs have been widely used as extraction solvents during the dispersive liquid-liquid micro-extraction (DLLME) technique. For instance, Wang et al. (2016) used IL-DLLME technique to pre-concentrate benzoylurea insecticides in honey samples. Gure et al. (2015) used vortex-assisted IL-DLLME for the analysis of sulfonylurea insecticides in wine samples.

An alternative to ILs are deep eutectic solvents (DESs) introduced in 2003 (Abbott et al., 2004). DESs are generally formed by mixing a hydrogen bonding acceptor with a hydrogen bonding donor after continuous heating and stirring (Chen et al., 2016). This eutectic mixture has comparable characteristics to ILs but are cheaper to produce due to lower cost of the raw materials, less toxic, and often biodegradable (Piemontese et al., 2017). Due to their green aspects, DESs have gained the attention of researchers as potential green replacements of organic solvents (Chen et al., 2016; Morais et al., 2018; Piemontese et al., 2017). The DESs are slowly finding applications as extraction solvents during analysis of pesticides. For instance, Florindo et al. (2017) successfully used DESs for the extraction of pesticides in wastewater. Pirsaheb and Fattahi (2018) used liquid-phase micro-extraction based on freezing of a DES during the analysis of pesticides in environmental water. However, the high viscosity and solid state of most DES at room temperature act as obstacles in their application as extraction solvents (Morais et al., 2018).

To overcome the drawbacks of DESs, natural deep eutectic solvents (NADES) were developed (Bajkacz and Adamek, 2018; Katarzyna et al., 2016). NADES are obtained exclusively from natural components (sugars, organic acids, amino acids) which are commonly present in the cells of living organisms, unlike ordinary DESs (Bajkacz and Adamek, 2018). They fully represent green chemistry principles and offer many advantages, including readily available components, low costs, simple preparation, a low toxicity profile and sustainability. These solvents are characterized by very good physicochemical properties: negligible volatility, a liquid state even at temperatures far below 273 K, adjustable viscosity, a wide polar range and a high degree of solubilisation strength for different compounds (Katarzyna et al., 2016). NADES offer the possibility of combining various molecules, leading to the preparation of tailor-made solvents. Thus, NADES are often considered as “designer solvents”. This made them to gain recognition in analytical chemistry as greener and safer alternatives to organic solvents (Sut et al., 2017). Currently, the use of NADES as extraction solvents for phenolic compounds is the most studied application by far (Alañón et al., 2018; Gu et al., 2014; Paradiso et al., 2016). Bajkacz and Adamek (2018) developed a method based on NADES for the extraction of flavonoids from food samples. Bosiljkov et al. (2017), on the other hand, developed an ultrasound-assisted method for the extraction of wine lees anthocyanin using NADES.

### *1.1.3. Solvent-free techniques*

The development of new pre-concentration techniques for the food industries has received a lot of attention lately due to the environmental restrictions, reducing wastewater, and the need for minimizing the energy costs in line with the goals of green chemistry (Xu et al., 2016). Recently, as a response to environmental restrictions, green techniques that are based on sorptive extraction have gained popularity from researchers during trace analysis of pesticides in food matrices. These techniques include solid phase micro extraction (SPME) and stir bar sorptive extraction (SBSE). Indeed, sorptive extraction has proven to be an interesting pre-concentration technique during pesticide analysis in food matrices, as it requires a little quantity of the aqueous sample and no organic solvents (Souza-Silva et al., 2015). The “greenness” of SPME and SBSE, therefore, is largely due to their solvent-free nature as well as their miniaturized state.

One of most widely used sorbent in SPME and SBSE is polydimethylsiloxane (PDMS). This plays the role of adsorbing the pesticides from the food matrices. The main difference between SPME and SBSE is the much larger volume of PDMS used in the latter, which results in higher recoveries and higher sample capacity. After the pre-concentration step, the pesticides can be introduced quantitatively into the analytical system by thermal desorption or by liquid desorption (Margoum et al., 2013). Liquid desorption, however, requires the use of small volumes of organic solvents.

## 2. Green pre-concentration techniques for pesticide analysis in food samples

Green technology is the need of present time in order to keep environment healthy, pollution free and sustainable for future generations (Singh et al., 2015). Thus, currently the selection of a pre-concentration technique during analysis of pesticides in food matrices should be informed, among other things, by the following attributes of green chemistry: use of safer solvents and less or zero emission of hazardous chemicals into the environment (Chanshetti, 2014). Moreover, the technique should strive to maximize the incorporation of all the chemicals used into the final product and a general drive towards real time analysis to minimize pollution of the environment (Dhanani et al., 2015). Many green pre-concentration techniques have been applied during pesticide analysis in food matrices and these include QuEChERS, DLLME, SPME, HF-LPME and MASE

### *2.1. QuEChERS*

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is one of the green pre-concentration techniques that has gained tremendous popularity among researchers during pesticide analysis of food and agricultural samples. It is a two-step green pre-concentration method composed of an extraction step and a clean-up step (Fig 2) (Nantia et al., 2017). The extraction step is based on partitioning of the analyte between an aqueous and an organic layer while the clean-up step involves the use of combinations of $MgSO_4$ with different sorbents, such as $C_{18}$, graphitized carbon black (GCB) or primary-secondary amines (PSA), to remove interfering substances (Rizzetti et al., 2016). It is effective for cleaning-up food samples such as fruits and vegetables during analysis of pesticides (Table 1). The green aspects of the technique stem from the fact that QuEChERS is inexpensive and uses smaller volumes of organic solvents as compared to conventional pre-concentration techniques. Moreover, it is a very simple pre-concentration technique that can be quickly performed yielding high enrichment factors (Abdel-Ghany et al., 2016). Due to all its advantages, the QuEChERS pre-concentration method has been increasingly used and adopted as recommended method for determination of pesticide residues in food matrices.

With the appropriate sorbents and extraction reagents at hand, one will be ready to perform the QuEChERS technique to analyse pesticides in food matrices. The first step, before analysing real food samples, is optimisation of the QuEChERS extraction parameters. Optimisation involves the investigation of most of the parameters that influence the QuEChERS extraction efficiency of pesticides from food matrices to pave way for the use of optimal conditions during analyses and to achieve the best possible extraction results (Muhammad et al., 2017). Thus, the importance of optimisation cannot be over-emphasised during pesticide analysis using the QuEChERS methodologies. The parameters to be optimised during pesticide analysis include type and volume of extraction solvent, quantity of sorbents, pH of the sample, centrifugation time and speed, and quantity of extraction salts (Ferreira et al., 2016a; Han et al., 2016; Wang et al., 2017). It should be noted that although the original publications for all the standard QuEChERS methods indicate specific amounts of reagents, it is important to optimize each step in QuEChERS for the compounds under investigation, as the optimal parameters may vary by analyte and matrix.

There are three common standard procedures when performing the QuEChERS technique. These are the original (unbuffered) QuEChERS method, Association of Analytical Committee (AOAC) QuEChERS method and the buffered European (EN) QuEChERS method. These methods are referred to as standard methods because international regulatory bodies such as the European Committee and the AOAC in the United States accept them. The basic steps are the same for all the three standard methods: a liquid-liquid extraction between an organic phase and water using salts for liquid–liquid partitioning. The sample is stirred, centrifuged, and an aliquot of the organic extract is removed and subjected to a d-SPE clean up using a sorbent such as primary secondary amine (PSA). The sample can then be analysed using GC or LC. The differences among the methods are mainly in the type of buffers used as well as the type and amounts of sorbents used. The QuEChERS method has recently found a lot of application during the analysis of pesticides in food matrices such as cowpea (Han et al., 2016), honey samples (Tette et al., 2016) and cucumber samples (Dankyi et al., 2015) (Table 1).

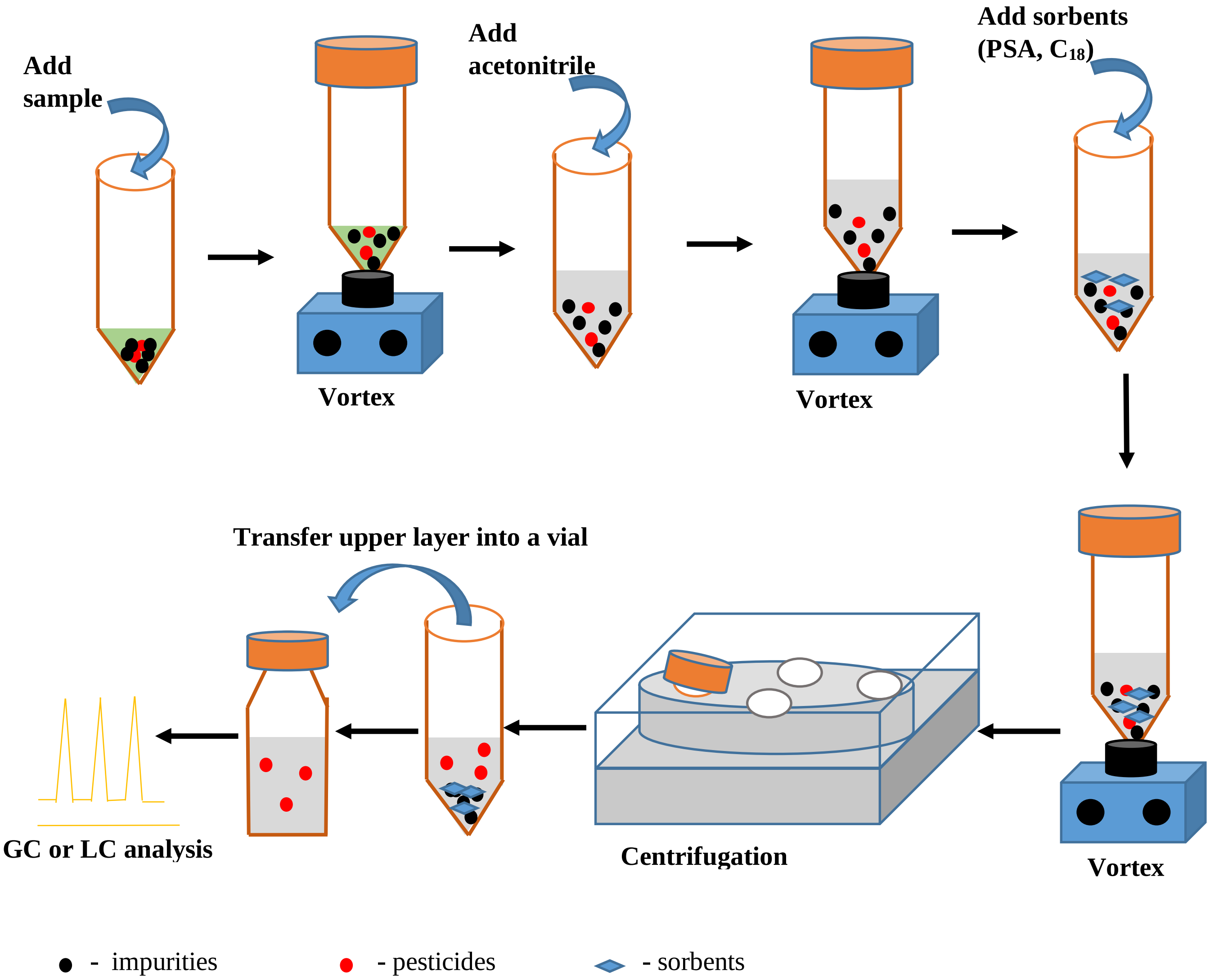

**Fig. 2.** Schematic diagram for the QUEChERS technique during pesticide analysis

**Table 1**

Pesticides analysed using QuEChERS technique

| Analyte | Pre-concentration technique | Extraction solvent | Clean-up process | Matrix | LOD (mg kg$^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| Organochlorines | QuEChERS-DLLME-SFO | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | Fish | 0.00065 – 0.00158 | Wang et al., 2017 |
| Multiclass pesticides | QuEChERS combined with freeze out using dry ice. | 1% acetic acid in acetonitrile | d-SPE using $MgSO_4$ and, $C_{18}$ and PSA sorbents | Coconuts | *0.01 | Ferreira et al., 2016 |
| Multiclass pesticides | QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and, $C_{18}$, PSA and GCB | Tobacco | *0.025 -0.075 | Bernardi et al., 2016 |
| Organochlorines | QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and PSA sorbents | Raw food (beans, carbages, beef and fish) | ng | Nuapia et al., 2016 |
| Multiclass pesticides | AOAC QuEChERS | 1% acetic acid in 10ml of acetonitrile : ethyl acetate (70:30 v/v) | d-SPE using $MgSO_4$ and , Florisil and PSA sorbents | Honey | 0.005 | Tette et al., 2016 |
| Herbicides | EN QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | Brown rice | 0.003- 0.015 | Lee et al., 2016 |
| Neonicotinoids | EN QuEChERS | Acetonitrile | d-SPE using $MgSO_4$ and $C_{18}$ sorbents | Cucumber | 0.08 – 6.06 | Abdel-Ghany et al., 2016 |
| Multiclass pesticides | QuEChERS | Acetonitrile | r- DSPE using multi-walled carbon nanotubes | Cowpea | 0.001 – 0.03 | Han et al., 2016 |

**Note**: ng – not given; * - converted from $\mu g\ kg^{-1}$ to mg kg$^{-1}$; r-DSPE - reversed- dispersive solid phase extraction

### 2.2. Dispersive liquid-liquid micro-extraction

Rezaee et al. (2006) introduced the green pre-concentration technique called dispersive liquid–liquid micro-extraction (DLLME). It is a simple and fast pre-concentration technique based on the use of an appropriate extraction solvent and a dispersion solvent. A few micro-litres of an organic extraction solvent with a higher density than water is used during trace analysis of pesticides in food matrices (Fig 3a). Suitable solvents to use for extraction of pesticides in food matrices include tetrachlorometane, chloroform, carbon disulphide, nitrobenzene, bromobenzene, chlorobenzene or 1,2-dichlorobenzene (Hou et al., 2014). A disperser solvent, on the other hand, should be highly miscible with both the extraction solvent and the aqueous phase. Methanol, ethanol, acetonitrile and acetone are the dispersion solvents commonly used during pre-concentration of pesticides in food matrices.

During the DLLME procedure, a mixture of the extraction and dispersion solvents is rapidly injected into the aqueous sample (Wang et al., 2016). This results in the production of a high turbulence in the aqueous sample. This turbulent mixture gives rise to the formation of small droplets, which are dispersed throughout the aqueous sample (Moema et al., 2012). Emulsified droplets have large interfacial area, which facilitates mass transfer and increase reaction rate (Wang et al., 2017). The nature of the emulsifier (dispersion solvent) can have an influence on droplet size distribution, the mean droplet size, and on emulsion viscosity (Zhang et al., 2014).

One major drawback of DLLME, however, is the availability of a limited number of extraction solvents that have a density higher than that of water. To overcome this limitation and to increase the applicability of DLLME, researchers have recently robbed in low density solvents during the pre-concentration process using a mode called DLLME with solidification of floating organic drop (DLLME-SFO) (Fig 3b). For instance, Hou et al. (2014) used DLLME-SFO for the determination of pyrethroid pesticides in tea samples. (Wang et al., 2017) determined organochlorine pesticides in fish using DLLME-SFO coupled with QuEChERS.

DLLME is a well-established method for the pre-concentration of pesticides in aqueous food samples (Chen et al., 2016; Wang et al., 2017) during pesticide analysis (Table 2). The widespread use can be attributed, among other things, to its green aspects. The green attributes of DLLME include simplicity, low cost and high enrichment factors. Above all, DLLME uses very low volumes of organic solvents and, as a result, it is environmentally friendly.

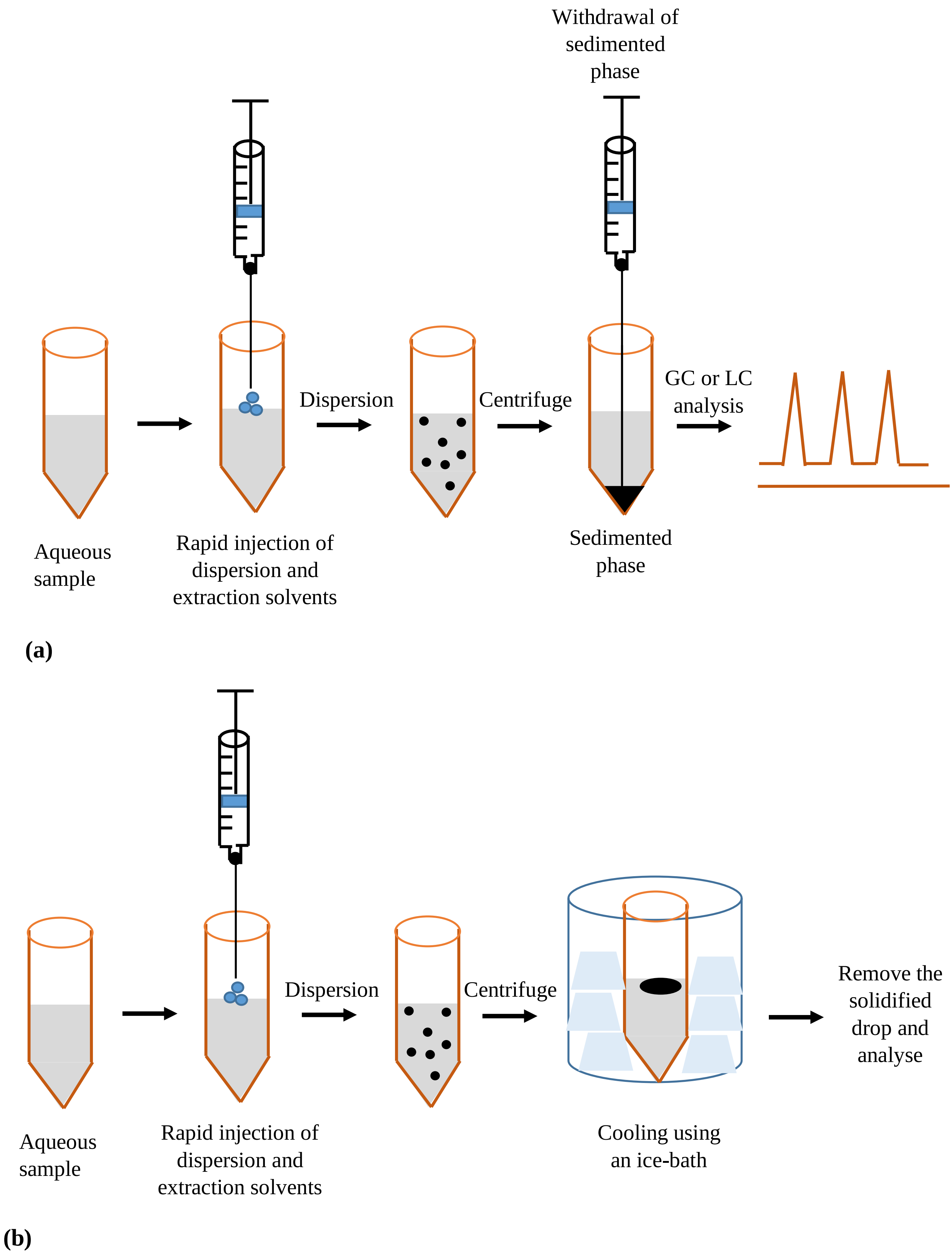

**Fig. 3.** Schematic diagram for DLLME procedure. (a) Using high-density extraction solvents. (b) Using low-density extraction solvents.

**Table 2**

Pesticides in food matrices analysed using DLLME techniques

| Pesticide class | Pre-concentration technique | Extraction solvent | Dispersive solvent | Matrix | LOD ($\mu g\ L^{-1}$) | Ref |
|---|---|---|---|---|---|---|
| Organophosphates | DLLME | Trichloroethane | Acetonitrile | Hawthorn juice | *0.05 – 0.1 | Ren and Xia, 2016 |
| triazine herbicides | MSPD-MIL-DLLME | 1-butyl-3-methylimidazolium tetrachloroferrate | nr | oilseeds | ng | Wang et al., 2015 |
| Pyrethroids | QuEChERS -DLLME | Carbon tetrachloride | Acetone | Fruit juices | 0.2 – 2 | Zhang et al., 2014 |
| Pyrethroids | UA- DLLME-SFO | 1-dodecanol | Ethanol | Tea | ng | Hou et al., 2014 |
| Sulfonylurea | VA-IL-DLLME | 1-hexyl-3-methylimidazolium hexafluorophosphate | Methanol | Wine | 3.2- 6.6 | Gure et al., 2015 |
| Herbicides | DLLME | Chloroform | Acetonitrile | Wine | 0.025- 0.88 | Chen et al., 2016 |
| Fungicides | UDSA-DLLME | 1-octanol | ng | Wine | 0.007- 0.025 | Chu et al., 2015 |
| Mixture of pesticides | CCSHLIE-DLLME | 1,1,2,2-tetrachloroethane | Acetonitrile | Fruit juices | 2.0 – 12 | Farajzadeh et al., 2016 |
| Benzoylurea | IL-DLLME | Tetrabutyl ammonium hexafluorophosphate | ng | Honey | 2.1 – 4.2 | Wang et al., 2016 |
| Benzoylurea | In-syringe SIL-DLLME | Tricaprymethyl ammonium hexafluorophosphate | ng | Tea beverages | 0.29 – 0.59 | Wang et al., 2017 |

**Note**: ng – not given; nr- not required; MSPD-MIL-DLLME- matrix solid-phase dispersion combined with magnetic ionic liquid dispersive liquid-liquid micro-extraction; IL- ionic liquid; VA – vortex-assisted; UA – ultrasound-assisted; CCSHLIE - counter current salting-out homogenous liquid–liquid extraction; UDSA – up and down shaking-assisted; *- converted from ng mL$^{-}$1 to $\mu g\ L^{-1}$.

### *2.3. Solid phase micro-extraction*

One of the most significant developments in sample pre-concentration has been solid phase micro-extraction (SPME), a technique first described by Arthur and Pawliszyn in 1990 (Kenessov et al., 2016). SPME is an advanced, green sample pre-concentration technique integrating sampling, extraction, concentration and sample introduction into a single step (Zhang et al., 2017; Wu et al., 2016; Wang et al., 2013) during pesticide analysis. One of the most important aspect that makes it to be appealing to researchers is the fact that it is a solvent free pre-concentration technique (Saraji et al., 2016; Wang et al., 2016). This makes the SPME process to be environmentally friendly and green.

SPME is based on the partition of analytes between the sample phase and a solid extracting phase (the sorbent) (Saraji et al., 2016). It consists of a coated polymeric fibre that is exposed to a sample in order to extract the pesticides from food matrices. The fibres can be exposed to the pesticides in food samples in two different operation modes: direct immersion (DI) and headspace (HS) approach (Bordagaray et al., 2016; Farajzadeh et al., 2014) (Fig 4). However, the sample components with matching polarity might adsorb on the fibre creating a capacity problem in DI conditions. HS sampling, on the other hand, provides a kind of selectivity as the volatiles and semi volatiles can only be collected on the fibre thus, providing a long life and reproducible performance (Pelit et al., 2015).

Different parameters affect the SPME extraction efficiency during pre-concentration of pesticides in food samples, among them: type of coating, extraction time, extraction temperature, salt addition, pH, agitation, sample volume and headspace volume (Bordagaray et al., 2016). These variables, therefore, should be optimized during SPME method development when analysing pesticides in food samples.

SPME is an equilibrium based extraction method. The extraction efficiency in SPME is determined by the distribution of the pesticides between the food matrix and the fibre coating (Liang et al., 2017). Therefore, the fibre coating material plays the most important role in SPME (Zhang et al., 2017). One of the major analytical limitations of SPME is the availability of a limited number of commercially coated fibres. Some of the few commercially available fibres with different coating and thickness include those with polyacrylate (PA), polydimethylsiloxane (PDMS), and divinylbenzene (DVB) sorbent materials (Aulakh et al., 2005). To get around this limitation some researchers have developed SPME methodologies for the pre-concentration of pesticides in food matrices using other sorbents. For instance, Jafari et al. (2016) used halloysite nanotube-titanium dioxide fibre as SPME fibre during the pre-concentration of diazinon and parathion in fruit juices. Liang et al. (2017) pre-concentrated pesticides in tomatoes and cucumbers using barley husk carbon as the SPME fibre coating. Zhang et al. (2017), on the other hand, used nanoporous carbon –coated fibres for the SPME pre-concentration of pyrethroids in fruits and vegetables. The possibility of using other sorbents during the SPME technique as well as its convenience, speed and green aspect, have made SPME to be implemented by many researchers during pre-concentration of pesticides in food matrices (Li et al., 2016; Wu et al., 2016; Pelit et al., 2015; Zhang et al., 2017). Applications of SPME during the analysis of pesticides in food samples are summarised in Table 3.

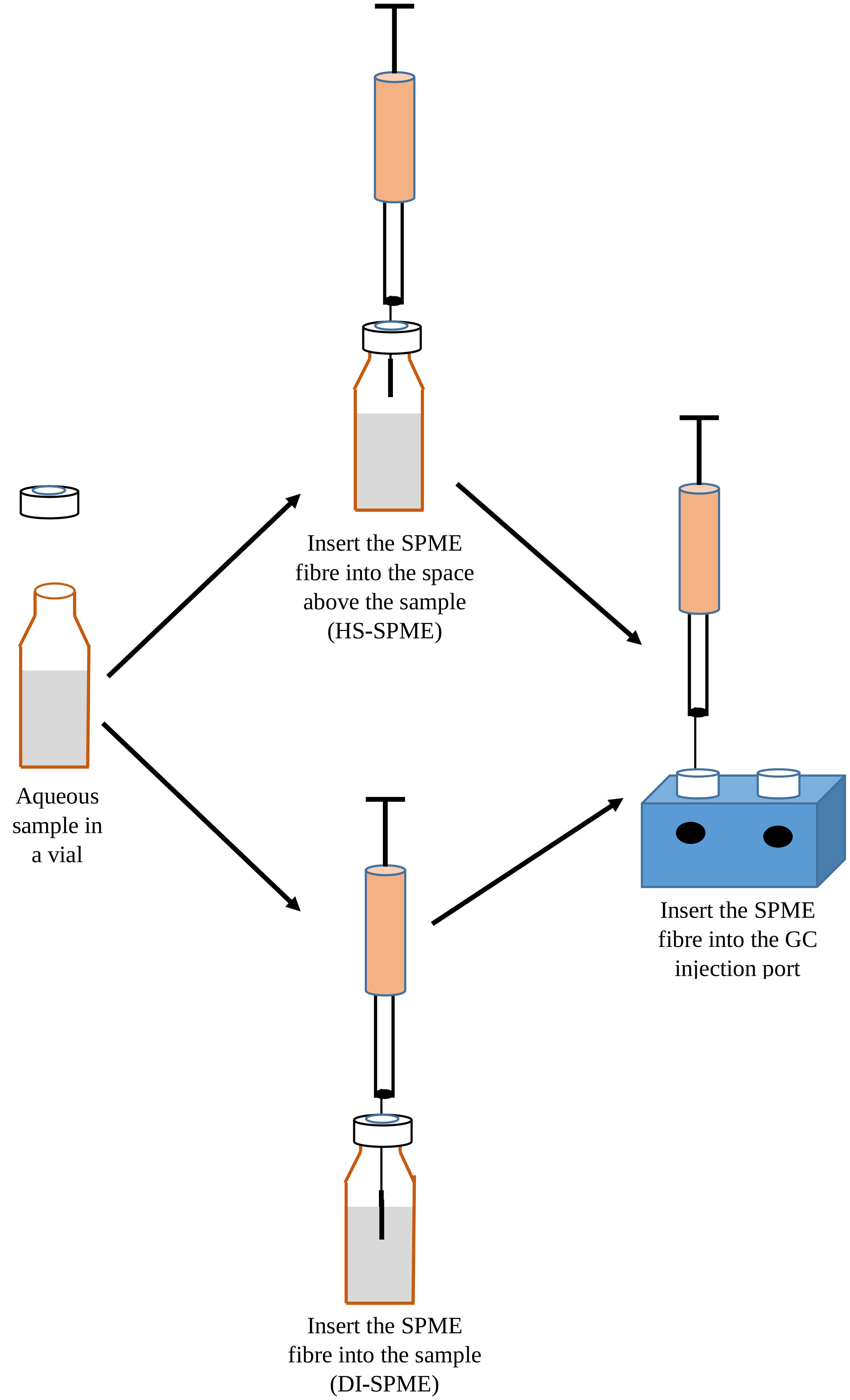

**Fig. 4.** Schematic diagram for the pre-concentration of pesticides in food matrices using SPME

**Table 3**

Application of SPME for the analysis of pesticides in food samples

| Pesticide class | Food matrix | Fibre type | Mode of application type | Chromatographic technique | LOD ($\mu$g L$^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| Organophosphates | Apples and pineapples | Calixarene MIP fibre | HS-SPME | GC-NPD | 0.0051-0.065 | Li et al., 2016 |
| Benzoylurea insecticides | Fruit juices | Poly (MA-co-ED) monolithic fibre | MMF-SPME | HPLC-DAD | 0.053-0.29 | Mei et al., 2015 |
| Pyrethroids | Fruits and vegetables | ZIF-90-NPC fibre | HS-SPME | GC-ECD | 0.1-0.5 | Zhang et al., 2017 |
| Carbamates | Apple and lettuce | PEDOT-PIL/MWCNTs | DI-SPME | GC-FID | 15.2- 27.2 | Wu et al., 2016 |
| Diazinon and parathion | Fruit juices | Halloysite nanotubes–titanium dioxide | DLLME-SPME | GC-CD-IMS | 0.005-0.007 | Jafari et al., 2016 |
| Organophosphates | Fruits and vegetables | Carbon nanotubes–silicon dioxide | SPME | GC-CD-IMS | 0.005 – 0.020 | Saraji et al., 2016 |
| 12 pesticides | Tomato and cucumber | Barley husk carbon | DI-SPME | GC-MS | 0.01- 0.05 | Liang et al., 2017 |
| Multi-class pesticides | Grape juice | polythiophene – ionic liquid modified clay | HS-SPME | GC-ECD | ng | Pelit et al., 2015 |

**Note**: ng – not given; MOF- metal-organic frameworks; poly (MA-co-ED)- poly(methacrylic acid-co-ethylene dimethacrylate); MMF- multiple monolithic fiber; NPC- nanoporous carbon; ZIF- zeolitic imidazolate framework; MIPs- molecularly imprinted polymers; PEDOT-PIL/MWCNTs- poly(3,4-ethylenedioxythiophene)-ionic liquid polymer functionalized multiwalled carbon nanotubes; CD-IMS - corona discharge ion mobility spectrometric detection

### *2.4. Hollow-fibre liquid-phase micro-extraction*

Hollow-fibre liquid-phase micro-extraction (HF-LPME) was developed by Pedersen-Bjergaard and Rasmussen (2008) and has been used by many researchers in recent years to pre-concentrate pesticides in food matrices. Its widespread use can be attributed to its green attributes such its low cost, which enables the rejection of the hollow fibre after use, eliminating problems of cross-contamination (Puri, 2014) or low reproducibility as well as its decreased consumption of organic solvents. Moreover, the process is simple and a clean-up step is not necessary and can be applied to a variety of food matrices reaching high enrichment factors (Menezes et al., 2016). The HF-LPME method display higher extraction efficiency when compared to other liquid-phase micro-extraction methods and this can be attributed to the enhancement of mass transfer process as a result of vigorous stirring during the pre-concentration process (Alsharif et al., 2017).

During the HF-LPME procedure, when pre-concentrating pesticides in food matrices, one end of a piece of a porous hollow fibre is attached to a pipette tip and the other end is mechanically sealed (Gjelstad and Pedersen-Bjergaard, 2013). Typically, this porous hollow fibre is made of polypropylene. Prior to HF-LPME, the hollow fibre is impregnated with an organic solvent such as 1-octanol. Impregnation is done through soaking the hollow fibre in the organic solvent. The organic solvent is immediately immobilized in the pores of the hollow fibre by capillary forces, forming a supported liquid membrane (SLM) (Alsharif et al., 2017). The internal lumen of the hollow fibre is then filled with an acceptor solution, and the hollow fibre is placed in the aqueous food sample. With strong agitation or stirring of the sample, the pesticides in the food sample are extracted from the sample, through the SLM, and into the acceptor solution. After equilibration, the acceptor phase is removed from the hollow fibre and injected into HPLC or GC for the final analysis.

The extraction efficiency of HF-LPME during pre-concentration of pesticides in food matrices is affected by various parameters such as the extraction mode, volume and type of extraction solvents, agitation, salt addition, and extraction time (Menezes et al., 2016). The selection of a suitable organic solvent is a critical step for efficient pesticide pre-concentration. The selected solvent should present the highest possible capacity of interaction with the pesticides. At the same time, the extraction solvent should have the potential to extract all of the pesticides in the food sample simultaneously, maintaining features such as a high affinity with the membrane and low volatility, toxicity and water solubility. The volumes of the donor and acceptor phases also have a direct effect on the extraction efficiency during pre-concentration of pesticides in food matrices using the HF-LPME technique. The pH condition influences the solubility, dissociation and equilibrium of the basic or acidic pesticides. Sample agitation is used to accelerate the extraction kinetics and to increase extraction efficiency. The agitation causes continuous exposure of the acceptor phase to the donor phase. Therefore, thermodynamic equilibrium can be achieved in a shorter time and migration of the pesticides from the donor phase via the SLM to the acceptor phase is enhanced. During the pre-concentration the ionic strength of the donor phase may be altered through the addition of salts to ensure that there are optimal conditions for the salting out effect (Bedendo et al., 2012).Thus, the importance of optimizing of these parameters during pre-concentration of pesticides in food sample should not be over-emphasised.

There are two commonly used configurations of the HF-LPME technique when pre-concentrating pesticides in food matrices: two-phase HF_LPME and the three-phase HF_LPME. In the two-phase HF-LPME configuration, the acceptor solution can be the same organic solvent as used for the SLM (Puri, 2014). In this HF-LPME configuration, pesticides are extracted from an aqueous food sample into the organic solvent based on classical partition. After the pre-concentration, since the acceptor phase is organic, it is directly injected into the GC injector port. Therefore, two-phase HF-LPME is well suited for low and medium polar analytes which are to be analysed by GC.

In another configuration, the acceptor solution can be an aqueous solution, providing a three-phase extraction system. For HF-LPME of basic pesticides, pH in the sample is made alkaline (to suppress ionization of the basic pesticides), whereas the aqueous acceptor solution is acidified (Menezes et al., 2016). Thus, the basic pesticides are extracted from the aqueous food sample and into the SLM in their neutral state, whereas protonation occurs when entering the acceptor solution. The protonation ensures a high solubility in the acceptor solution, and prevents back-extraction into the SLM (Alsharif et al., 2017). For extraction of acidic pesticides, the pH-gradient is reversed; the food sample is acidified, whereas the acceptor solution is alkaline. Since the acceptor solutions in three-phase HF-LPME are aqueous, they can be injected directly into HPLC. Three-phase HF-LPME is ideally suited for acidic or basic pesticides, which are to be analysed by HPLC.

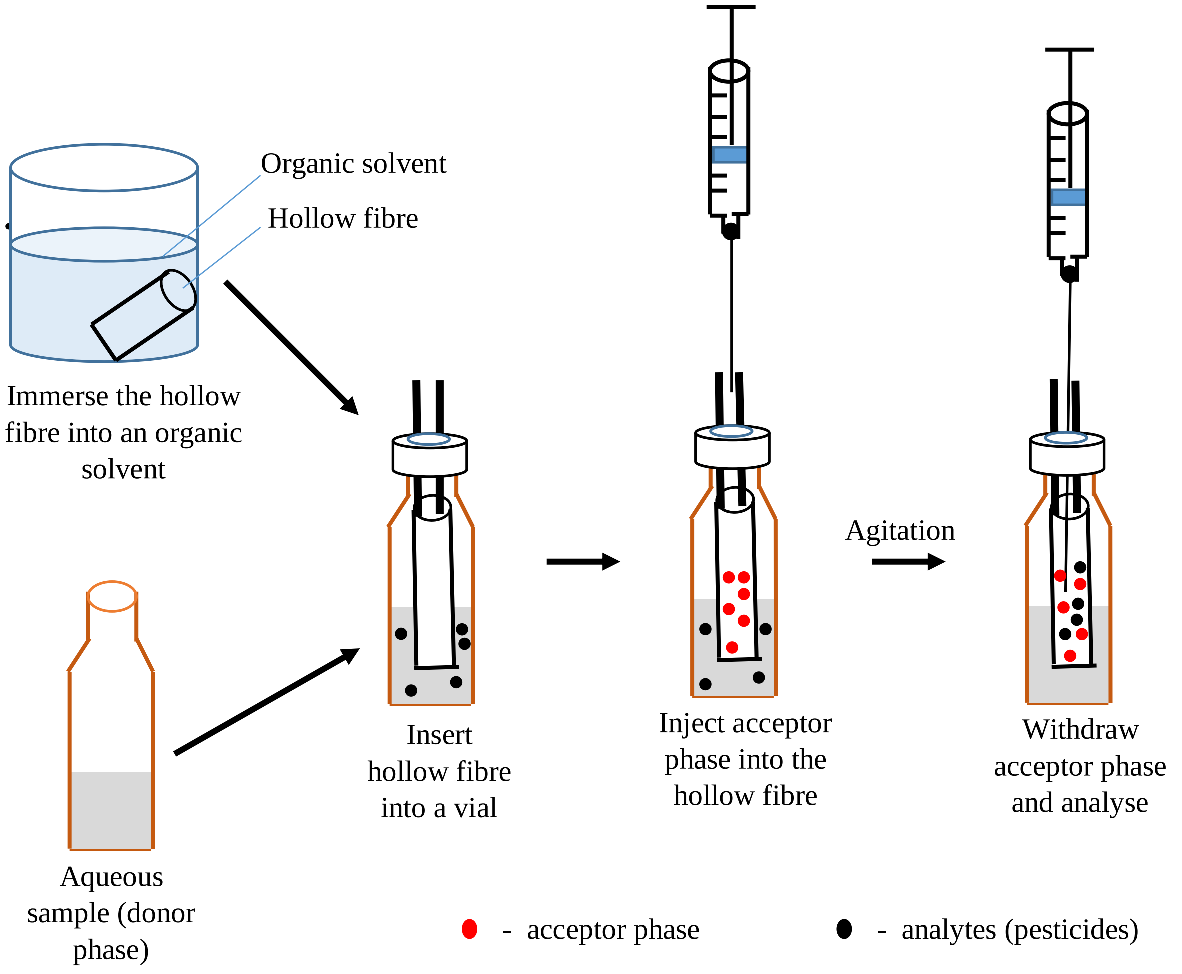

**Fig. 5.** Schematic diagram for the pre-concentration of pesticides in food matrices using HF-LPME.

**Table 4**

Application of HF-LPME for the analysis of pesticides in food samples

| Pesticides | Matrix | SLM | Analytical instrument | LOD | Ref. |
|---|---|---|---|---|---|
| Carbamates | Fruit samples | 1-octanol | HPLC-DAD | 0.2 – 1.0 ng $g^{-1}$ | Ma et al., 2014 |
| Pyrethroids | Fruits and vegetables | 1-octanol | LC-MS | 0.02– 0.07 ng $mL^{-1}$ | Alsharif et al., 2017 |
| Multi-class pesticides | Cucumber | 1-octanol | LC-MS/MS | 0.01 – 0.31 $\mu$g $kg^{-1}$ | Wang et al., 2012 |
| Organophosphates | Fish tissue | o-Xylene | GC-MS | 2.1 – 4.5 ng $g^{-1}$ | Sun et al., 2011 |
| Herbicides | Pear | Chloroform | HPLC-UV | 7.15 – 8.26 ng $g^{-1}$ | Shi et al., 2014 |
| Organophosphates | Fruit juices | 1-octanol | GC-MS | 0.018 – 0.096 $\mu$g $L^{-1}$ | Wu et al., 2015 |
| Carbamates | Vegetables | 1-octanol | GC-MS | 0.2 – 0.6 ng $g^{-1}$ | Ma et al., 2015 |

## 3. Challenges and future work

Tremendous strides have been made by researchers to make the pre-concentration process during pesticide analysis environmentally friendly and greener. This has been made possible mainly through miniaturisation of the pre-concentration techniques and, to a lesser extent, by the use of environmentally friendly solvents. The challenge is, however, that most of the miniaturized techniques still use toxic organic solvents although in small volumes. There is limited use of natural solvents such as natural deep eutectic solvents during the pre-concentration of pesticides in food samples. Moreover, research on trace analysis of pesticides in food samples is not driving towards the development of more solvent-free pre-concentration techniques such as SPME. Thus, research on trace analysis of pesticides in food matrices should take a new turn; a turn that emphasis the use of natural solvents and solvent-free pre-concentration processes. Such a move will make pre-concentration of pesticides in food samples greener.

## 4. Conclusion

As is clearly highlighted in this review paper, the extent of the green aspect of a pre-concentration technique during trace analysis of pesticides depends largely on miniaturisation and the use of environmentally friendly solvents such as ionic liquids and natural deep eutectic solvents. Researchers should take a conscious step to move away from the use of pre-concentration techniques that choke the environment with large volumes of toxic organic solvents. Instead, they should gravitate towards miniaturized pre-concentration techniques that are more effective and environmentally friendly. Several such pre-concentration techniques have been successfully applied during trace analysis of pesticides in different food matrices. Their main analytical merits being the reduction of pre-concentration time, sample manipulation, solvent consumption, and use of toxic solvents, in accordance with the green analytical chemistry concepts.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## 4.4. Paper IV

This paper "Analysis of heavy metals from vehicular emission, using vortex-assisted dispersive liquid-liquid micro-extraction, on roadside dust as well as fruits and vegetables" is a manuscript in preparation. The impact of emissions from vehicles on the concentration of heavy metals in roadside dust, and fruits and vegetables was investigated in this paper.

# Analysis of heavy metals from vehicular emission, using vortex-assisted dispersive liquid-liquid micro-extraction, on roadside dust as well as fruits and vegetables

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## ABSTRACT

The effect of vehicular emissions of Cd, Cr, Ni and Pb on roadside dust as well as fruits and vegetables sold on roadside markets along a major highway with high traffic density have been investigated. The samples were pre-concentrated using dispersive liquid-liquid micro-extraction (DLLME) prior to analysis with flame atomic absorption spectroscopy (FAAS). Dithizone, chloroform and methanol were used as chelating agent, extraction solvent and dispersion solvent respectively during the DLLME technique. The pH of the sample was adjusted to around 8 using sulphuric acid or sodium hydroxide solution in order to facilitate complexation. The influential DLLME parameters such as pH, type and volume of extraction solvent, and concentration of dithizone, were optimized prior to the application of the developed method to real samples (roadside dust, fruits and vegetables). The limit of detection (LOD) were 0.15, 0.26, 0.19 and 0.59 mg kg$^{-1}$ for $Cr^{3+}$, $Cd^{2+}$, $Ni^{2+}$and $Pb^{2+}$ respectively while the limit of quantification (LOQ) were 0.52, 0.87, 0.64 and 1.96 mg kg$^{-1}$ for $Cr^{3+}$, $Cd^{2+}$, $Ni^{2+}$and $Pb^{2+}$ respectively. The correlation coefficients ($R^2$) were 0.9982, 0.9987, 0.9987 and 0.9991 for $Cr^{3+}$, $Cd^{2+}$, $Ni^{2+}$and $Pb^{2+}$ respectively. The developed method was applied to real samples and the concentration of these heavy metals ranged from 0.5 – 1.0 mg kg$^{-1}$ in roadside dust while their concentration in fruits and vegetables ranged from 0.07 – 0.63 mg kg$^{-1}$.

*Keywords*

Heavy metals, vehicular emission, roadside dust, fruits and vegetables

## 1. Introduction

Food safety is an aspect that is of public interest in the modern world. Thus, during the past decades, the increasing demands for food safety have prompted research targeting the assessment of the risk associated with consumption of food products contaminated by toxic materials such as heavy metals as well as the determination of their concentration levels (Al-Saidi and Emara, 2014; Ferreira et al., 2015; Ghorbani et al., 2013; Seeger et al., 2017). Heavy metals are among the major contaminants of fruits and vegetables as well as roadside dust (Mortatti and Probst, 2010). Heavy metals are not biodegradable, have long biological half-lives and are therefore persistent (Jaishankar et al., 2014). They also have the potential for bio-accumulation in the different body organs leading to unwanted, adverse effects (Jaishankar et al., 2014; Mohmand et al., 2015; Osakwe and Okolie, 2016).Most heavy metals are extremely toxic, and because of their solubility in water, contamination may readily reach toxic levels. Due to their persistent nature, food chain contamination is one of the most important pathways for the entry of heavy metals into the human body (Yan et al., 2012). Sources of heavy metals in the environment include anthropogenic contamination from agricultural and industrial activities as well as vehicular emissions (Bortey-Sam et al., 2015).

Vehicular emissions, in recent years, have emerged as one of the major anthropogenic activities that causes the release of particulate matter into the environment that contain a significant amount of heavy metals (Lin et al., 2015). The heavy metals from vehicular emissions can be significant threats to humans and the environment because they have adverse effects on ecosystems inducing contamination of air, water, and soil (Cheng et al., 2015). The heavy metals generated during the operation of vehicles are derived from a number of sources, such as wearing of breaking systems, tires and clutch plates, erosion of the active layer of the catalytic converter and combustion of fossil fuels (Adamiec et al., 2016; Lin et al., 2015).

Automotive emissions, therefore, may contaminate fruits and vegetables as well as roadside dust with heavy metals. This in turn, would expose the consumers of these fruits and vegetables to a higher concentration of heavy metals. On the other hand, the heavy metals from vehicular emissions may be washed into water bodies by rain resulting in the pollution of water bodies. This might prove toxic to aquatic life as well as organisms that consume aquatic plants and animals (Fang and Zhu, 2014). In general, the presence of heavy metals in high concentrations in the environment result in health hazards such as adversely affecting the nervous, blood forming, cardiovascular, renal and reproductive systems (Christoforidis and Stamatis, 2009; Guerra et al., 2012). Other adverse health effects of heavy metals include reduced intelligence, attention deficit and behavioural abnormality, as well as their contribution to cardiovascular disease in adults (Mohmand et al., 2015)

The analytical techniques that are commonly used to determine heavy metal residues in various samples are inductively coupled plasma optical emission spectrometry (Gaubeur et al., 2015; Sadeghi et al., 2018; Yao et al., 2017), capillary zone electrophoresis (Liu et al., 2018), graphite furnace atomic absorption spectrometry (Ghorbani et al., 2013), flame atomic absorption spectrometry (FAAS) (Elik et al., 2017; Özdemir et al., 2014; Sorouraddin et al., 2018), and inductively coupled plasma-mass spectrometry (Chen et al., 2018; Sager et al., 2018; Seeger et al., 2017; Türkmen and Budur, 2018; Zhang et al., 2018). The FAAS is a powerful and useful technique for determination of heavy metals, in terms of speed, higher accessibility and fairly low operational cost. However, the FAAS have an insufficient limit of detection and sensitivity for the determination of trace levels of heavy metals as compared to other methods such as ICP, because of the matrix interferences and low concentration levels of them. Thus, sample preparation forms an integral part during the trace analysis of heavy metals using FAAS. Complexation is also a basic requirement during the pre-concentartion of heavy metals using these techniques and the chelates increase the environmental footprint of these pre-concentration methods.

Several preparation methods including liquid–liquid extraction (LLE) (Kokare et al., 2017; Sorouraddin et al., 2017), co-precipitation (Ipeaiyeda and Ayoade, 2017; Javadian et al., 2018; Yang et al., 2018), cloud point extraction (CPE) (Altunay and Gürkan, 2015; Khan et al., 2017) and solid phase extraction (SPE) (AlSuhaimi et al., 2018; Du et al., 2018; Tuzen et al., 2016) have been used for the separation and pre-concentration of heavy metals from different samples. However, these methods have some well-known drawbacks such as high cost, unsatisfactory enrichment factors, high time consumption, multistage operations and production of large amounts of waste as well as the use of large volumes of toxic organic solvents.

To remedy these drawbacks, a current fashion in the world of analytical chemistry is the use of advanced micro-extraction procedures for the extraction of heavy metals, with particular emphasis on simplicity, miniaturization and time efficiency. These micro-extraction techniques include hollow fibre liquid phase micro-extraction (HF-LPME) (Margui and Hidalgo, 2016; Wang et al., 2015), solid phase micro-extraction (SPME) (Kazemi et al., 2017), ultrasound-assisted emulsification micro-extraction (USAEME) (Doroudi and Niazi, 2018; Yao et al., 2018) and vortex assisted liquid-liquid micro-extraction (VA-LLME) (Khan et al., 2014).

A pre-concentration technique that has gained the attention of many researchers, in recent years, is dispersive liquid-liquid micro-extraction (DLLME) (Du et al., 2018; Sadeghi et al., 2018; Seeger et al., 2017; Sorouraddin et al., 2018). DLLME is a ternary solvent system and its acceptor-to-donor phase ratio is greatly reduced compared with other methods (Ghorbani et al., 2013). In DLLME, the appropriate mixture of the extraction and disperser solvents is rapidly injected by syringe into aqueous samples containing heavy metals and a suitable chelating agent. The interaction of these reagents causes a cloudy solution to form. In fact, the cloudy state results from the formation of fine droplets of the extraction solvent which disperse in the sample solution. Then, this cloudy solution is centrifuged and the fine droplets become sediment at the bottom of the conical test tube. The determination of the heavy metals in the sedimented phase can then be performed by instrumental analysis. In this extraction method the heavy metals in the aqueous sample, derivatized through chelation, interacts with the fine droplets of the extraction solvent and consequently gets extracted from the initial solution and concentrates in the small volume of the sedimented phase. Simplicity of the operation, rapidity, low sample volume, low cost, high recovery and high enrichment factor are some advantages of DLLME (Yao et al., 2017).

In the current study, a simple and eco-friendly DLLME procedure was developed to assess the impact of vehicular emissions on the concentrations of heavy metals (lead, nickel, chromium and cadmium) on roadside dust as well as selected fruits and vegetables from roadside markets. Heavy metals can negatively affect the health of humans even at trace levels. Thus, simple and effective pre-concentration techniques should be developed to facilitate the monitoring of heavy metals in the environment. A simple, cheap, effective and novel method was developed in this study that would enable the quick pre-concentration of trace heavy metals in fruit and vegetable samples prior to analysis by FAAS.

## 2. Experimental

### *2.1. Chemicals and instruments*

All solutions were prepared using de-ionised water. The stock solutions of lead, chromium, cadmium and nickel (1000 mg $L^{-1}$ for atomic spectroscopy standard) as well as the chelating agent, dithizone, were purchased from Sigma-Aldrich (Johannesburg, South Africa). Working standard solutions were prepared by serial dilutions of the stock solution with de-ionised water prior to analysis. All the extraction and disperser solvents such as ethanol, methanol, acetone, acetonitrile, dichloromethane, tetrachloromethane and chloroform were bought from Sigma-Aldrich (Johannesburg, South Africa). Fruits and vegetables were purchased at local roadside markets in Thohoyandou, Limpopo province, South Africa.

The vortex used for mixing the chemicals during DLLME was bought from Sigma-Aldrich (Johannesburg, South Africa) as well as the centrifuge and the centrifuge tubes. The measurements were performed using a PerkinElmer PinAAcle 900T atomic absorption (AA) spectrometer (PerkinElmer Inc., Shelton, CT, USA), equipped with the intuitive Syngistix™ AA software for sample analysis, data reporting, and archiving results.

### *2.2. Sampling*

Fruit and vegetable samples (apples, pears, tomatoes and cucumber) were purchased from the local fruit and vegetable market. The roadside dust was collected along one of the busy roads, with a high traffic volume, near the local fruit and vegetable market in Thohoyandou, Limpopo province, South Africa.

### *2.3. Washing of dust on fruit samples*

To determine the concentration of Cd, Ni, Cr and Pb in the adhered dust on fruits and vegetables, the dust was collected from each fruit sample sampled from the roadside market. Samples were washed thrice with distilled water and the washing residues were collected in a beaker maintaining the final volume up to 200 mL. All beakers were heated on hot plate up to complete dryness. The dry residues were weighed and kept for Cd, Ni, Cr and Pb analysis using DLLME for their pre-concentration.

### *2.4. Acid digestion of the samples*

One gram of the sample was placed in a 250 mL digestion tube and 15 mL of concentrated nitric acid and 5 mL hydrochloric acid in the ratio 3:1(v/v) was added. The samples were heated for 45 min at 90$^{o}$C; later, the temperature was increased to 150$^{o}$C at which the samples were boiled for at least 2–3 hours until a clear solution was obtained. More concentrated nitric acid and hydrochloric acid (3:1 v/v) was added at least three times and digestion continued until the volume was reduced to about 1 mL. The interior walls of the digestion tube were washed down with a little distilled water and the tubes were swirled periodically throughout the digestion to keep the wall clean and prevent the loss of the samples. After cooling, 5 mL of 1% $HNO_3$ was added to the sample. Thereafter, the solution was filtered using Whatman No. 42 filter paper.

The filtrate was then transferred to a 25 mL volumetric flask and volume was made up to the mark using distilled water.

### *2.5. DLLME procedure*

In DLLME method, 2 mL of aqueous sample was placed in a 50 mL screw cap centrifuge tube. Then, a mixture of 300 µl of methanol (as disperser solvent) and 700 µl of chloroform (as extraction solvent) was rapidly injected into the sample solution, containing 300 µl of 1.0 mg $L^{-1}$ dithizone (as chelating agent), by using micro-pipette. A cloudy solution was formed and separation of the phases was achieved by centrifugation at 3500 rpm for 3 min. After this process, chloroform, impregnated with the complex, was sedimented at the bottom of centrifuge tube. Then 500 µl of the sedimented phase was withdrawn into a micro-syringe and was then analysed using FAAS.

## 3. Results and discussions

In this research, a DLLME procedure for the pre-concentration of heavy metals from vehicular emissions was developed and the pre-concentrated heavy metals were then analysed using FAAS. Chelation of the heavy metals using dithizone was done to facilitate the interaction of heavy metals with the organic extraction solvent, chloroform. Chloroform was used as extracting solvent due to its low water solubility and low vapour pressure. Furthermore, it has higher density than water, which is one of the most important requirements when choosing an extraction solvent for a DLLME procedure. On the other hand, methanol was used as a disperser solvent. In order to obtain high enrichment factors and good extraction recoveries, the main parameters affecting the complex formation and extraction conditions were optimized.

### *3.1. Effect of type of extraction solvent*

The first step in the development of the DLLME method was to select a proper extraction solvent. The extraction solvent should possess some special properties, such as high extraction capability of the dithizone-metal complex and low solubility in water. To investigate the effect of extraction solvent, chloroform, carbon tetrachloride, and dichloromethane were tested (Fig. 1). From the results, it was observed that chloroform had the highest enrichment factors for most of the heavy metals studied. Thus, chloroform was selected as the extraction solvent. Many researchers have used chloroform as the extraction solvent during the pre-concentration of heavy metals in different matrices using DLLME (Özdemir et al., 2014; Salahinejad and Aflaki, 2014; Sánchez-Rojas and Bosch-Ojeda, 2017). The complex formed between dithizone and heavy metals is neutral and non-polar. Thus, it effectively interacts with the non-polar organic solvents such as chloroform through Van der waals forces resulting in high extraction efficiencies. Chloroform has relatively lower solubility in water (8.09 g $L^{-1}$) as compared to the other organic solvents, like dichloromethane with a solubility of 17.5 g $L^{-1}$, used during the evaluation of the effect of extraction solvents on enrichment factors. Owing to its lower solubility, chloroform impregnated with dithizone-metal complexes, can be easily separated from the aqueous medium during DLLME. Chloroform has a higher density (1.50 g $mL^{-1}$) than water (0.999 g $mL^{-1}$) and this facilitates its sedimentation during the centrifugation stage in the DLLME procedure. Its density is also higher than that of dichloromethane (1.33 g $mL^{-1}$) and therefore, chloroform has an edge over dichloromethane during sedimentation on centrifugation. Tetrachloromethane, however, has a higher density (1.59 g $mL^{-1}$) as well as lower solubility in water (1.16 mg $L^{-1}$) than chloroform but the enrichment factors obtained when it was used as an extraction solvent were lower than those of chloroform. The bulky nature of tetrachloromethane probably prevented the close packing of it with the dithizone-metal complex thereby weakening the intermolecular forces between them. This probably hampered the extraction process when tetrachloromethane was used resulting in lower enrichment factors.

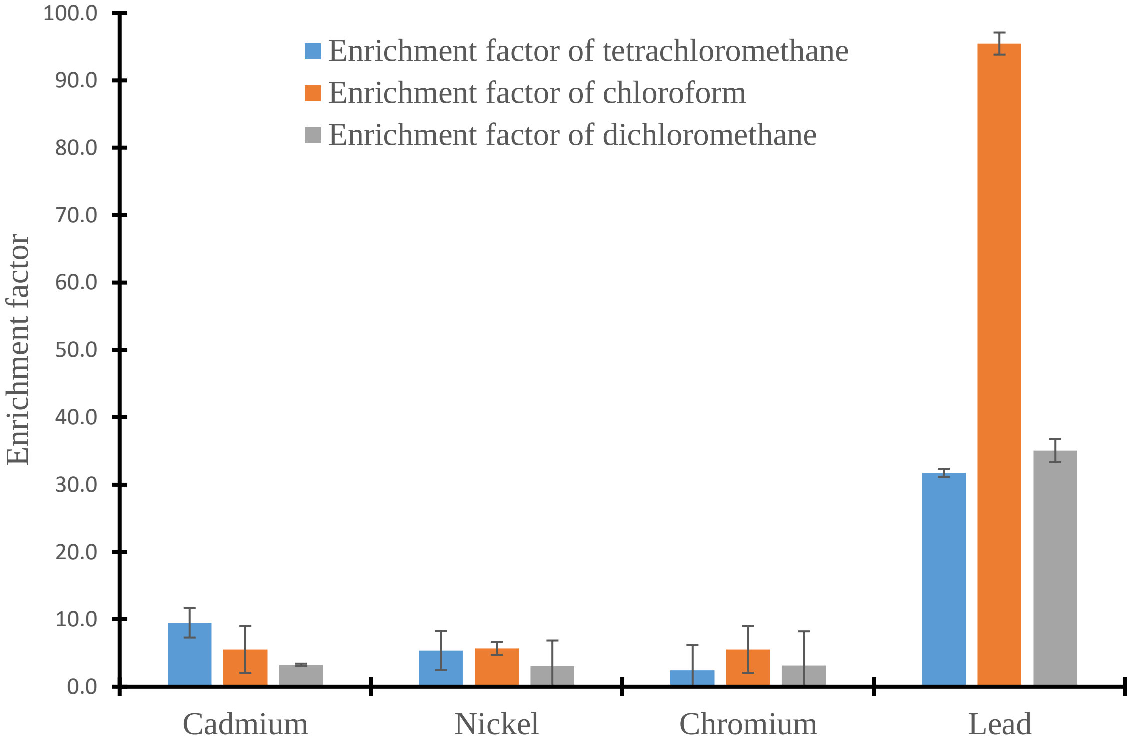

**Fig. 1:** Effect of type of extraction solvent on the enrichment factor of Cd, Cr, Ni and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg L$^{-1}$; sample volume, 2.0 mL; disperser solvent, methanol; extraction solvent (chloroform, tetrachloromethane and dichloromethane) volume, 1000 µL; disperser solvent (methanol) volume, 900 µL; dithizone concentration, 10 mg L$^{-1}$; dithizone volume, 500 µL; pH of sample, 7; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### *3.2. Effect of type of disperser solvent*

Disperser solvent should be miscible in both extraction solvent and aqueous sample. For this purpose, different solvents such as acetone, acetonitrile and methanol were tested. A series of sample solutions were studied with different types of disperser solvents containing 1000 µL of the extraction solvent and 900 µL of dithizone solution (chelating agent) (Fig. 2). Optimum enrichment factors were obtained when methanol was used as the dispersion solvent and, consequently, it was selected as the disperser solvent for use in subsequent experiments. The use of methanol as a disperser solvent is consistent with what other researchers used during the pre-concentration of heavy metals with DLLME. For instance, Gaubeur et al. (2015) used methanol as a disperser solvent to pre-concentrate heavy metals in beverages using DLLME. Elik et al. (2017) also successfully pre-concentrated Mn (II) and Cd (II) in food samples using methanol as the DLLME disperser solvent. Acetone and acetonitrile form dipole-dipole interactions with water in the aqueous sample while methanol forms hydrogen bonds with the water molecules. Hydrogen bonds are stronger intermolecular forces than the dipole-dipole interactions. These stronger interactions with water through hydrogen bonding probably enabled methanol to permeate the aqueous sample and to partition effectively the extraction solvent (chloroform) into tiny droplets. The tiny droplets of the extraction solvent have a larger surface area exposed to the dithizone-metal complex and this probably caused the enhancement of the extraction process and higher enrichment factors. Methanol is miscible with chloroform used as the extraction solvent in this research. Thus, it satisfies the requirements needed for a solvent to be used as a disperser solvent.

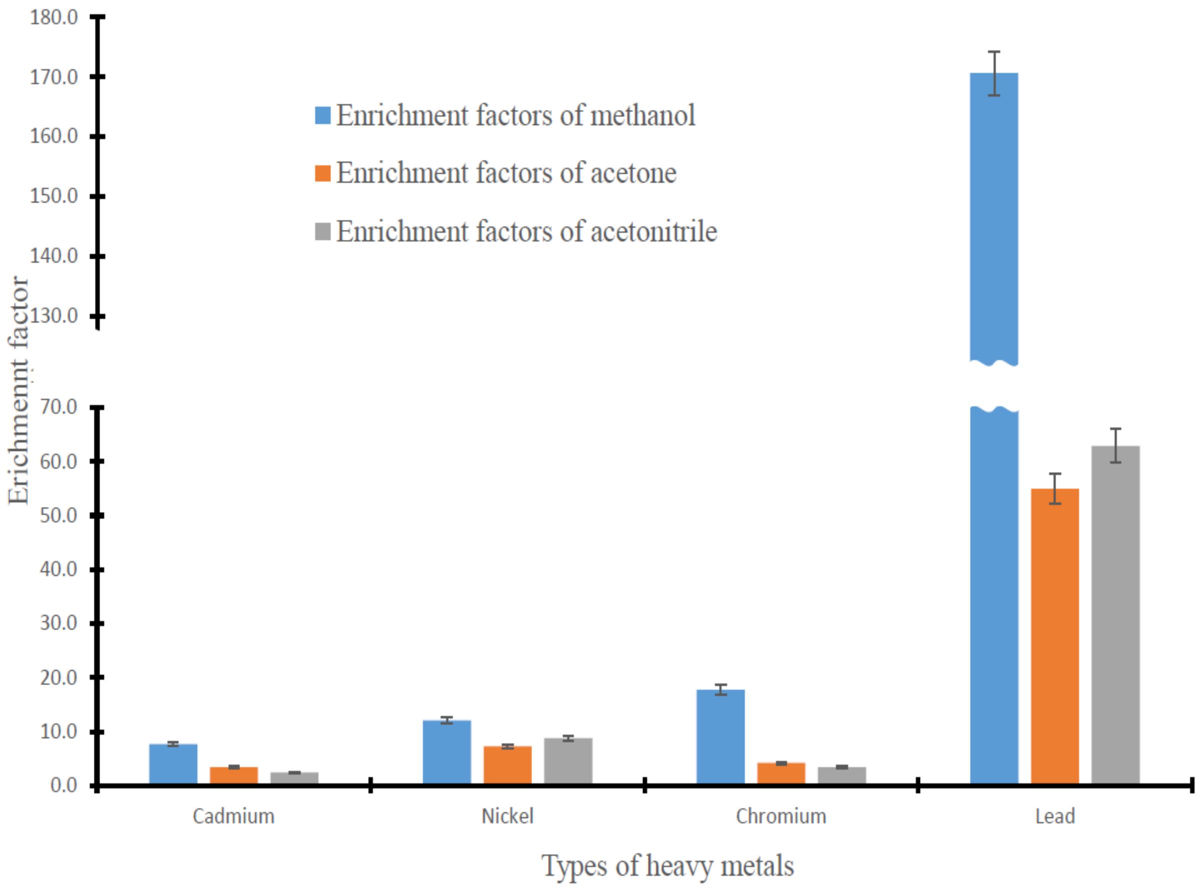

**Fig. 2:** Effect of type of disperser solvent on the enrichment factors of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg L$^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; extraction solvent (chloroform) volume, 1000 µL; disperser solvent (methanol) volume, 900 µL; dithizone concentration, 10 mg L$^{-1}$; dithizone volume, 500 µL; pH of sample, 7; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### 3.3. *Effect of volume of extraction solvent*

To examine the effect of the extraction solvent volume on the performance of the presented DLLME procedure, solutions containing different volumes of chloroform (500, 700, 900 and 1200 µL) with a constant concentration and volume of disperser agent (methanol) were used with the same DLLME procedure (Fig. 3). When the volume of the extraction solvent used was 700 µL most of the metals had high enrichment factors and this volume was selected for use in the subsequent experiments. Özdemir et al. (2014), and Salahinejad and Aflaki (2014) used 300 µL and 200 µL of chloroform (extraction solvent) respectively during the pre-concentration of heavy metals using DLLME. There is a large difference between the optimised volume and the volume of chloroform used by other researchers. The volume of the extraction solvent (chloroform) varies with the matrix and the volume of sample used. The enrichment factor of $Pb^{2+}$ generally decreased with an increase in the volume of chloroform used (Fig. 3). As the volume of chloroform was increased, the amount of droplets of the extraction solvent formed on dispersion also increased. At higher volumes of the chloroform extraction solvent, probably overcrowding of the droplets occurred resulting in their overlapping and fusion into larger drops with reduced surface area. As a result, the extraction efficiency of chloroform was reduced and the enrichment factors decreased. At lower volumes of the extraction solvent, probably fewer droplets were formed on dispersion causing a reduction in surface area of chloroform exposed to the dithizone-metal complex and lower enrichment factors. This could be the probable explanation for the increase that was observed between 500 and 700 µL in the cadmium graph (Fig. 3). Variation in the volume of chloroform had no significant effect on the enrichment factors of chromium and nickel. The enrichment factors of nickel and chromium were generally low and almost constant (Fig. 3). Probably the formation constants of the dithizone-nickel and the dithizone-chromium complexes are very low. Thus, these dithizone-metal complexes are not stable and as result, there was poor extraction and lower enrichment factors.

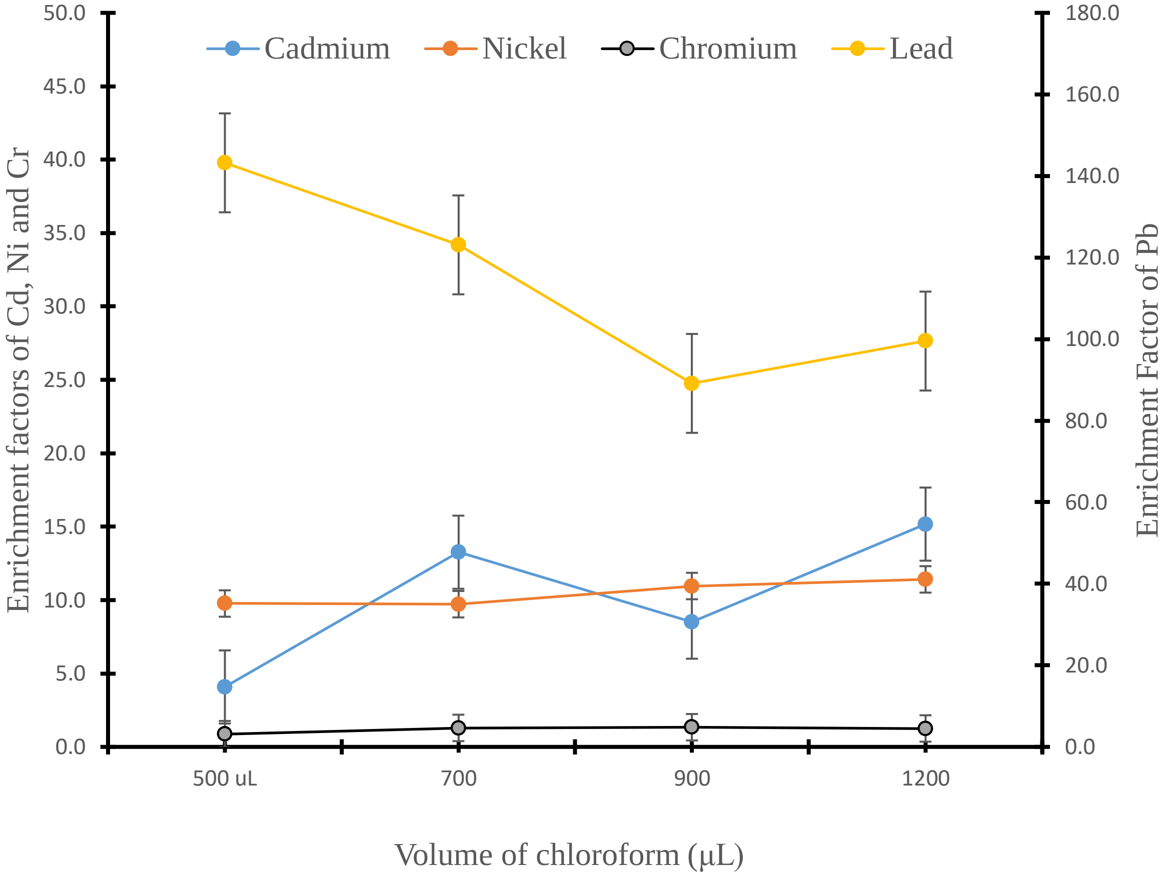

**Fig. 3:** Effect of volume of extraction solvent on the enrichment factors of Cd, Ni and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg L$^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; disperser solvent (methanol) volume, 900 μL; dithizone concentration, 10 mg L$^{-1}$; dithizone volume, 500 μL; pH of sample, 7; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### *3.4. Effect of volume of disperser solvent*

The influence of the volume of disperser solvent on the extraction efficiency was also examined. Various experiments were performed with different volumes of disperser solvent (300 – 900 μL) keeping all the other DLLME parameters constant (Fig. 4). From the results, it can be observed that the maximum enrichment factor was obtained when the volume of the disperser solvent (methanol) was 300 μL and this was taken as the optimum volume of methanol to be used in subsequent experiments. (Gaubeur et al., 2015) used 150 μL of methanol during the DLLME technique to pre-concentrate heavy metals while (Elik et al., 2017) used 200 μL of methanol for the pre-concentration of heavy metals in beverage samples using DLLME. The difference between the optimised volume and the volumes used by other researchers is not much. Thus, the optimised volume is consistent with volumes of disperser solvent (methanol) used by other researchers. There was a general decrease in enrichment factors as the volume of methanol was increased (Fig. 4). Thus, the extraction efficiency decreased with increase in the volume of the disperser solvent. This could be attributed to the fact that methanol forms stronger interactions (hydrogen bonds) with water than with the extraction solvent, chloroform. Large volumes of methanol, therefore, made the aqueous medium to be less polar and more soluble to the dithizone-metal complexes. As a result, extraction of the complex from the aqueous medium became more difficult at higher methanol volumes and the enrichment factors were reduced. From the results in Fig. 4, dithizone seemed to have a higher affinity and more sensitive to lead as compared to the other metals used in this study. This is supported by the extremely high enrichment factors of the dithizone-lead complex experienced during the experiment. On the other hand, the affinity and sensitivity of dithizone for chromium seemed to be very low as evidenced by the low enrichment factors encountered during the extraction of the dithizone-chromium complex. Chromium is a hard acid and therefore it forms weak interactions with dithizone. Dithizone, on the other hand, forms strong interactions with soft acids like $Pb^{2+}$, $Ni^{2+}$ and $Cd^{2+}$ (Sadeghi et al., 2018). This explains the lower enrichment factors of chromium and the higher enrichment factors of $Pb^{2+}$, $Ni^{2+}$ and $Cd^{2+}$ during DLLME pre-concentration involving complexation with dithizone. Since a mixture of the ions was used during optimisation, the lower enrichment factors of chromium might also be due to the presence of co-existing ions that could compete with chromium during complexation with dithizone. Probably dithizone had a higher affinity for these competing, interfering metal ions resulting in lower enrichment factors for chromium.

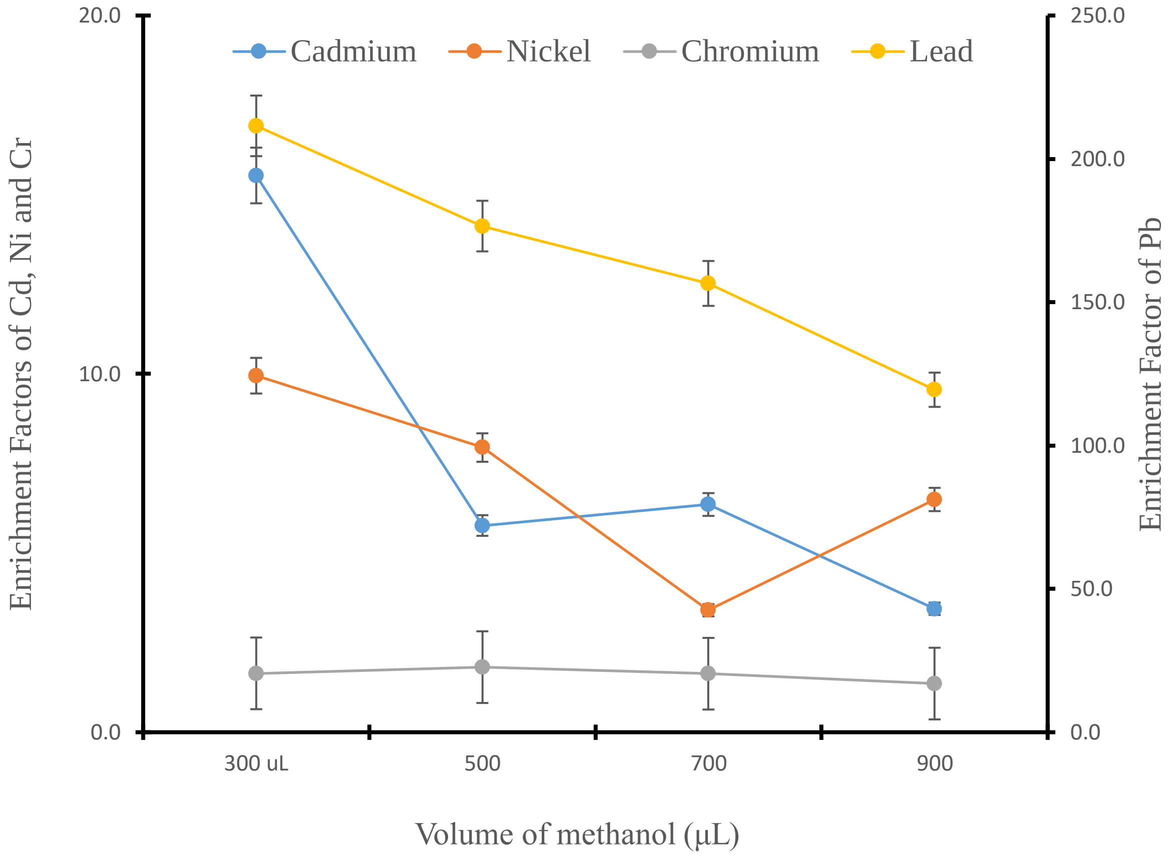

**Fig. 4:** Effect of volume of disperser solvent on the enrichment factors of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg $L^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 μL; dithizone concentration, 10 mg $L^{-1}$; dithizone volume, 500 μL; pH of sample, 7; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### *3.5. Effect of concentration of a chelating agent*

Different concentrations of dithizone ranging from 1 to 15 mg $L^{-1}$ were prepared and used to investigate the effect of concentration of the chelating agent on the enrichment factors of $Pb^{2+}$, $Cd^{2+}$, $Cr^{3+}$ and $Ni^{2+}$ when using the DLLME technique for their pre-concentration (Fig. 5). The highest enrichment factors were obtained when 1.0 mg $L^{-1}$ of dithizone was used for chelation and this was taken as the optimum concentration of dithizone in subsequent experiments. Heydari (2014) used 1.0 mg $L^{-1}$ of dithizone during the pre-concentration of cadmium in saffron samples using DLLME. Sadeghi et al. (2018), however, used 0.1 mmol $L^{-1}$ of dithizone as complexing agent during the pre-concentration of heavy metals from food and environmental samples. Thus, the concentration of dithizone used probably depends on the matrix in which the heavy metals are found. Dithizone is a sulphur containing non-polar, organic compound. It is a good bidentate ligand and it forms hydrophobic complexes with many metals such as lead, cadmium, chromium and nickel. The complexes formed can be easily extracted, due to their hydrophobic nature, from aqueous medium since dithizone is more soluble in organic solvents such as chloroform as compared to water. From Fig. 5, there was a general decrease in the enrichment factors when the concentration of dithizone was increased beyond the optimum. For a fixed volume, the optimum concentration of dithizone corresponds with the right stoichiometric amount required to react completely with the metal ions in the aqueous medium. Thus, maximum enrichment factors were obtained when the optimum concentration (1 g $L^{-1}$) of dithizone was used. The use of concentrations of dithizone higher than the optimum, however, negatively affected the enrichment factors. The excess dithizone probably interacted more with the organic extraction solvent during DLLME than the aqueous phase due to its non-polar nature. Thus, during DLLME, chloroform was impregnated probably with both the dithizone- metals complex as well as the excess dithizone at concentration levels above the optimum. With a density higher than that of water (1.2 g $mL^{-1}$), the excess dithizone was probably sedimented together with the dithizone-metal complex during the centrifugation stage of the DLLME technique. This probably resulted in larger volumes of the sedimented phase than expected and decreased enrichment factors.

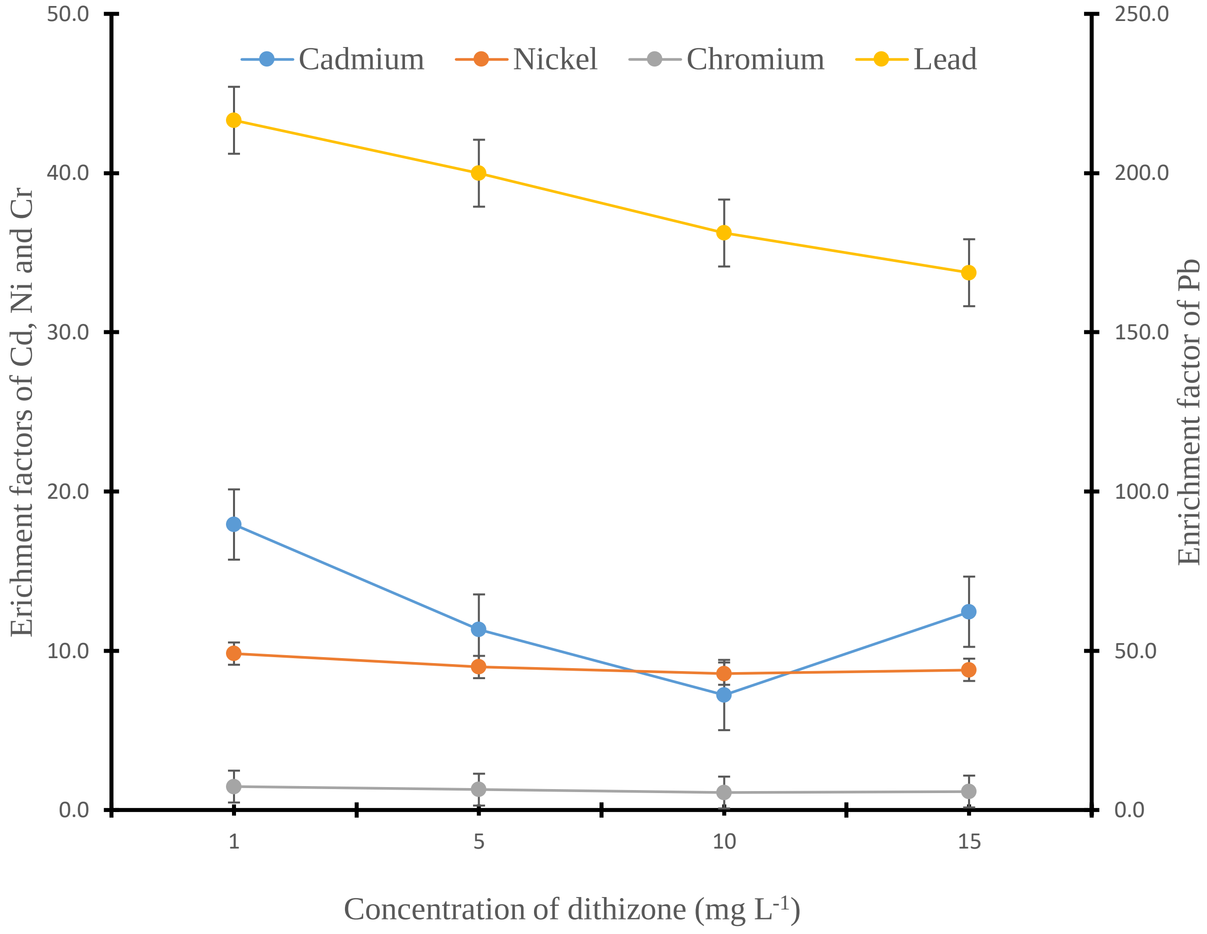

**Fig. 5:** Effect of concentration of dithizone (chelating agent) on the enrichment factor of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg $L^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 µL; disperser solvent (methanol) volume, 300 µL; dithizone volume, 500 µL; pH of sample, 7; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### *3.6. Effect of volume of chelating agent (dithizone)*

The effect of the volume of the chelating agent was investigated by varying it from 200 to 800 µL while keeping all the other DLLME parameter constant (Fig. 6). From the results, it was observed that the maximum enrichment factors were obtained when the volume of dithizone was 300 µL and this was taken as the optimum volume of the chelating agent to be used in subsequent experiments. This volume of dithizone is consistent with what other researchers used during the DLLME technique. For instance, Heydari (2014) used 200 µL of dithizone as chelating agent during the pre-concentration of cadimium in saffron samples using DLLME. At the optimum volume of dithizone, the stoichiometric amount of the chelating agent required for complete reaction with the metals would have been added. Thus, the maximum amount of the dithizone-metal complex was formed resulting in higher enrichment factors. At volumes less than the optimum, the moles of dithizone added were less than the stoichiometric amount required for complete reaction. As a result lower enrichment factors were obtained. When the volume of dithizone exceeded the optimum, there was a general decrease in enrichment factors with an increase in the volume of dithizone. The excess dithizone, since it has a density greater than that of water and is more soluble in chloroform than water, was probably sedimented together with the dithizone-metal complex. This caused an increase in the volume of the sedimented phase and a reduction in enrichment factors.

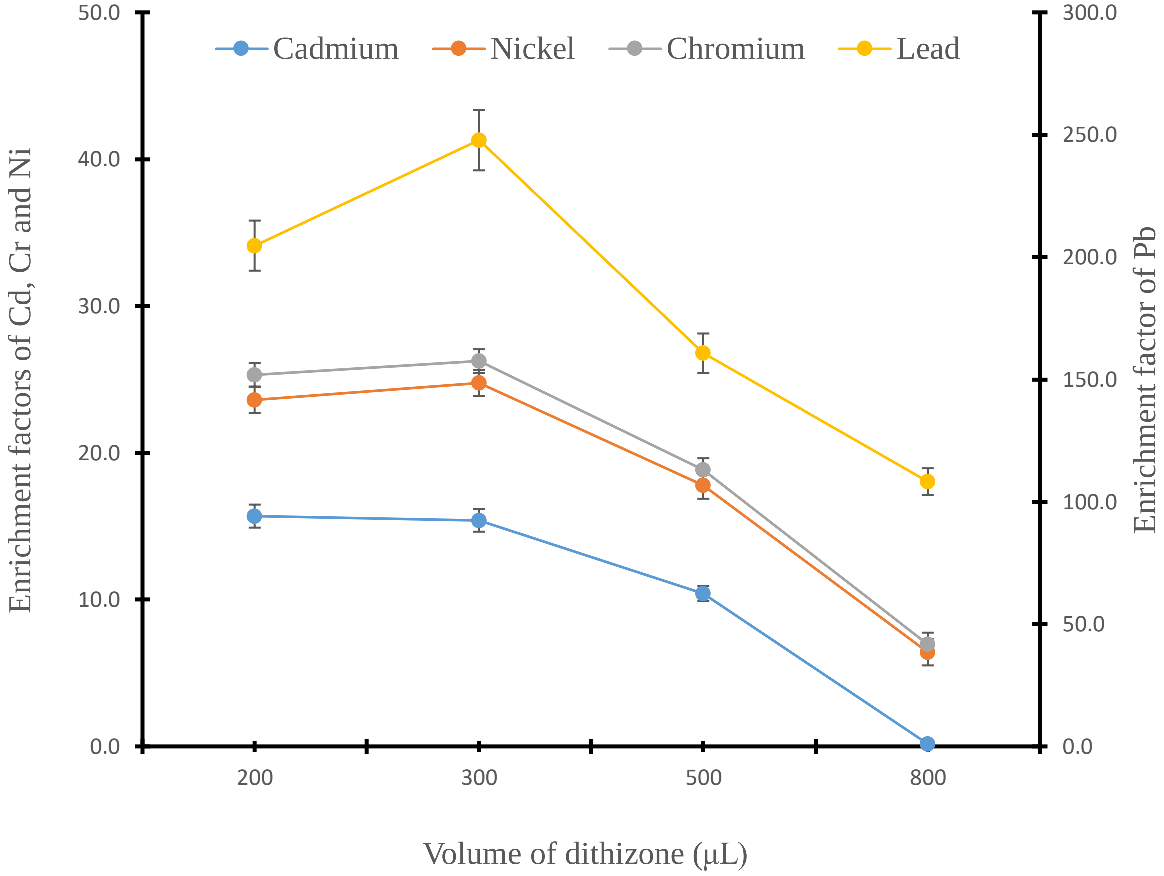

**Fig. 6:** Effect of concentration of dithizone (chelating agent) on the enrichment factor of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg L$^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 µL; disperser solvent (methanol) volume, 300 µL; dithizone concentration, 1.0 mg L$^{-1}$; pH of sample, 7; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### *3.7. Effect of pH*

In the extraction of metal ions, the complexing and extraction efficiency are closely related to the pH of the solution system. In general, $Pb^{2+,}$ $Cd^{2+}$, $Cr^{3+}$ and $Ni^{2+}$ should be converted into the complex form, using chelating agents like dithizone, to make the extraction process with organic solvents like chloroform possible. In order to obtain the favourable complexing and pre-concentration efficiency, the pH values were examined in the range of 2.0–10.0, using concentrated sodium hydroxide and sulphuric acid for their adjustment (Fig. 7). Maximum enrichment factors were obtained when the pH of the sample was adjusted to 8 and this was taken as the optimum pH to be used in subsequent experiments. Sadeghi et al. (2018) successfully pre-concentrated $Ni^{2+}$, $Cd^{2+}$ and $Pb^{2+}$ in food and environmental samples at pH 7.5 using DLLME with dithizone as the chelating agent while Bişgin et al. (2016) used the same chelating agent at pH 6.5 to pre-concentrate $Ni^{2+}$, $Cd^{2+}$ and $Cu^{2+}$ in food and water samples using DLLME. Thus, the optimised pH of 8 is very close to the commonly used pH values when dithizone is used as a chelating agent during DLLME. Soft metals such as $Pb^{2+,}$ $Cd^{2+}$ and $Ni^{2+}$ formed complexes with dithizone at pH values around 8. This phenomenon can be explained by using the two tautomers of dithizone (Naeemullah et al., 2016). As pH was increased, the dithizone tended to exist in enol form, which means that ligand property changed from weak to strong field ligand as pH of the medium change from acidic to slightly basic. At this slightly basic pH, soft acids such as cadmium, lead and nickel ions formed hydrophobic complexes with the enol form of dithizone, which is a strong field ligand resulting in higher enrichment factors (Cheng et al., 2012). Thus, the pH of 8 was selected as the optimum for the subsequent experiments.

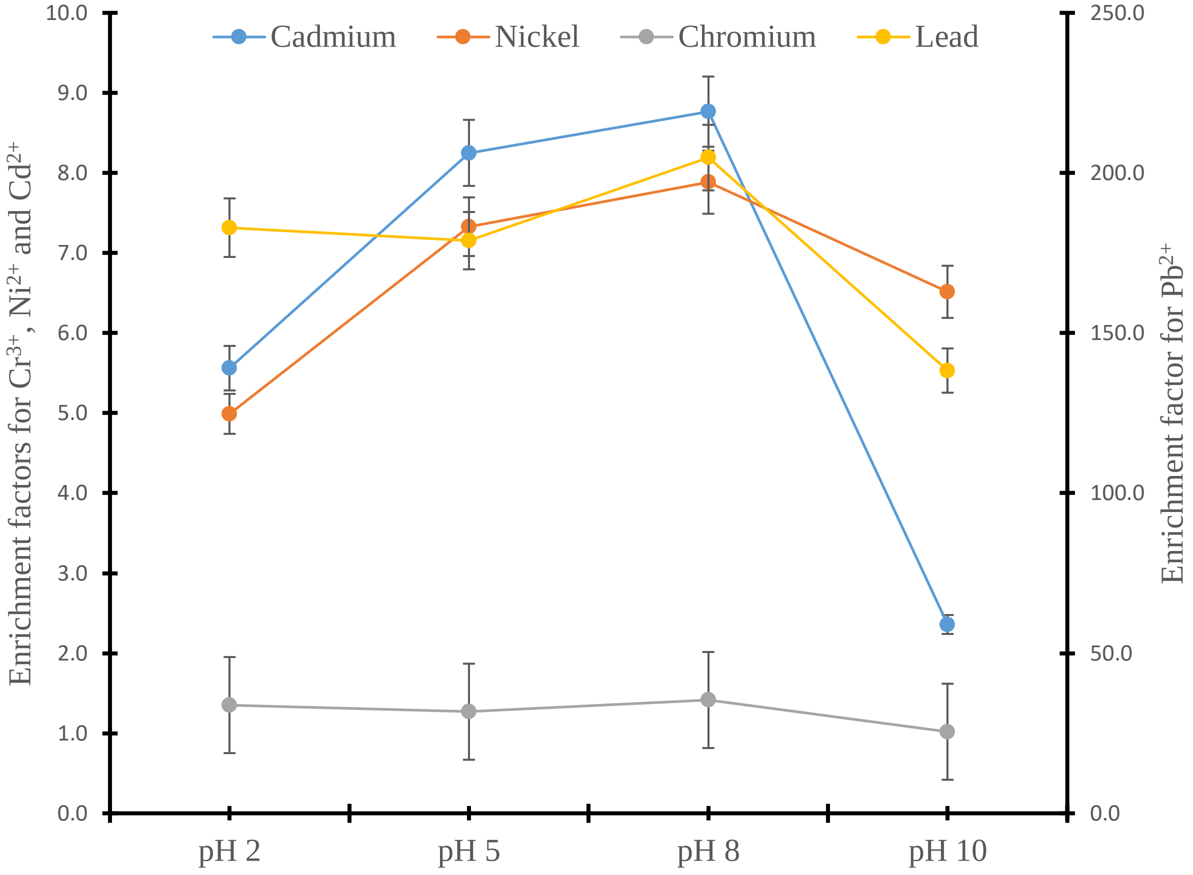

**Fig. 7:** Effect of pH of sample on the enrichment factors of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg L$^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 µL; disperser solvent (methanol) volume, 300 µL; dithizone concentration, 1.0 mg L$^{-1}$; dithizone volume, 300 µL; centrifugation rate, 5000 rpm; centrifugation time, 5 min

### *3.8. Effect of the centrifugation rate*

One of the most important parameters for the DLLME procedures is centrifugation rate. For this purpose, a series of experiments were made at different centrifugation rates varying from 3500 to 5000 rpm with all the DLLME parameters being kept constant (Fig. 8). The maximum enrichment factors were obtained at a centrifugation rate of 3500 rpm. This optimised value of centrifugation rate is consistent with what other researchers have used. For instance, Peng et al. (2015) used a centrifugation rate of 3500 rpm during the pre-concentration of pesticides in water samples while Gaubeur et al. (2015) used a centrifugation rate of 3000 rpm during the DLLME pre-concentration of heavy metals in water. The enrichment factors generally decreased with an increase in centrifugation rate. During centrifugation, the process of diffusion will be opposing the process of sedimentation (Antonopoulou et al., 2018). Optimising the centrifugation rate would ensure that an appropriate rate where sedimentation will be occurring faster than diffusion is used. Thus, if the centrifugation rate is increased beyond the optimum then probably the diffusion speed would surpass the sedimentation speed. More dithizone-metal complex would diffuse from the sediments into the aqueous phase causing a decrease in the enrichment factors.

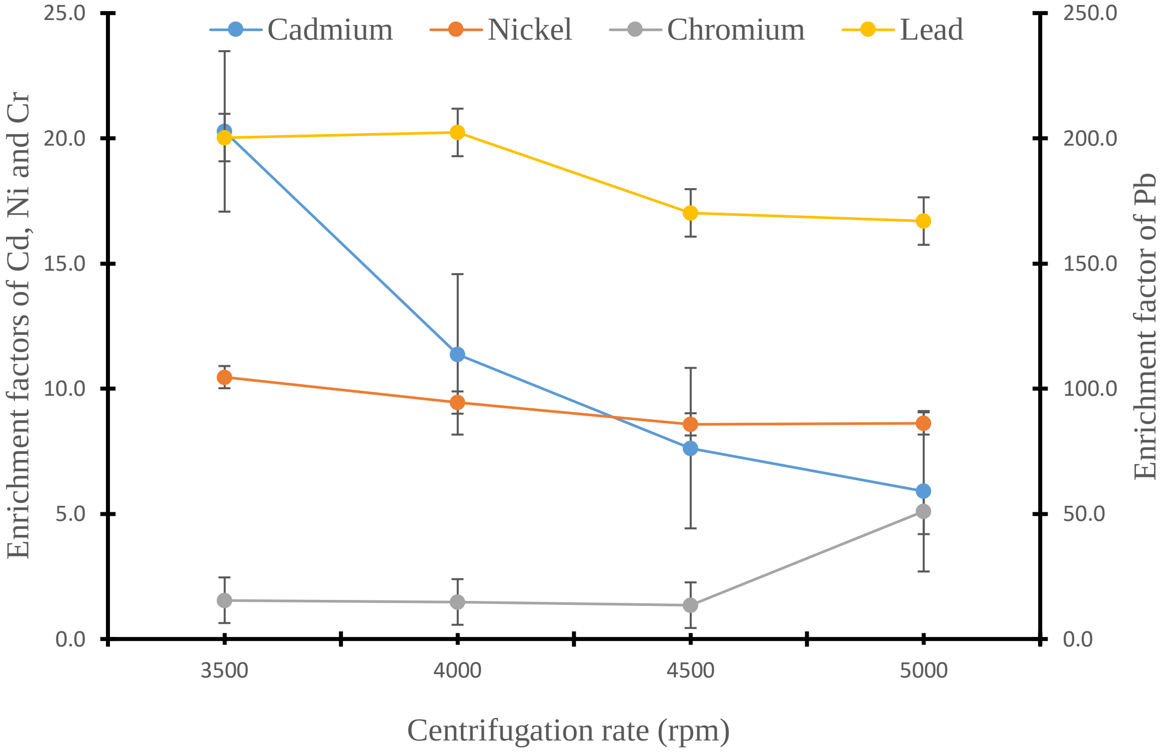

**Fig. 8:** Effect of centrifugation rate on the enrichment factors of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg $L^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 µL; disperser solvent (methanol) volume, 300 µL; dithizone concentration, 1.0 mg $L^{-1}$; dithizone volume, 300 µL; pH of samples, 8; centrifugation time, 5 min

### *3.9. Effect of the centrifugation time*

The influence of centrifugation time in dispersive liquid–liquid micro-extraction of lead, nickel, chromium and cadmium using dithizone chelating reagent was also studied. The effect of centrifugation time was studied over the range of 1–5 min, while the other experimental conditions were kept constant (Fig. 9). From Fig. 9, it can be observed that there was generally a gradual decrease in enrichment factors as the centrifugation time was increased with the exception of lead. The time of 3 min was selected for use as the centrifugation time in the subsequent experiments as most metals had fairly high enrichment factors when this time was used. Gaubeur et al. (2015) used centrifugation time of 3 min during pre-concentration of heavy metals using DLLME while Sadeghi et al. (2018) used centrifugation time of 5 min to pre-concentrate heavy metals using the same technique. Thus, the optimised centrifugation time falls within the range and is consistent with what other researchers used. At centrifugation times lower than the optimum (graph of lead in Fig. 9), an increase in centrifugation time caused an increase in the enrichment factors. Thus, an increase in centrifugation time caused an increase in sedimentation time, which translated to an increase in the enrichment factor. At the optimum centrifugation time (3 min), the dithizone-metal complexes were allowed enough time for their complete sedimentation. Hence, the increase in the enrichment factors experienced at 3 min. The enrichment factors of lead decreased when the centrifugation time was above the optimum. During centrifugation, there are two competing processes, which are sedimentation and diffusion (Antonopoulou et al., 2018). Prolonged centrifugation probably promoted the diffusion of the dithizone-metal complex from the sediment into the aqueous phase thereby causing a decrease in the enrichment factors. For the other graphs (graph of Cr, Cd and Ni in Fig. 9), it was observed that an increase in centrifugation time had no significant influence on the enrichment factors of the dithizone-metal complexes as evidenced by the gradual changes in enrichment factors. This gradual decrease in the enrichment factors was probably due to an increase in the diffusion rate of the dithizone-metal complex from the sediments into aqueous medium at higher centrifugation times.

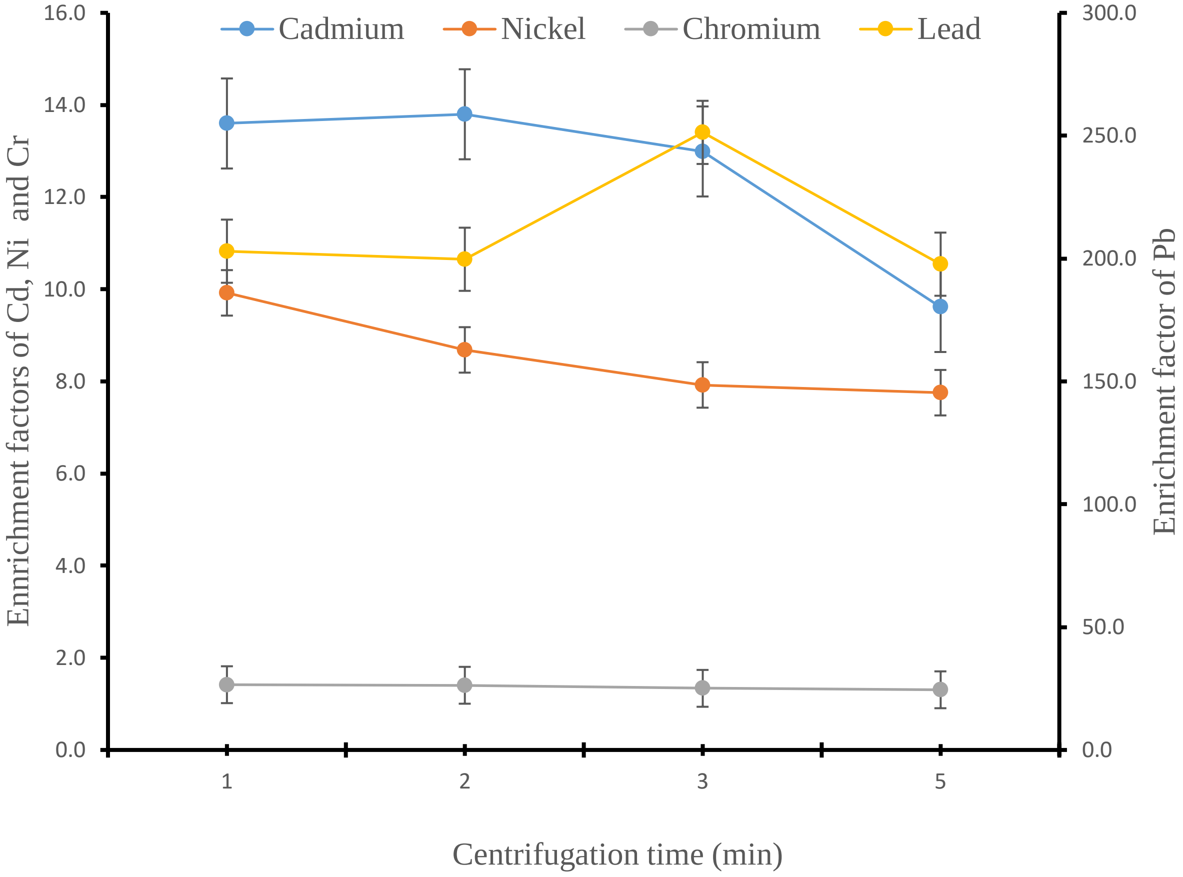

**Fig. 9:** Effect of centrifugation rate on the enrichment factors of Cd, Ni, Cr and Pb (n = 3). Experimental conditions: Concentrations (Cd, Ni, Cr and Pb), 3 mg L$^{-1}$; sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 µL; disperser solvent (methanol) volume, 300 µL; dithizone concentration, 1.0 mg L$^{-1}$; dithizone volume, 300 µL; pH of samples, 8; centrifugation rate, 3500 rpm.

### *3.2. Validation and application of the developed method*

The analytical performance of the developed method was investigated before application to real samples in terms of linearity, limit of detection (LOD) and limit of quantification (LOQ). The calibration curves were linear in the range 4 –10 mg L$^{-1}$ Pb$^{2+}$, 0.5–5 mg L$^{-1}$ Cr$^{3+}$, 1–10 mg L$^{-1}$ Cd$^{2+}$ and 0.5–10 mg L$^{-1}$ Ni$^{2+}$ under the optimum conditions of the proposed method. The determination coefficients (R$^2$) for Cr$^{3+}$, Cd$^{2+}$, Pb$^{2+}$ and Ni$^{2+}$ were 0.9982, 0.9987, 0.9987 and 0.9991 respectively. The LOQ values for Cr$^{3+}$, Cd$^{2+}$, Ni$^{2+}$ and Pb$^{2+}$ were calculated as ten times the standard deviation of the y-intercept, divided by the slope of the calibration curve and they were found to range from 0.52 to 1.96 mg kg$^{-1}$ (Table 1). The LOD values for Cr$^{3+}$, Cd$^{2+}$, Ni$^{2+}$ and Pb$^{2+}$ were calculated as three times the standard deviation of the y-intercept, divided by the slope of the calibration curve and they ranged from 0.15 - 0.59 mg kg$^{-1}$ (Table 1). The enrichment factors, defined as the ratio of the metal ion concentration in the final organic phase to the initial concentration of the sample of metal ion, were 1.4, 13.8, 9.9 and 251.4 for $Cr^{3+}$, $Cd^{2+}$, $Ni^{2+}$ and $Pb^{2+}$ respectively.

The developed method was then applied to real samples, which included roadside dust, fruits (apple and grapes) and vegetables (tomato and cucumber). The roadside dust had relatively higher concentrations of heavy metals compared to fruits and vegetables (Table 1 and Fig. 10). The high concentration of heavy metals in roadside dust can be attributed largely to vehicular emissions. The combustion of leaded petrol in car engines as well as the wear and tear of the car brakes and other metallic parts of the vehicle contribute significant amounts of heavy metals to the environment. These emitted heavy metals are adsorbed in the humic materials contained in the dust and, with continuous deposition; their concentrations may reach toxic levels. The concentration of heavy metals from the washings of fruits and vegetables were generally lower than those from roadside dust. This was probably due to the fact that fruits and vegetables are constantly wiped and cleaned at the markets resulting in the removal of most of the adhered dust and heavy metals from their surface. Also constant movement of the fruits and vegetables assist to shake off some of the dust and heavy metals from their surface. From the results, the fruits and vegetables contained a significant amount of heavy metals (Fig. 10). The concentrations of most of the heavy metals in the selected fruits and vegetables were lower than the maximum allowable limits set up by WHO (1996). The only exception was cadmium, which had higher concentrations in all the studied fruits and vegetables.

**Table 1**

Concentrations of $Cr^{3+}$, $Cd^{2+}$, $Ni^{2+}$ and $Pb^{2+}$ in roadside dust and selected fruits and vegetables and the figures of merit of the developed DLLME method

| Heavy metal | Mean concentration (mg kg$^{-1}$) ± SD | | | | | MAL (mg kg$^{-1}$) (WHO, 1996) | | LOD (mg kg$^{-1}$) | LOQ (mg kg$^{-1}$) | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | Roadside dust | Apple | Grape | Tomato | Cucumber | Soil | Fruits and vegetables | | | |
| Chromium | 0.5±0.01 | ND | ND | 0.4±0.02 | ND | 100 | 1.30 | 0.15 | 0.52 | 0.9982 |
| Nickel | 1.0±0.02 | 0.4±0.025 | 0.631±0.03 | 0.2±0.022 | 0.22±0.03 | 80.0 | 10.0 | 0.19 | 0.64 | 0.9987 |
| Cadmium | 0.8±0.02 | 0.07±0.01 | ND | 0.25±0.03 | 0.2±0.02 | 3.00 | 0.02 | 0.26 | 0.87 | 0.9987 |
| Lead | 0.6±0.03 | 0.2±0.02 | 0.4±0.02 | 0.51±0.04 | 0.1±0.01 | 100 | 2.00 | 0.59 | 1.96 | 0.9991 |

**Note**: Each concentration value is a mean of three replicates (n = 3)±SD; SD- standard deviation; ND- not detected; LOD- limit of detection; LOQ- limit of quantification; MAL- maximum allowable limit

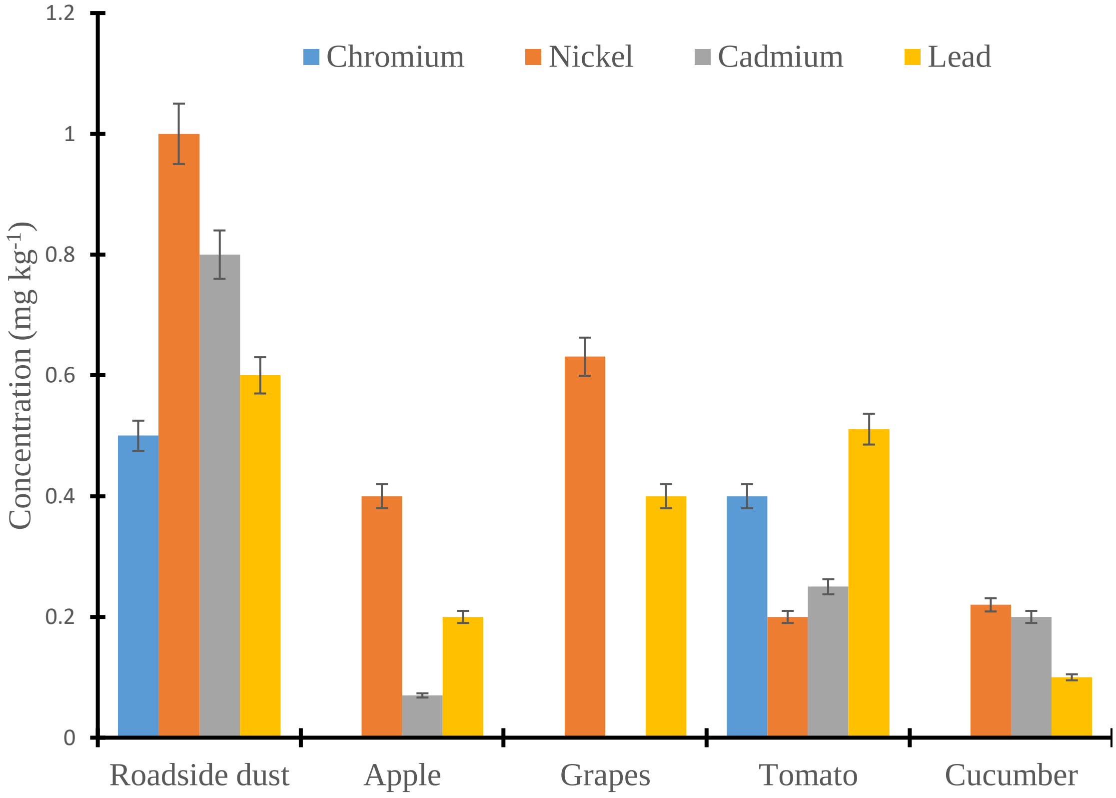

**Fig. 10:** Application of the developed DLLME method on real samples (n = 3 ) Experimental conditions: Sample volume, 2.0 mL; extraction solvent, chloroform; disperser solvent, methanol; extraction solvent (chloroform) volume, 700 µL; disperser solvent (methanol) volume, 300 µL; dithizone concentration, 1.0 mg L$^{-1}$; dithizone volume, 300 µL; pH of samples, 8; centrifugation rate, 3500 rpm; centrifugation time, 3 min

## 4. Conclusion

In the present study, methanol and chloroform were used as disperser and extraction solvents respectively with dithizone as the chelating agent during pre-concentration of $Cr^{3+}$, $Cd^{2+}$, $Pb^{2+}$ and $Ni^{2+}$ using the DLLME technique. This combination was successfully applied to the extraction and pre-concentration of heavy metals from different fruit and vegetable samples as well as roadside dust prior to analysis by FAAS. The developed method provides good precision, multi-element enrichment capability, good recovery and low detection limits within a relatively short time compared to other techniques. The other benefits of the developed method were consumption of low volume of the organic solvent, low cost and good accuracy and selectivity. The developed method yielded good enrichment factors for soft metal ions like

$Cd^{2+}$, $Pb^{2+}$ and $Ni^{2+}$. However, the chelating agent used, dithizone, does not form stable complexes with hard metal ions like $Cr^{3+}$. Thus, the enrichment factors of chromium were very low on application of the developed method.

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## Conflict of interests

There is no conflict of interests

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## 4.5. Paper V

This paper "1,5-diphenylcarbazide based dispersive liquid-liquid micro-extraction for the pre-concentration and speciation of chromium in fruits and vegetable" is a manuscript in preparation. Speciation studies of chromium were performed using dispersive liquid-liquid micro-extraction for pre-concentration of chromium (VI) and 1,5-diphenylcarbazide as a chelating agent in this paper.

# 1,5-diphenylcarbazide based dispersive liquid-liquid micro-extraction for the pre-concentration and speciation of chromium in fruits and vegetables

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## ABSTRACT

Chromium is an environmental pollutant resulting mainly from industrial activities that include electroplating, wood preservation, leather tanning and steel industries. The main species of chromium found in the environment are trivalent chromium (Cr (III)) and hexavalent chromium (Cr (VI)). In this study, the species of chromium in fruits and vegetables were studied. The chromium in fruit and vegetable samples was pre-concentrated using dispersive liquid-liquid micro-extraction (DLLME) prior to analysis with flame atomic absorption spectroscopy (FAAS). The dye (1,5- diphenylcarbazide (DPC)), was used as a chelating agent while salting out of the complex from the aqueous medium into the organic phase was done using sodium acetate. Chloroform and methanol were used as extraction and dispersion solvents respectively. The important parameters (pH, sample volume, extraction solvent and complexing agent) that influence the efficiency of the DLLME technique were optimized using the univariate approach prior to FAAS analysis. For total chromium, the trivalent chromium was oxidised using acidified KMnO4 to hexavalent chromium before performing the DLLME technique. The concentration of chromium (III) was determined from the difference between total chromium and concentration of chromium (VI). The limits of detection and quantification were 0.36 and 1.20 mg $L^{-1}$ respectively. After optimization, the developed method was applied to selected fruit and vegetable samples and the concentration of chromium (VI) ranged from 0.01 to 0.03 mg $L^{-1}$.

*Keywords*

Chromium, speciation, dispersive liquid-liquid micro-extraction, fruits and vegetables

## 1. Introduction

Elemental speciation refers to various physico-chemical forms in which elements occur in a given environment and, in some cases, includes the presence of different oxidation states with contrasting environmental biogeochemistry (Aharchaou et al., 2018). Speciation analysis is important because physiological effects of a metal in biological systems have been linked to its chemical forms rather than the total concentration (Salihu and Bakar, 2018). Thus, there is need of prioritizing speciation analysis over the total concentration in matrices when assessing the bioavailability and eco-toxicity of a metal (Rahbar et al., 2018).

One of the elements whose speciation have been extensively studied is chromium (Akhtar et al., 2018; Arain et al., 2018; Dalkıran et al., 2017; Fernandez et al., 2018; Hamilton et al., 2018; Liu et al., 2016). Speciation of chromium is always important because of the bioavailability and toxicity of some of its chemical forms (Aharchaou et al., 2018; Fernandez et al., 2018). Even though there are many oxidation states of chromium, the most commonly found are Cr(III) and Cr(VI) ( Huang et al., 2018), especially as environment pollutants. These two chemical forms of chromium have contrasting environmental and biological behaviour (Cui et al., 2017). The hexavalent chromium, a recognized human carcinogen, forms negatively charged chemical species, interacts little with colloidal and particulate material, has a high environmental mobility, and easily crosses biological membranes. Apart from its carcinogenic nature, hexavalent chromium also has mutagenic and teratogenic potentials in living organisms (Rahbar et al., 2018). In contrast, the trivalent species preferentially forms positively charged chemical species, tends to associate with colloids or suspended particulate matter, and is considered of less eco-toxicological concern. Trivalent chromium is an essential element that plays a significant role in regulating the blood sugar levels by improving the way human bodies use insulin (Munonde et al., 2017). Chromium occurs naturally in the earth crust; however, anthropogenic activities in diverse industrial processes such as textile dyeing, electroplating, mining, leather tanning, and wood preservatives has led to an extensive spread of associated contaminations with this metal in the environment (Tahmasebi and Davarani, 2016). It may also be introduced into drinking water supplies through the corrosion inhibitors applied on water pipes and containers.

Sensitivity and selectivity are paramount aspects when choosing an analytical technique for speciation analysis. Such sensitive and selective analytical technique commonly used for chromium speciation analysis include atomic absorption spectrometry (AAS) (Cui et al., 2017; Dokpikul et al., 2018; Porto et al., 2016), inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Munonde et al., 2017), inductively coupled plasma mass spectrometry (ICP-MS) (Huang et al., 2018) and high performance liquid chromatography (HPLC) with ICP-MS detector (Marcinkowska et al., 2017). To further increase selectivity through minimizing matrix interferences, some separation, extraction and/or pre-concentration steps usually precede analysis. Thus, various pre-treatment methodologies such as cloud point extraction (López-García et al., 2017), liquid-liquid extraction and solid phase extraction (Herrero-Latorre et al., 2018) have been developed for the trace analysis of chromium species. However, these conventional analytical methods usually are time consuming and involve the use of large volumes of toxic organic solvents as well as having low enrichment factors (Altunay et al., 2018; Bahadir et al., 2016). Thus, conventional pre-concentration techniques have been surpassed, in recent years, by miniaturized approaches that involve low amounts of organic solvents, or even none, resulting in environmentally friendly procedures.

Recently, dispersive liquid-liquid micro-extraction (DLLME), a new mode of liquid phase micro-extraction technique, has aroused great interest in analytical community (Amoli-Diva et al., 2017; Ayala-Cabrera et al., 2016; da Silva et al., 2017; Doroudi and Niazi, 2018; Liang et al., 2017; Rahmani et al., 2016; Sorouraddin et al., 2018) and has also found application in chromium speciation. The advantages of the DLLME method are simplicity of operation, rapidity, low cost and high enrichment factors (El-Shahawi and Al-Saidi, 2013; Farajzadeh et al., 2016). This method has been successfully applied for the pre-concentration of inorganic species such as chromium in environmental samples (Du et al., 2018; Horstkotte et al., 2017; Kokare et al., 2017; Li et al., 2015).

Many strategies are used during pre-concentration techniques such as DLLME in order to convert inorganic analytes into a form compatible with a particular method. One such technique is derivatization through chelation (Onchoke and Sasu, 2016; Salihu and Bakar, 2018). The common chelating agents used by researchers during trace analysis of heavy metals using DLLME include ammonium pyrrolidine dithiocarbamate (Baig et al., 2012; Porto et al., 2016; Salihu and Bakar, 2018; Taziki et al., 2013), 2-thenoyltrifluoroacetone (Dokpikul et al., 2018), rhodamine 6G hydrochloride (Ahmad et al., 2016) and diethyldithiocarbamate (Gaubeur et al., 2015).

Recently, a chelating agent that has gained the attention of many researchers during chromium speciation is 1,5-diphenylcarbazide (DPC) (Abadi et al., 2013; Sobhi et al., 2018). DPC is highly sensitive and selective for hexavalent chromium. It forms a cationic complex with chromium (VI). The chemical reaction of Cr(VI) with DPC comprises of two steps, which are the reduction of Cr(VI) to Cr(III), and the formation of an intense red-violet Cr(III) complex (Tahmasebi and Davarani, 2016).

In this current work, speciation analysis of trace chromium was performed using DLLME as a pre-concentration technique. Chromium (VI) can affect human health even at trace levels. Thus, it is imperative that cheap and sensitive pre-concentration techniques should be developed to effectively monitor the concentration levels of chromium species in the environment. The developed method involves the chelation of chromium (VI) with DPC prior to its pre-concentration using DLLME. This is a sensitive and novel method for the pre-concentration of chromium (VI) during speciation analysis. The concentration of chromium, after pre-concentration, was then determined using a flame atomic absorption spectrometer (FAAS).

## 2. Experimental

### *2.1. Chemicals and instruments*

All reagents and solvents used were of analytical quality grade. Stock solutions of 1000 mg $L^{-1}$ of Cr (VI) and Cr (III) ions were prepared by dissolving appropriate amounts of $K_2Cr_2O_7$ and $CrCl_3 \cdot 6H_2O$ both from Sigma-Aldrich (Johannesburg, South Africa). Working solutions of Cr (VI) and Cr (III) were obtained daily by appropriate dilution of stock solutions. The pH of solutions was adjusted by using 32% sulphuric acid or 98% sodium hydroxide purchased from Sigma-Aldrich (Johannesburg, South Africa). Dispersion and extraction solvents such as acetonitrile, acetone, methanol, ethanol, carbon tetrachloride and chloroform were also purchased from Sigma-Aldrich (Johannesburg, South Africa). The chelating agent, 1,5- diphenylcarbazide (DPC), was also from Sigma-Aldrich (Johannesburg, South Africa).

The centrifuge and centrifuge tubes were bought from Sigma-Aldrich (Johannesburg, South Africa) as well as the vortex machine used for mixing. Ultrapure water (MilliQ Plus system, Millipore Corp.) was used for dilutions and standard preparations in all experiments. The measurements were performed using a PerkinElmer PinAAcle 900T atomic absorption (AA) spectrometer (PerkinElmer Inc., Shelton, CT, USA), equipped with the intuitive Syngistix™ AA software for sample analysis, data reporting, and archiving results.

### 2.2. DLLME procedure

The DLLME procedure was used to isolate and pre-concentrate the chromium species. The hexavalent chromium concentration in the samples was determined by DLLME after chelation by DPC. Subsequently, total chromium concentration in the samples was analysed. The concentration of the trivalent chromium was found from the difference between the concentration of Cr (VI) and that of the total chromium.

#### 2.2.1. Determination of Cr (VI)

For Cr (VI) extraction, 2 mL of the aqueous fruit and vegetable samples were placed in a centrifuge tubes and the pH of solutions were adjusted to 2 with 0.1 M $H_2SO_4$. Then, 300 μL of 1.0 g $L^{-1}$ DPC in acetone was added to each sample followed by 400 μL of the dispersion solvent (methanol). Thereafter, 1.0 mL of chloroform extraction solvent was rapidly injected into the sample solution using a syringe and was followed by the addition of 2 mL of a saturated solution of sodium acetate. The mixture was vortexed for 1 min. After waiting for 5 min at room temperature, the cloudy solution was centrifuged at 4000 rpm for 2 min to obtain phase separation. The sedimented organic phase settled at the bottom of the tube (which contained the Cr–DPC complex) was then collected using a micropipette followed by analysis using FAAS.

#### 2.2.2. Determination of total chromium

Total chromium was determined as Cr (VI) by the method described above after oxidizing Cr (III) to Cr (VI) using acidified potassium permanganate. During oxidation, 4 or 5 drops of acidified 0.02 mol $L^{-1}$ $KMnO_4$ solution were added into a beaker containing 20 mL of sample solution containing both Cr (VI) and Cr (III). The beaker was covered with a watch glass and heated without boiling at a temperature of 70°C for 15 min. The solution was cooled and 1% (w/v) sodium azide solution was added dropwise, waiting a few seconds after the addition of each drop, until the mixture became colourless. The resultant mixture was subjected to the DLLME procedure and then analysed using FAAS in order to get the total chromium.

## 3. Results and discussion

### 3.1. Optimization of DLLME parameters

#### 3.1.1. Type of extraction solvent

The selection of an appropriate extraction solvent is of paramount importance during the DLLME technique as this would enable the attainment of higher signals for the chromium species during analysis. The extraction solvent should have unique properties such as homogeneity with disperser solvent as well as extremely low solubility in water. In this study, three commonly used organic solvents namely chloroform, carbon tetrachloride, and dichloromethane were evaluated for their efficiency as extraction solvents during chromium speciation. Each extraction solvent (1.0 mL) was combined with 400 µL of methanol (disperser solvent) during the DLLME technique, and the enrichment factor was calculated (Fig. 1).

The highest enrichment factor (4.5) was found when chloroform was used and therefore it was selected as the best extraction solvent to be used in subsequent experiments. The effectiveness of an organic solvent as an extraction solvent depends among other factors, on its solubility in water. Generally, the lower the solubility in water the more efficient an organic solvent is as an extraction solvent during the DLLME technique. Three extraction solvents were evaluated in this study: chloroform, dichloromethane, tetrachloromethane, and their solubilities in water at 25°C are 8.02 g $L^{-1}$, 17.5 g $L^{-1}$ and 1.13 mg $L^{-1}$ respectively. Although terachloromethane has the lowest solubility in water, the enrichment factor of chromium was the lowest. This anomaly was probably due to the fact that the sedimentation of the DPC-chromium complex in terachloromethane was incomplete during centrifugation as the complex particles could be observed in the aqueous phase in the centrifuge tube on visual inspection. Consequently, the highest enrichment factor was obtained when chloroform, with a slightly lower solubility in water, was used as the extraction solvent.

Chloroform is one of the extraction solvents widely used during pre-concentration of different analyte using DLLME. Sánchez-Rojas and Bosch-Ojeda (2017) successfully used chloroform as an extraction solvent during the pre-concentration of nickel ions in water samples using DLLME. Özdemir et al. (2014) also used chloroform as an extraction solvent and successfully pre-concentrated palladium and gold ions in environmental samples using DLLME. In the same vein, Salahinejad and Aflaki (2014) pre-concentrated lead ions in water samples using chloroform as the extraction solvent during the DLLME procedure.

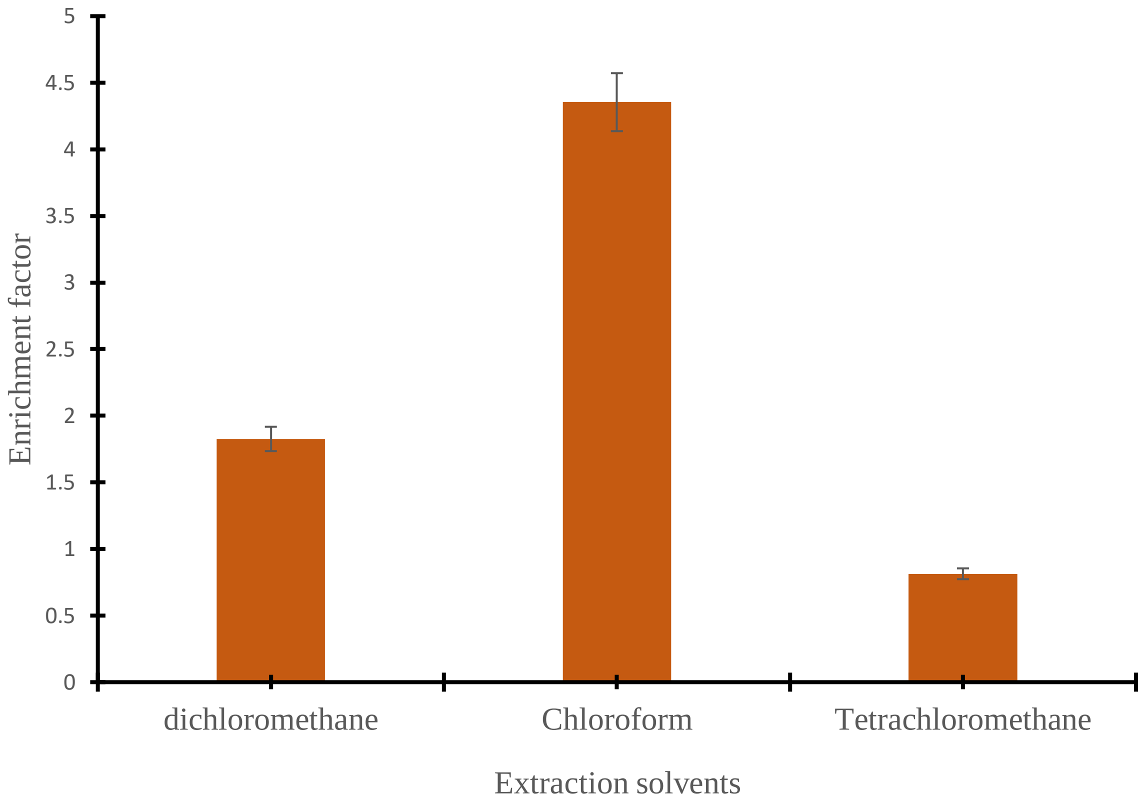

**Fig. 1:** Effect of type of extraction solvent on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg L$^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 400 µL; extraction solvent volume, 1000 µL; DPC concentration, 1.0 mg L$^{-1}$; DPC volume, 500 µL; centrifugation rate, 5000 rpm; centrifugation time, 2 min; pH during complex extraction, 6.8; sodium acetate volume, 2.0 mL.

### *3.1.2. Effect of salt addition*

The complex formed in the reaction of DPC with hexavalent chromium is not readily extractable out of aqueous solutions into organic phase during the DLLME technique. Only a fraction of the DPC-chromium complex is extracted into chloroform extraction solvent. This is because the DPC-chromium complex is cationic and therefore hydrophilic (Yousefi and Shemirani, 2013). Thus, it is difficult to extract it using non-polar solvents like chloroform used in this study. As a result, salting out using salts like sodium chloride or sodium acetate can facilitate the extraction process. The anions from the salts will combine with the cationic complex resulting in the formation of a neutral complex or a complex with reduced polarity that can be easily extracted using an organic solvent like chloroform.

In this study, the effects of three salts (sodium chloride, sodium bromide and sodium acetate) were studied (Fig. 2). A saturated solution (2 mL) of each salt was added to the sample solution during the DLLME procedure and in each case, the enrichment factor was calculated. From Fig. 2, it can be observed that the highest enrichment factor was obtained when sodium acetate was used and this was taken as the optimum salt for use in the subsequent experiments. The salt that is commonly used by researchers for salting out is sodium chloride. For instance, Sorouraddin et al. (2017) used sodium chloride for the salting out effect during the pre-concentration of heavy metals from aqueous samples. Bolzan et al. (2015) also used sodium chloride for salting out effect during the pre-concentration of pesticides using DLLME. In this study, however, sodium acetate proved to be more efficient than sodium chloride as a salting out reagent. This was probably because the acetate ion is bulky as compared to the chloride and bromide ions. Probably the bulky nature of the acetate ion makes it possible for it to mask completely the positive charge on the chromium complex cation. Thus, enabling complete dissolution and interaction of the DPC-chromium complex with the non-polar organic solvents like chloroform.

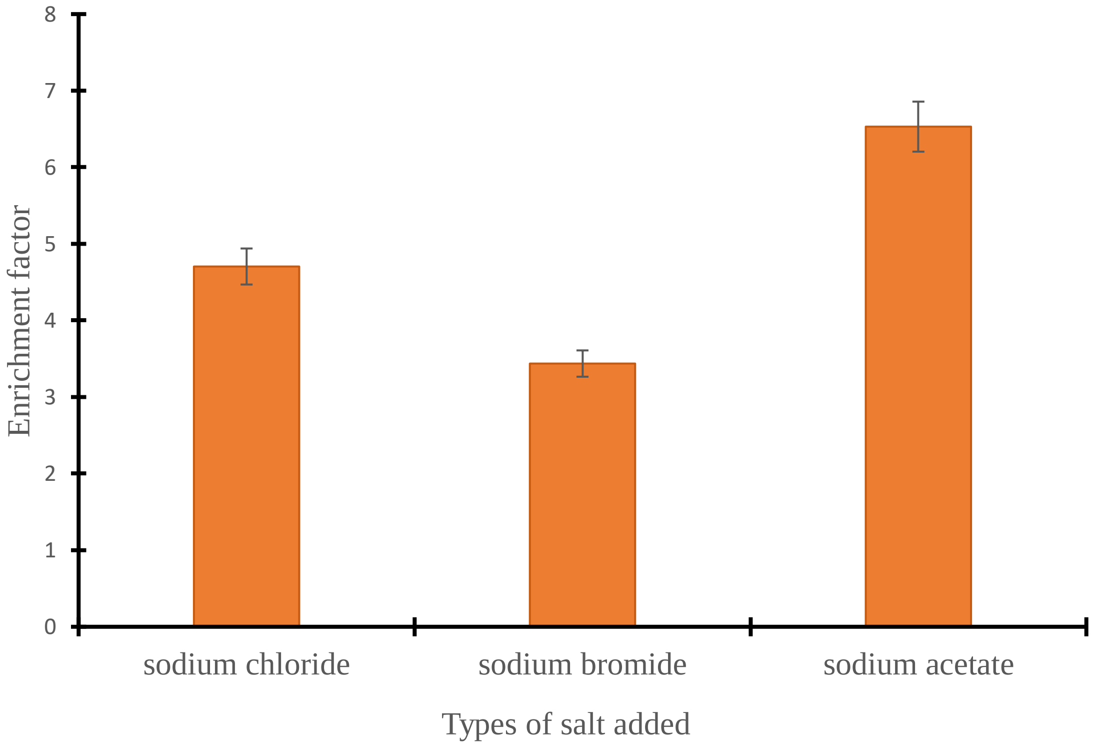

**Fig. 2:** Effect of salt addition on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 400 µL; extraction solvent, chloroform; extraction solvent (chloroform) volume, 1000 µL; DPC concentration, 1.0 mg $L^{-1}$; DPC volume, 500 µL; centrifugation rate, 5000 rpm; centrifugation time, 2 min; pH during complex extraction, 6.8; salt solution volume, 2.0 mL.

### *3.1.3. Type of disperser solvent*

A disperser solvent plays an important role in decreasing the interfacial tension between water and the extracting solvent during the DLLME technique resulting in the formation of tiny droplets of the extraction solvent impregnated with the chromium species. The major selection criterion for the disperser solvent hinges on its ability to be highly miscible with both water and the extraction solvent. In order to select the disperser solvent for chromium speciation in this study, three solvents, namely acetone, acetonitrile and methanol were examined while the volume of chloroform (extraction solvent) was kept constant at the level of 1 mL (Fig. 3).

The highest enrichment factor was obtained when methanol was used as the dispersion solvent and consequently it was selected as the dispersion solvent to be used in the subsequent experiments. Both acetone and acetonitrile are polar solvents and as a result, they form dipole-dipole interactions with analyte-containing aqueous medium during the DLLME method. Methanol, on the other hand forms hydrogen bonds with the water molecules in the sample. The hydrogen bonds formed by methanol and water are stronger intermolecular forces than the dipole-dipole interaction formed with acetone and acetonitrile. Thus, due to these strong interactions with water, methanol can effectively permeate the aqueous sample resulting in the rapid dispersion and partitioning of the extraction solvent. More tiny droplets of the extraction solvent were formed when methanol was used as a disperser solvent causing increased surface area to be exposed to the DPC-chromium complex. Hence, a higher enrichment factor of chromium was obtained when methanol was used to cause dispersion.

Many researchers have used methanol as a disperser solvent during the pre-concentration of different analytes from a wide variety of matrices. For instance, Elik et al. (2017) successfully used methanol as a disperser solvent during the DLLME pre-concentration of Mn (II) and Cd (II) in foodstuffs. Gaubeur et al. (2015) used the same disperser solvent to pre-concentrate heavy metals in beverages using DLLME. Asensio-Ramos et al. (2011), on the other hand, used methanol as a disperser solvent during the pre-concentration of pesticides in soil samples using the DLLME technique.

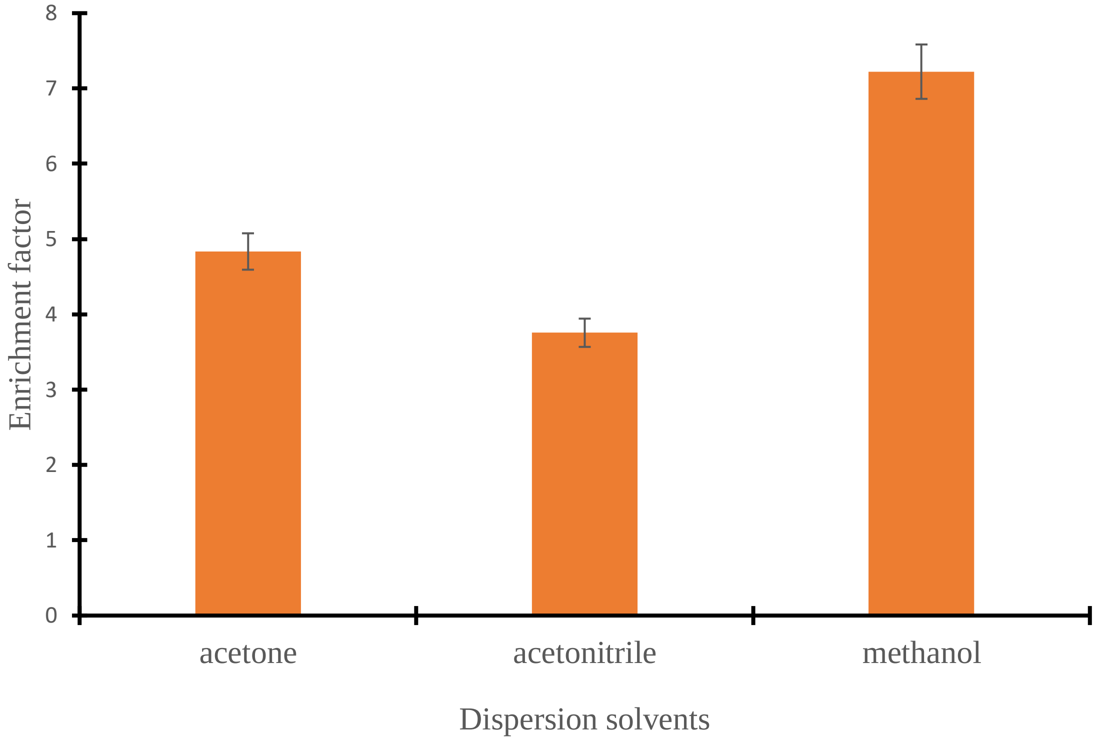

**Fig. 3:** Effect of type of dispersion solvent on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg L$^{-1}$; sample volume, 2.0 mL; dispersive solvent volume, 400 µL; extraction solvent, chloroform; extraction solvent (chloroform) volume, 1000 µL; DPC concentration, 1.0 mg L$^{-1}$; DPC volume, 500 µL; centrifugation rate, 5000 rpm; centrifugation time, 2 min; pH during complex extraction, 6.8; sodium acetate volume, 2.0 mL.

### *3.1.4. Influence of extraction solvent volume*

To examine the effect of the extraction solvent volume during chromium extraction, different volumes of chloroform (600, 800, 1200 and 1500 µL) were investigated (Fig 4). The enrichment factor reached a peak when the volume of chloroform was 800 µL. Thus, 800 µL of chloroform was used during the extraction of chromium in this study. The volume of chloroform used must be adequate to permeate completely the sample to ensure maximum solvent and DPC-chromium complex interaction. The chloroform should not get to its saturation point before extracting the entire chromium complex in the sample. Thus, the volume of chloroform should be such that there is always a slight excess in order for it to extract completely the DPC-chromium complex. If the volume of chloroform used is too low (less than the experimentally determined optimum of 800 µL), then it will not be able to completely extract the DPC-chromium complex leading to low enrichment factors and poor recoveries. At volumes higher than the optimum, many tiny droplets of extraction solvent are formed during the dispersion process and the volume would be limited. Thus, overcrowding of the extraction solvent droplets might probably cause their fusion into larger drops with reduced surface area. This could be the explanation of the observed decrease in enrichment factors of chromium at volumes beyond the optimum.

There are some variations in the volumes of chloroform used by researchers when pre-concentrating different analytes using DLLME. Salahinejad and Aflaki (2014), for instance, used 200 μL of chloroform as extraction solvent when pre-concentrating lead ions in water samples using DLLME. The same volume of chloroform was used by Sánchez-Rojas and Bosch-Ojeda (2017) when applying the DLLME technique to pre-concentrate nickel ions in water samples. Özdemir et al. (2014), on the other hand, used 300 μL of chloroform for palladium and gold ions pre-concentration using DLLME while the optimised volume in this study was 800 μL of chloroform for the pre-concentration of chromium in fruits and vegetables using DLLME.

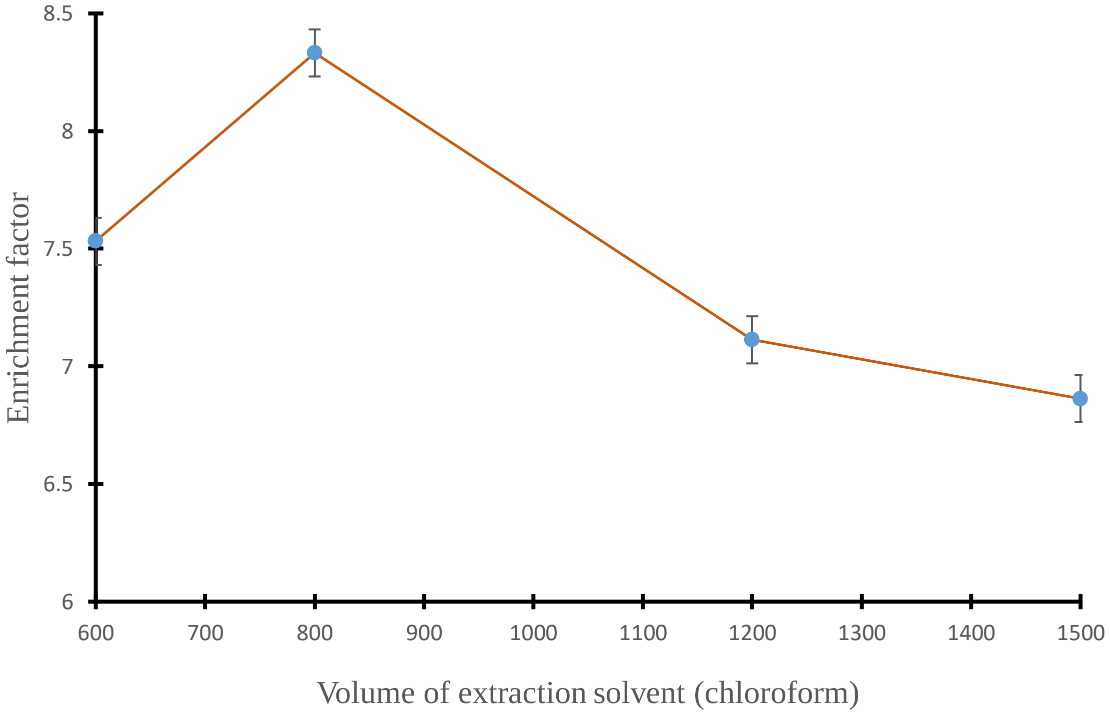

**Fig. 4:** Effect of volume of extraction solvent (chloroform) on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 400 μL; extraction solvent, chloroform; DPC concentration, 1.0 mg $L^{-1}$; DPC volume, 500 μL; centrifugation rate, 5000 rpm; centrifugation time, 2 min; pH during complex extraction, 6.8; sodium acetate volume, 2.0 mL.

### *3.1.5. Effect of volume of disperser solvent*

The influence of the volume of the disperser solvent (methanol) on the extraction efficiency of chromium (VI) was also investigated. Various experiments were performed with different volumes of methanol (200, 400, 500 and 700 µL) containing 800 µL of the extraction solvent and 300 µL of DPC chelating agent. Maximum enrichment factor (Fig. 5) was found when 200 µL of the dispersion solvent was used and as a result, this was selected as the optimum volume of the methanol to be used in subsequent experiments.

During the DLLME technique, a mixture of the dispersion solvent (methanol) and the extraction solvent (chloroform) were rapidly injected into the aqueous sample. This resulted in the partitioning and formation of tiny droplets of chloroform throughout the sample. These tiny droplets of chloroform increased its surface area facilitating the efficient extraction of chromium complex from the fruit and vegetable samples. If the volume of the dispersion solvent used is less than the optimum (200 µL), then partial dispersion of the extraction solvent occurs resulting in poor extraction of the chromium complex and therefore lower enrichment factors. Thus, lower disperser volumes result in an unsatisfactory partitioning of the chloroform as well ineffective droplet formation, ultimately decreasing the extraction efficiency. Volumes that are larger than the optimum, on the other hand, may adversely affect the extraction of the chromium complex. Excessive volumes of the dispersion solvent may increase the solubility of the analyte in the aqueous medium thereby hampering the extraction process resulting in lower enrichment factors.

The solubility of the analyte increases at higher disperser volumes reducing the polarity of the aqueous phase, and ultimately the analyte distribution becomes less in the extraction solvent affecting negatively the extraction efficiency (Ahmad et al., 2016). Thus, the optimum volume of the dispersion solvent should be used to ensure more efficient extraction of the chromium complex and have good enrichment factors. The optimised volume of disperser solvent (methanol) is consistent with the volumes of disperser solvents used by other researchers. Gaubeur et al. (2015), for instance, used 150 µL of methanol as disperser solvent when pre-concentrating heavy metals in beverages using DLLME while Elik et al. (2017) used 200 µL of methanol to pre-concentrate Mn (II) and Cd (II) in foodstuffs.

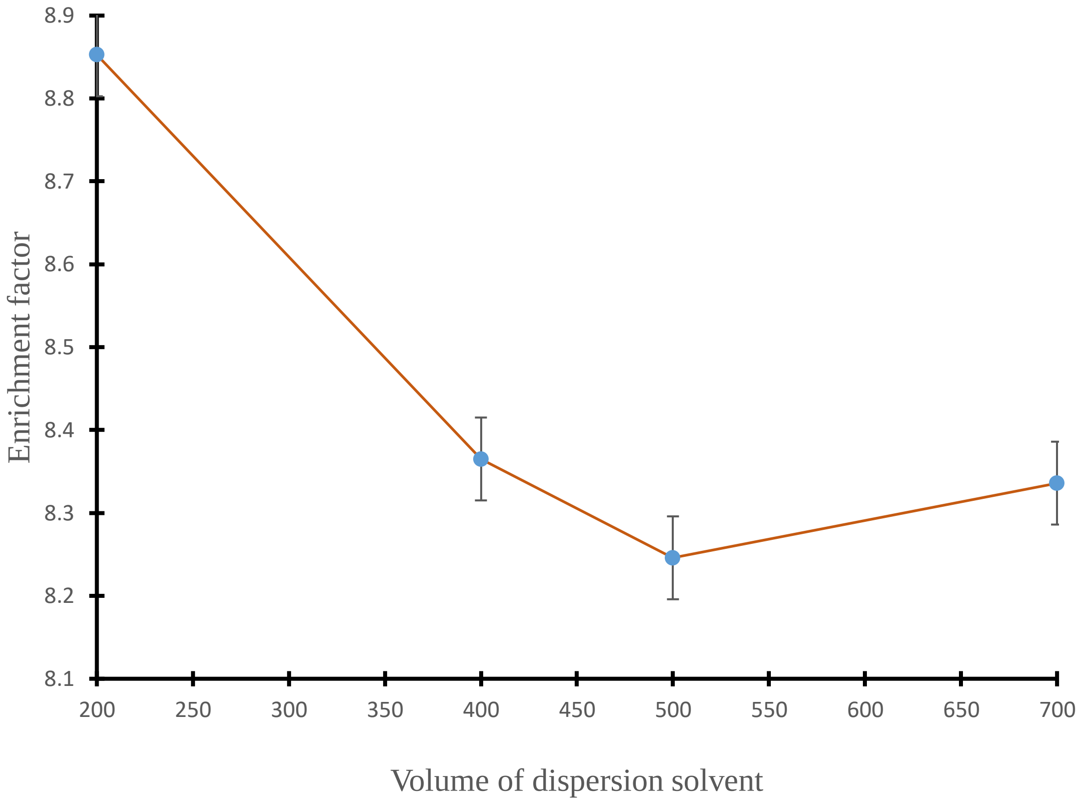

**Fig. 5:** Effect of volume of dispersion solvent (methanol) on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 400 µL; extraction solvent, chloroform; extraction solvent (chloroform) volume, 800 µL; DPC concentration, 1.0 mg $L^{-1}$; DPC volume, 500 µL; centrifugation rate, 5000 rpm; centrifugation time, 2 min; pH during complex extraction, 6.8; sodium acetate volume, 2.0 mL.

### *3.1.6. Effect of pH during complex formation*

The formation of the DPC-chromium complex is pH dependent. Thus, the effect of pH during complex formation was investigated in the range pH 1 to 8 (Fig. 6). The optimum enrichment factor was at pH 2 and consequently the sample pH was adjusted to 2 to facilitate rapid complex formation. From the results it was observed that chromium (VI) reacts with 1,5-diphenylcarbazide (DPC) in acidic medium. According to equation (1), DPC is converted into 1,5-diphenylcarbazone by the oxidation capability of Cr(VI) and then Cr(III)-diphenylcarbazone complex is formed to give a violet solution:

$$2CrO_4^{2-} + 3C_{13}H_{10}ON_4H_4 + 8H^+ \rightleftharpoons [Cr(C_{13}H_{10}ON_4H)_2]^+ + Cr^{3+} + C1_3H_{12}ON_4H_2 + 8H_2O \quad (1)$$

(Yousefi and Shemirani, 2013)

Variations in pH has a direct impact on the formation of the DPC-chromium (VI) complex. Below the optimum pH of 2, an increase favoured the formation of the DPC-chromium complex. Thus, there was an increase in enrichment factors between pH 1 and pH 2 (Fig. 8). At pH 2, probably enough $H^+$ ions, required for the reaction between DPC and chromium (VI), were added in the sample mixture. Thus, there was maximum reaction between chromium (VI) and DPC at this optimum pH resulting in the attainment of equilibrium. An increase in pH above 2, would probably disrupt the equilibrium position causing negative impacts on the enrichment factors.  This was probably due to the fact that the concentration of $OH^-$ ions in the samples increased with an increase in pH. The additional $OH^-$ ions would react with the $H^+$ ions, required during complex formation, in a neutralisation reaction. The removal of the $H^+$ ions would promote the dissociation of the DPC-chromium complex. Thus, pH values above the optimum, reduce the formation of the DPC-chromium complex causing also a reduction in the enrichment factors.

The adjustment pH, in order to promote the complex formation, is a common feature in the DLLME techniques that involve complexation. For instance, Seeger et al. (2017) used ammonium pyrrolidinedithiocarbamate and sodium diethyldithiocarbamate as chelating agents during pre-concentration of arsenic and cadmium in sugar using DLLME and the pH was adjusted to 2 in order to promote complex formation. Bahadir et al. (2016) also adjusted the pH of the sample to 2 during pre-concentration of chromium using DLLME so as to promote complex formation when ammoniumpyrrolidinecarbodithioate was used as the chelating agent. Thus, the optimised pH falls within the commonly used values during complexation of metals.

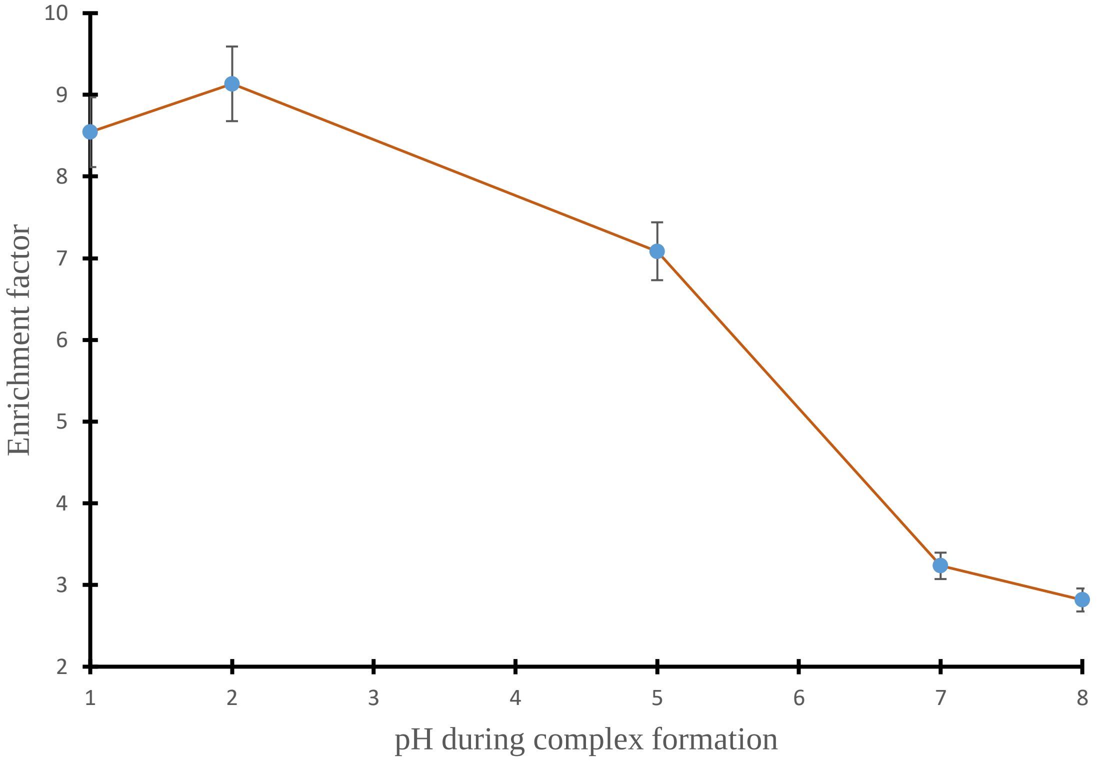

**Fig. 6:** Effect of pH during complex formation on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 µL; extraction solvent (chloroform) volume, 800 µL; DPC concentration, 10 mg $L^{-1}$; DPC volume, 500 µL; centrifugation rate, 5000 rpm; centrifugation time, 5 min; pH during complex extraction, 6.8; sodium acetate volume, 2.0 mL.

### *3.1.7. Effect of pH during complex extraction*

The extraction of the DPC-chromium complex is pH dependent (Fig. 7). The enrichment factors in Fig.7 are increasing with an increase in pH up to pH 8 followed by a decrease thereafter. This can be attributed to the fact that the DPC-chromium complex is cationic and basic conditions will assist to make the complex neutral thereby facilitating its extraction by chloroform. The cationic nature of the DPC-chromium (VI) complex makes it hydrophilic and more soluble in polar solvents. Thus, there is poor interaction between the DPC-chromium (VI) complex with non-polar and hydrophobic solvents like chloroform. Appropriate strategies, targeted at reducing the polarity, should be employed if the DPC-chromium complex is to be extracted using organic solvents like chloroform.

One such strategy involves the elevation of pH to such a level that would reduce the polarity of the DPC-chromium complex. As pH is increased, so does the amount of the hydroxide ($OH^-$) ions in the sample mixture. The negative changes on the hydroxide ions will reduce the polarity of the DPC-chromium complex, thereby facilitating its extraction with chloroform. Thus, there was an increase in enrichment factors of chromium (VI) when the pH was increased from 2 to an optimum value of 8 (Fig. 7). At pH 8, the maximum possible amount of $OH^-$ ions, which does not affect the stability of the complex, would be in the sample mixture. Any further increase in pH, above the optimum pH of 8, would negatively affect the stability constant of the complex. Thus, at pH values above 8, the DPC-chromium (VI) complex would be probably less stable as evidenced by the rapid decrease in enrichment factors (Fig. 7).

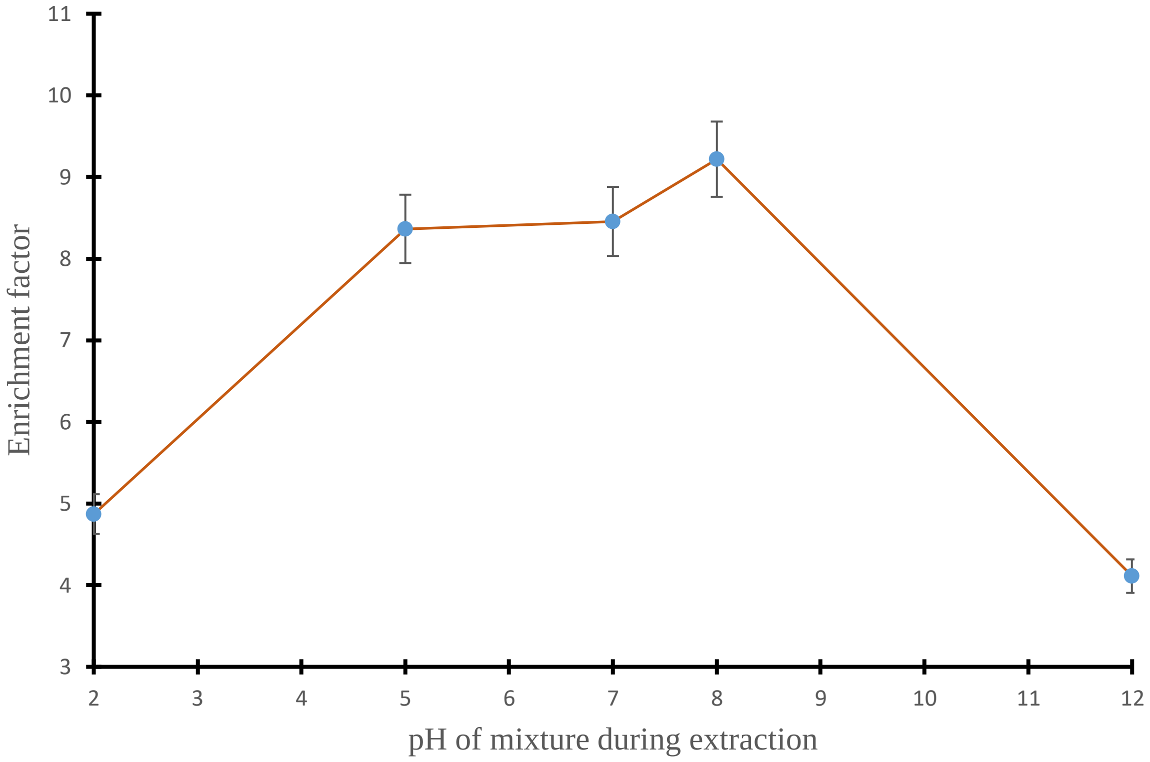

**Fig. 7:** Effect of pH during complex extraction on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 µL; extraction solvent (chloroform) volume, 800 µL; DPC concentration, 10 mg $L^{-1}$; DPC volume, 500 µL; centrifugation rate, 5000 rpm; centrifugation time, 5 min; pH during complex formation, 2; sodium acetate volume, 2.0 mL.

### *3.1.8. Effect of the centrifugation rate*

Centrifugation rate is one of the most important parameters during the DLLME procedures. For this purpose, a series of experiments were made at different centrifugation rates varying from 3000 to 5000 rpm for 2 min and the enrichment factor was measured in each case.

The highest enrichment factor was found when the centrifugation rate was 4000 rpm (Fig.8). As a result, the centrifugation rate of 4000 rpm was taken as the optimum in this study and this was used in subsequent experiments.

The optimised centrifugation rate (4000 rpm) is consistent with what other researchers have used. Ghorbani et al. (2013), for instance, centrifuged water samples at 3000 rpm during the pre-concentration of copper and zinc using DLLME. The same centrifugation rate was used by Gaubeur et al. (2015) during the pre-concentration of heavy metals in water samples using DLLME while Peng et al. (2015) used 3500 rpm as the centrifugation rate when pre-concentrating pesticides in water by DLLME.

The sedimentation of particles during centrifugation is mainly a result of the centripetal force from the centrifuge (Romanò, 2018). The centripetal force should be large enough to cause the sedimentation process. Hence, the need to optimise the centrifugation speed. At centrifugation rate below the optimum, the centripetal force may not be adequate to cause the complete sedimentation of DPC-chromium complex. Thus, at centrifugation rates below the optimum, the enrichment factors of chromium (VI) were low. During centrifugation of the DPC-chromium complex, there are two competing processes: sedimentation and diffusion (Antonopoulou et al., 2018). The centrifugation conditions should be carefully chosen such that the rate of sedimentation of the analyte of interest (DPC-chromium (VI) complex) always surpasses its diffusion rate from the sediment. From Fig. 8, it can be observed that the enrichment factor of chromium (VI) gradually decreased as the centrifugation rate exceeded the optimum (4000 rpm). Above the optimum centrifugation rate, probably the rate of diffusion of the DPC-chromium complex from the sedimented phase would be greater than the sedimentation rate. Consequently, less DPC-chromium complex was in the sedimented phase causing a decrease in the enrichment factor.

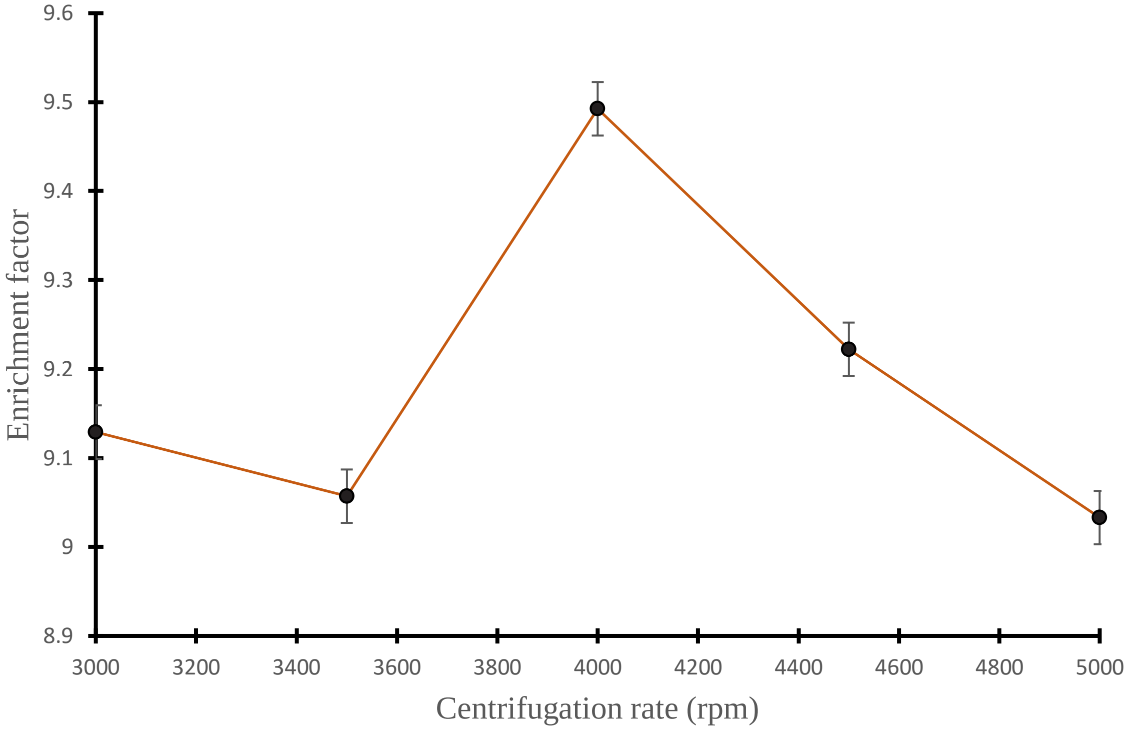

**Fig. 8:** Effect of centrifugation rate on the enrichment factor of Cr (VI) (n = 3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 µL; extraction solvent (chloroform) volume, 800 µL; DPC concentration, 10 mg $L^{-1}$; DPC volume, 500 µL; centrifugation time, 2 min; pH during complex formation, 2; pH during complex extraction, 8; sodium acetate volume, 2.0 mL.

### *3.1.9. Effect of the centrifugation time*

The influence of centrifugation time in dispersive liquid–liquid micro-extraction of Cr (VI) using DPC chelating reagent was also studied. The effect of centrifugation time was studied over the range of 2–7 min, while the other experimental conditions were kept constant. The results indicated a maximum enrichment factor at 3 min and this was considered as the optimum centrifugation time to be used in subsequent experiments (Fig. 9). Most researchers use centrifugation time that ranges from 2-5 min during the pre-concentration of analytes in different matrices (Gaubeur et al., 2015; Ghorbani et al., 2013; Peng et al., 2015). Thus the optimised centrifugation time falls within the common time ranges used by other researchers.

During the DLLME technique, the DPC-chromium complex should be given enough time to undergo sedimentation when the centrifugation process is performed. The sedimentation time depends on the density of the DPC-chromium complex as well as the nature of the matrix. It is therefore imperative that the centrifugation time should be optimized to ensure complete sedimentation of the DPC-chromium complex. Less centrifugation time than the optimum would entail incomplete sedimentation of DPC-chromium complex resulting in lower enrichment factors of chromium. The sedimentation process is opposed by diffusion of the sedimented particles during centrifugation (Antonopoulou et al., 2018). The centrifugation time should be just enough to promote sedimentation of the DPC-chromium complex but not adequate to make the diffusion rate of the chromium complex surpass the sedimentation rate. Thus, the gradual decrease in enrichment factors encountered after 3 min were probably due to the increase in the diffusion rate of the DPC-chromium complex from the sedimented phase into the aqueous layer.

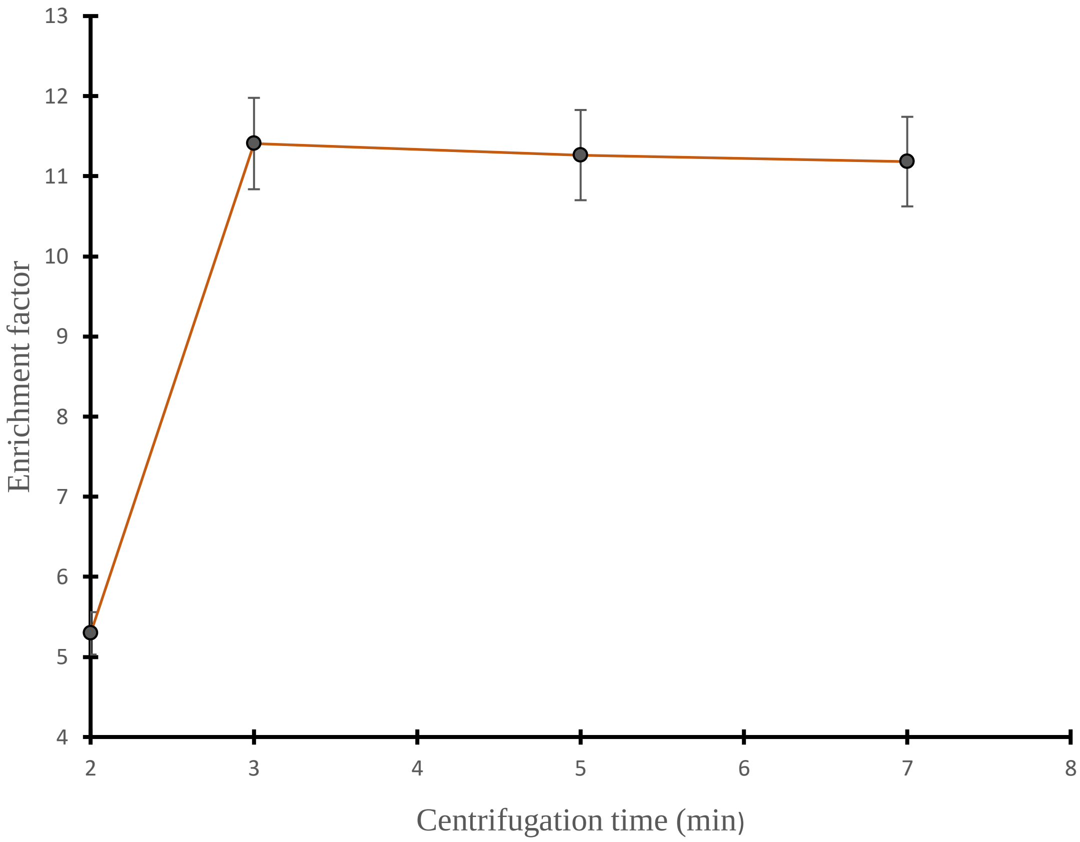

**Fig. 9:** Effect of centrifugation time on the enrichment factor of Cr (VI) (n=3). Experimental conditions: Cr (VI) concentration, 3.0 mg $L^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 µL; extraction solvent (chloroform) volume, 800 µL; DPC concentration, 1.0 g $L^{-1}$; DPC volume, 500 µL; centrifugation rate, 4000 rpm; pH during complex formation, 2; pH during complex extraction, 8; sodium acetate volume, 2.0 mL.

### *3.1.10. Effect of DPC concentration*

The concentration of the chelating agent (DPC) has a bearing on the extraction efficiency and enrichment factor. The concentration of DPC should be carefully controlled so as to favour the formation of the DPC-chromium complex instead of its decomposition. The effect of DPC concentration during chromium speciation analysis was investigated in the range of 0.1– 1 g $L^{-1}$. It was observed that the maximum enrichment factor was obtained when the concentration of DPC was 0.25 g $L^{-1}$ (Fig. 10). Thus, 0.25 g $L^{-1}$ was used as the optimum concentration of DPC in this study (Fig. 10) and this concentration was used in subsequent experiments. From the results (Fig, 10), there was an increase in enrichment factor as the concentration of DPC was increased from 0.1 to 0.25 g $L^{-1}$. However, there was a general decrease in the enrichment factors of chromium (VI) at concentration levels of DPC, which were above the optimum. For a fixed volume of DPC, the number of moles increased with an increase in concentration. At a concentration of 0.25 g $L^{-1}$, the right stoichiometric amount of DPC to complex completely with chromium (VI), would have been added to the sample resulting in the maximum enrichment factor. Thus, at concentration levels less than 0.25 g $L^{-1}$, the added DPC would be less than the stoichiometric amount required for complete complexation with chromium (VI) and this probably explains the lower enrichment factors experienced at levels below the optimum DPC concentration. Complexation between DPC and chromium (VI) is a reversible reaction. Higher concentrations of DPC would result in the presence of moles which surpass the required stoichiometric amount for its complete chelation with chromium (VI). The excess DPC would sediment together with the DPC-chromium (VI) complex since its density (1.31 g $cm^{-3}$) is greater than that of water (1 g $cm^{-3}$). The volume of the sedimented phase would be greater than expected causing a decrease in concentration of the complex and lower enrichment factors.

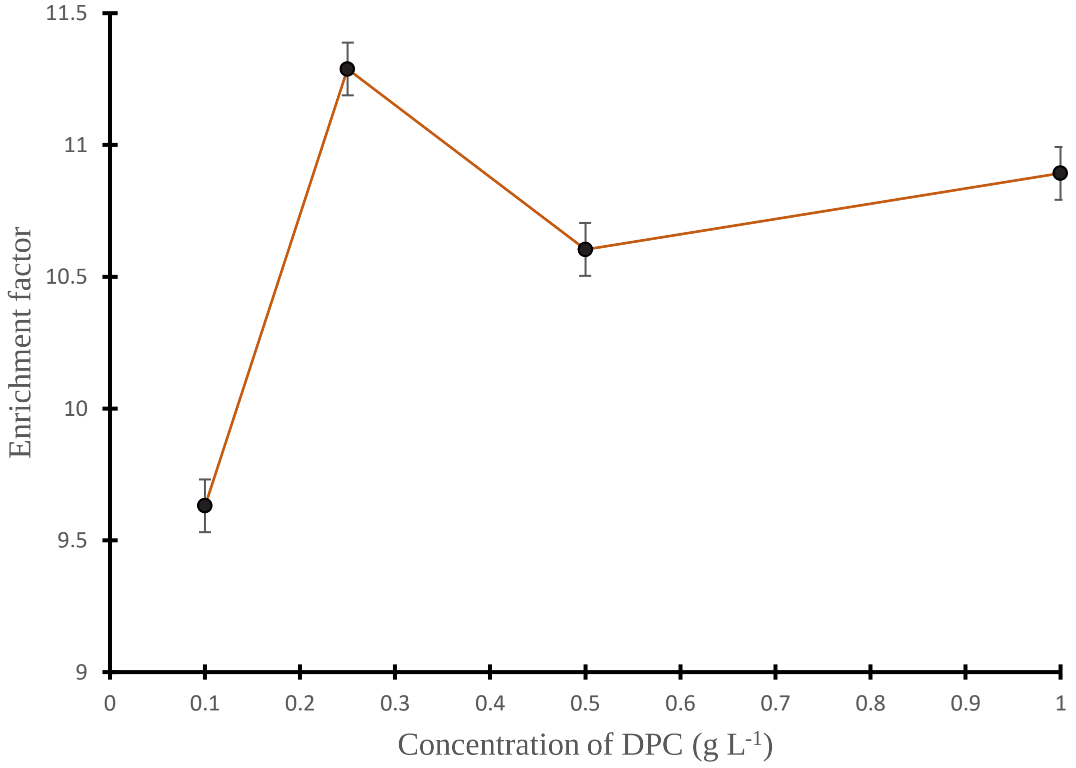

**Fig. 10:** Effect of concentration of DPC (chelating agent) on the enrichment factor of Cr (VI) (n=3). Experimental conditions: Cr (VI) concentration, 3.0 mg L$^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 µL; extraction solvent (chloroform) volume, 800 µL; DPC volume, 500 µL; centrifugation rate, 4000 rpm; centrifugation time, 3 min; pH during complex formation, 2; pH during complex extraction, 8; sodium acetate volume, 2.0 mL.

### *3.1.11. Effect of DPC volume*

The success of the DLLME technique hinges on the complete complexation of hexavalent chromium with DPC. Complete complexation depends on the volume of DPC used during the experiment. Thus, the effect of volume of DPC on the enrichment factor of chromium (VI) should be investigated. The effect of volume of DPC was examined in the range 200 to 800 µL (Fig. 11). The highest enrichment factor was found when the volume of DPC was 300 µL and this volume was used as the optimum volume of DPC in the subsequent experiments. During complexation, an adequate amount of DPC should be added to the aqueous samples to ensure that all the chromium (VI) ions in these samples are converted completely into the DPC-Cr (VI) complex. Thus, for a given concentration of DPC, the right volume of DPC should be used such that there is a slight excess of the DPC chelating agent. Chromium (VI) should be the limiting reagent during this complex formation reaction. Hence, the need to optimise the volume of DPC used during complexation with the chromium in the aqueous samples. Volumes of DPC lower than the optimum, therefore, would cause incomplete complexation and lower enrichment factors of chromium (VI). On the other hand, volumes higher than the optimum would result in excess DPC that might be sedimented together with the complex because its density (1.31 g cm$^{-3}$) is greater than that of water (1 g cm$^{-3}$). This would cause increase in volume of sedimented phase as well as decrease in concentration of the complex and lower enrichment factors.

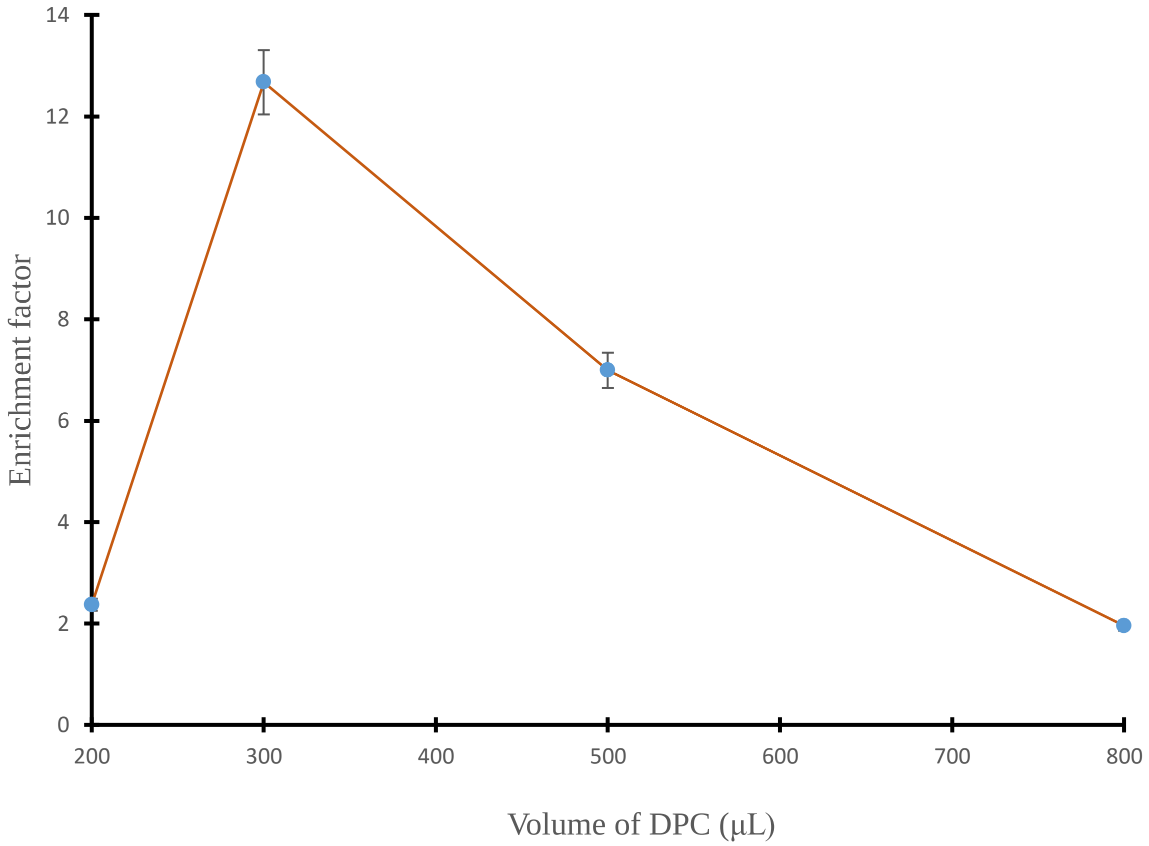

**Fig. 11:** Effect of volume of DPC (chelating agent) on the enrichment factor of Cr (VI) (n=3). Experimental conditions: Cr (VI) concentration, 3.0 mg L$^{-1}$; sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 µL; extraction solvent (chloroform) volume, 800 µL; DPC concentration, 0.25 g L$^{-1}$; centrifugation rate, 4000 rpm; centrifugation time, 3 min; pH during complex formation, 2; pH during complex extraction, 8; sodium acetate volume, 2.0 mL.

### *3.2. Method validation and application to real samples*

The developed method was validated before its application to determine chromium species in real fruit and vegetable samples. The following figure of merits were used during the validation process: linear range, linearity, limit of detection and limit of quantification. The linear range of the developed method was from 0.01 to 8.0 mg $kg^{-1}$. The limit of detection and limit of quantification were 0.36 mg $kg^{-1}$ and 1.20 mg $kg^{-1}$ respectively. Thus, the developed method is fairly sensitive to chromium (VI) and can be used during the speciation studies. The correlation coefficient ($R^2$) was found to be 0.9975 and therefore the calibration curve can be used for quantification.

To evaluate the applicability and accuracy of the proposed method in real fruit and vegetable samples, three fruit samples including apple juice, grape juice and orange juice as well as three vegetable samples including lettuce, tomato and cucumber, were considered. These fruit and vegetable samples were subjected to the optimal conditions of the developed method. All the fruits and vegetables analysed had trace amounts of chromium (Fig 12). From Fig. 12, it can be observed that the concentration of chromium (III) was higher than the concentration of chromium (VI) in all analysed fruit and vegetable samples. Chromium (III) plays vital biochemical roles in the body of living organisms (Salihu and Bakar, 2018). Hence, its higher concentration levels, in fruits and vegetables used in this study, are not a threat to human health. Chromium (VI), however, has detrimental effects on the health of living organisms. It is considered to be carcinogenic (Akhtar et al., 2018). Therefore, the concentration of chromium (VI) should be monitored so that it remains within acceptable limits. The maximum allowable limit of chromium (VI) set up by WHO for drinking water and foodstuffs, is 50 µg $L^{-1}$ (0.05 mg $L^{-1}$) (Sobhi et al., 2018). The concentration of chromium (VI) in all fruits and vegetables, analysed in this research, were below the maximum permissible limit. Thus, these fruits and vegetables are safe for human consumption.

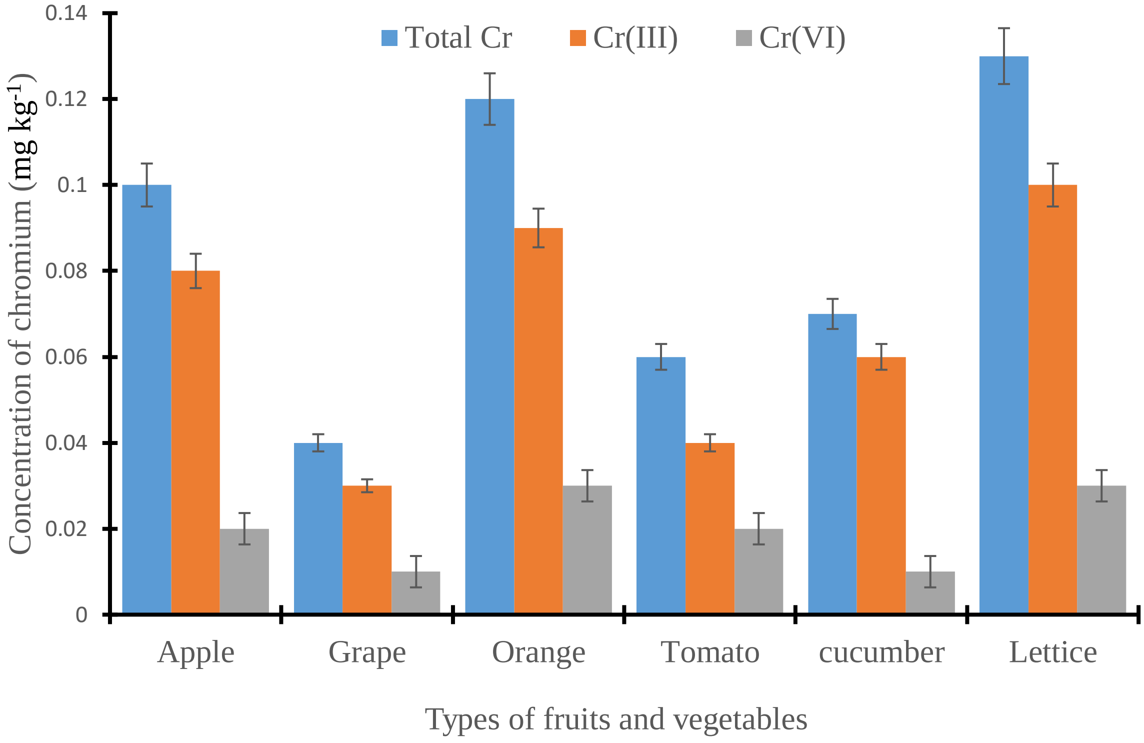

**Fig 12**: The concentration levels of Cr(III) and Cr(VI) in selected fruit and vegetable samples (n=3). Experimental conditions: sample volume, 2.0 mL; dispersive solvent (methanol) volume, 200 μL; extraction solvent (chloroform) volume, 800 μL; DPC concentration, 0.25 g $L^{-1}$; DPC volume, 300 μL; centrifugation rate, 4000rpm; centrifugation time, 3 min; pH during complex formation, 2; pH during extraction of complex, 8; sodium acetate volume, 2.0 mL.

## 4. Conclusion

In this study, a rapid, simple and inexpensive dispersive liquid-liquid micro-extraction method for the pre-concentration of the hexavalent chromium during trace speciation analysis was developed. Determination of the concentration levels of the trivalent and hexavalent chromium in selected fruits and vegetables was performed by FAAS. A univariate approach was applied to optimize the influential factors affecting the extraction efficiency of the DLLME method. Following the establishment of the optimized conditions, the method demonstrated to be efficient, accurate and precise in Cr (VI) determination. Thus, this simple and fast method has the potential to be easily implemented for environmental monitoring of chromium species in wide variety of samples. The concentration of chromium (III) was determined from the difference between concentration of total chromium and concentration of chromium (VI).

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## Conflict of interests

There is no conflict of interests

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# 4.6. Paper VI

This paper “Z-sep$^+$ based QuEChERS technique for the pre-concentration of malathion pesticide in fruits followed by analysis using UV-Vis spectroscopy” is a manuscript in making. In this paper malathion was pre-concentrated using QuEChERS with Z-sep$^+$/PSA sorbent combination being used for d-SPE clean-up. Colour development reactions were then performed on the extracted malathion followed by analysis with UV-Vis spectrophotometry.

# Z-sep$^+$ based QuEChERS technique for the pre-concentration of malathion pesticide in fruits followed by analysis using UV-Vis spectroscopy

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## ABSTRACT

Malathion is an organophosphate pesticide that is an irreversible inhibitor of the enzyme cholinesterase and is widely used in agriculture to control pests in fruits. In this work, the concentrations of malathion in fruits were determined using UV-Vis spectrophotometry prior to pre-concentration using QuEChERS. The Z-sep$^+$/PSA sorbent combination was used for the d-SPE clean-up and extraction was done using acetonitrile during QuEChERS. The extracted malathion was then hydrolysed under basic conditions followed by the reaction with potassium bromate. This reaction caused the development of an orange-yellow colour, thereby making the mixture UV-active. The absorbance of the mixture was then measured using a UV-Vis spectrophotometer at a wavelength of 415 nm. The QuEChERS parameters, which included type and volume of extraction solvent, type and mass of sorbents, and centrifugation rate, were optimised prior to application of the developed method to real fruit samples. The linear range was from 0.1 to 0.9 mg kg$^{-1}$ while the coefficient of determination ($R^2$) was 0.9999. The limit of detection (LOD) for malathion was found to be 0.017 mg kg$^{-1}$ and the limit of quantification was 0.05 mg kg$^{-1}$. The orange samples were found with no malathion residues when the developed method was applied to them while the concentrations of malathion in apple and pear samples were 0.07 mg kg$^{-1}$ and 0.09 mg kg$^{-1}$ respectively.

*Keywords*

QuEChERS, malathion, fruits, Z-sep$^+$ sorbent, PSA sorbent

## Introduction

Environmental pollution by pesticides is one of the serious predicaments of the modern world (Evangelou et al., 2016; Kim et al., 2017; Talebianpoor et al., 2017). These pesticides are widely applied in a variety of different ways during the production of fruits and other crops in general to control the growth of weeds and fungi or to prevent crop damage by insects, mites and rodents (Cheng et al., 2017; Mohammadi et al., 2015). They are also frequently used on fruits during the post-harvest period to prolong storage life and for quality enhancement. The ever-increasing trend of population growth and the subsequent higher rate of fruit consumption have compelled the producers to intensify their efforts to increase fruit production. Thus, there is repeated application of pesticides for the control of various pests leaving the consumers exposed to high levels of pesticide residues. These pesticide residues in food items in general and in fruits in particular, have been a concern to environmental and consumer groups for a long time.

Organophosphate pesticides are among the pesticides that have been widely used in fruits and vegetables to control pests so as to increase productivity (Cheng et al., 2017). This often results in the presence of trace amounts of organophosphate pesticides in fruits and vegetables. Organophosphates are harmful to human health because of their potential mutagenicity properties (Wang et al., 2013).

Malathion is one of the widely used organophosphate to control pests in fruits and vegetables. Just like the other organophosphates, malathion interferes with the activity of cholinesterase (Seebunrueng et al., 2014). When the cholinesterase enzyme cannot perform its normal function, the nerves in the body send “messages” to the muscles continuously leading to muscle twitching and weakness. If the poisoning is severe, the victim may have “fits” or convulsions, and may even die (Ren and Xia, 2016). Malathion is irreversible cholinesterase inhibitor (Pirsaheb et al., 2013). Without medical treatment, the level of enzyme activity will return to normal only after several days, weeks or even months. Additive effects of small repeated doses of malathion over time, such as in a spraying season, may finally cause poisoning. The effects of mild malathion poisoning in humans include fatigue, headache, and dizziness (Zhao et al., 2007). Moderate poisoning leads to inability to walk, weakness and chest discomfort. In severe cases, there will be unconsciousness, severe constriction of pupils and muscle twitching ultimately resulting in death (Sapahin et al., 2014). Malathion has adverse effects on humans even at trace levels. Therefore, there is an increasing demand to develop an accurate and sensitive analytical method for simultaneous determination of trace levels of organophosphates in general, and malathion in particular to facilitate risk assessment. In addition, effective pre-concentration techniques are required to ensure interference reduction during analysis in order to reach reproducible and accurate data, which are especially challenging for complex matrices (Madureira et al., 2014). Malathion, on the other hand, reacts with other chemicals in the presence of sunlight in the atmosphere to produce 40 times more toxic compound, malaoxon. Malaoxon, however, is not stable and it undergoes photodecomposition very quickly (Geed et al., 2016).

The determination of pesticide residues like malathion (Fig. 1) in fruit matrices is a challenge especially because of the low concentration of pesticides and large amounts of interfering substances (Muñoz et al., 2017; Sivaperumal et al., 2017; Suganthi et al., 2018). The interfering substances can be co-extracted with pesticides and, in most of the cases, adversely affect the analysis results (Tuzimski and Rejczak, 2014). Several techniques have been proposed for the pre-concentration of pesticide residues in fruits and fruit juices that include solid phase extraction (Huo et al., 2016; Pelajić et al., 2016; Samnani, 2015; Shamsipur et al., 2016; Zhao et al., 2016), solid phase micro-extraction (Kenessov et al., 2016; Liang et al., 2017; Saraji et al., 2016; Wang et al., 2013; Zhang et al., 2017), matrix solid phase dispersion (Albero et al., 2003; Chatzimitakos et al., 2018), pressurized liquid extraction (Du et al., 2012; Robinson et al., 2013), microwave assisted extraction (Herbert et al., 2006; Merdassa et al., 2013; Otake et al., 2012) and dispersive liquid-liquid micro-extraction (Jafari et al., 2016; Pastor-Belda et al., 2015; Pirsaheb et al., 2015; Wang et al., 2016; You et al., 2015).

While these methods are highly efficient, most of them generally require considerable investment in instrumentation and allow a limited scope of pesticides to be extracted under certain conditions. In this context, although they can be employed in some applications, they are far from ideal for multi-residue pesticide determinations in food, due to the wide variety of pesticides with different chemical properties. Since 2003, when the QuEChERS method was introduced by Anastassiades et al. (2003), there has been a revolution in sample preparation steps for pesticide residue analysis. The QuEChERS technique has been used by many researchers to pre-concentrate pesticides, including malathion, in different matrices (Abbas et al., 2017; Andraščíková and Hrouzková, 2016; Cheng et al., 2017; Guo et al., 2018; Kaczyński et al., 2017; Kim et al., 2018; Lawal et al., 2018; Norli et al., 2015; Viera et al., 2017; Zheng et al., 2017). This method was proposed in order to overcome the limitations of the methods mentioned above such as low enrichment factors and use of large volumes of organic solvents. Moreover, it supplies the necessary characteristics for a multi-residue method and thus ensures accurate and precise results and low limits of detection for a wide range of pesticides (Rizzetti et al., 2016).

The QuEChERS method is based on an extraction with acetonitrile and partitioning with salt addition (Anastassiades et al., 2003; Rejczak and Tuzimski, 2015). The clean-up step with a dispersive solid phase extraction (d-SPE) promotes cleaner extracts (Dankyi et al., 2015). The main sorbent used in d-SPE is a primary–secondary amine (PSA) which provides high capacity for the removal of sugars, organic and fatty acids and polar pigments (Nuapia et al., 2016; Wang et al., 2017). The $C_{18}$ silica sorbent is used in fat-containing samples (Dankyi et al., 2015; Lee et al., 2016). The graphitized carbon black sorbent can be added for the clean-up of highly pigmented samples (Bernardi et al., 2016). New sorbents have been developed and among them there is Z-sep and Z-sep$^+$. The Z-sep sorbent is a new commercially available sorbent based on zirconium oxide, which is useful for the analysis of hydrophobic pesticides in fatty matrices. Z-Sep$^+$ is a mixture of the two sorbents octadecyl ($C_{18}$) and silica coated with zirconium dioxide with a $ZrO_2$: $C_{18}$ ratio of 2: 5 (Lozano et al., 2014). The Z-Sep sorbents provide improved sample clean-up over traditional PSA/$C_{18}$ which can extend analytical column life and instrument throughput. They provide an advantage especially for analysis of more hydrophobic pesticides (Tuzimski and Rejczak, 2014).

After sample preparation, the determination of organophosphate pesticides such as malathion in different sample matrices was usually carried out by using gas chromatography mass spectrometry (GC−MS) (Chen et al., 2016; Shamsipur et al., 2016), gas chromatography flame photometric detector(GC-FPD) (Du et al. 2004) and gas chromatography coupled to electron capture (GC-ECD) (Jardim et al., 2014) as well as UV-Vis spectrophotometry. Most pesticides, including malathion, are not UV-active and consequently chemical modification, to ensure colour development, is always essential prior to their analysis using UV-Vis spectrophotometry.

In the current work, a simple method was developed for trace analysis of malathion in fruit samples using QuEChERS for pre-concentration and UV-Vis spectrophotometry for instrumental analysis. The method has two simple steps: QuEChERS procedure and colour development prior to analysis using UV-Vis spectrophotometry. This is a simple and novel technique for the determination malathion in fruit samples. The use of cheap and simple techniques increases the number of people who can participate in the monitoring of malathion in the environment. Thus, enabling the quick detection of malathion before its concentration reaches lethal levels.

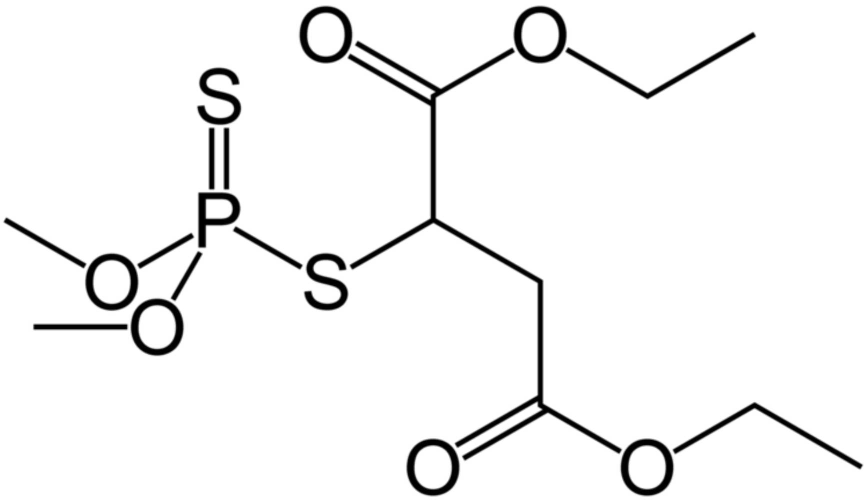

**Fig. 1:** Structure of malathion

## 2. Experimental

### *2.1. Chemicals and instruments*

All reagents and solvents used in this study were of analytical quality grade. The pesticide (malathion) used in this study was bought from Sigma-Aldrich (Johannesburg, South Africa) as well as nitric acid, potassium bromate, potassium hydroxide, copper sulphate, palladium chloride, acetone, acetonitrile and methanol. The sorbents: PSA, $C_{18}$ and Z-sep$^{+}$ were also from Sigma-Aldrich (Johannesburg, South Africa). All solutions were prepared using de-ionised water. The stock solution was prepared using malathion and working standard solutions were prepared by serial dilutions of the stock solution with de-ionised water prior to analysis.

The homogenised fruit samples were mixed during QuEChERS using an analog vortex mixer bought from Sigma-Aldrich (Johannesburg, South Africa). The centrifuge and centrifuge tubes were also bought from Sigma-Aldrich (Johannesburg, South Africa). The Jenway 7305 spectrophotometer, used during instrumental analysis, was also from Sigma-Aldrich (Johannesburg, South Africa).

### *2.2. Sampling*

The orange, apple and pear fruits were purchased from a local supermarket in Thohoyandou, Limpopo province, South Africa. They were homogenised using a blender and then stored in a refrigerator at 4$^{o}$C. Measured masses of the homogenised fruits were used each day during the QuEChERS technique.

### *2.3. QuEChERS procedure*

In the QuEChERS method, 10 g of each homogenised fruit sample was placed into a 50 mL falcon tube. Subsequently, 10 mL of acetonitrile solution were added to the tube, and it was vortexed for 2 min at 3000 rpm. The QuEChERS extraction salts (3.5 g anhydrous $MgSO_4$ and 1 g NaCl) were then added to the tube and it was vortexed again for another 2 min at 3000 rpm and then subjected to centrifuge at 4500 rpm for 3 min. Then, 3 mL of the supernatant was transferred to the dispersive centrifuge tube (15 mL) containing 150 mg of PSA, 150 mg of anhydrous $MgSO_4$ and 60 mg of Z-sep$^+$. The mixture was then stirred in vortex for 2 min followed by centrifugation at 4500 rpm for 3 min. An aliquot of 2 mL of the acetonitrile extract was then used during the colour development stage.

### *2.4. Colour development*

One millilitre of 2% alcoholic potassium hydroxide was added to 2 mL of the QuEChERS extract. This was followed by the addition of 10 mL of 0.1 M potassium bromate. To the mixture 0.5 mL of nitric acid and 2 mL of distilled were then added. The mixture was then vortexed for 1 min at 3000 rpm and an orange-yellow colour developed. In all the analysis, the solutions were then allowed to stand for 5 min before measuring the absorbance. The absorbance was measured at a wavelength of 415nm against reagent blank.

## 3. Results and discussion

Malathion is not conjugated and it is, therefore, not UV-active (Fig. 1). Many strategies, however, can be used to make malathion to be UV-active. For instance, malathion can be analysed using UV-Vis spectrophotometry through oxidizing it with excess N-bromosuccimide and determining the unreacted oxidant by reacting it with amaranth dye in hydrochloric acid (Ayman et al., 2010). Alkaline hydrolysis of the ester linkages in malathion using alcoholic potassium hydroxide followed by the addition of acidified potassium bromate produces an orange-yellow solution which is UV-active with an absorption maximum of 415 nm (Venugopal et al., 2013). Alternately, alkaline hydrolysis of malathion can be followed by chelation with copper resulting in the formation of a UV-active complex. In this study, alkaline hydrolysis followed by reaction with potassium bromate was used. This was the preferred method due to the fact that the reaction is fast and it results in the formation of a fairly stable coloured, UV-active product.

### *3.1. Optimization of QuEChERS parameters during pre-concentration of malathion in fruits*

#### *3.1.1. Effect of type of extraction solvent*

Four possible extraction solvents for malathion, namely ethyl acetate, methanol, acetone and acetonitrile, were investigated in this study (Fig. 2). Many aspects have to be considered when choosing an extraction solvent for malathion during QuEChERS. These include the ability of the solvent to cover the desired analytical spectrum (ranging from the polar to the non-polar compounds), the selectivity that can be reached during extraction, partitioning and clean-up, achieving separation from water, amenability to analytical separation techniques, cost, safety, environmental impact, and handling concerns (e.g., ease of evaporation and volume transfers) (Rejczak and Tuzimski, 2015). From Fig. 2, acetonitrile have the highest enrichment factor of 7.31 and therefore it was chosen as the extraction solvent of malathion in fruit samples. This is in agreement with other researchers as acetonitrile is the extraction solvent most commonly used during QuEChERS (Bargańska et al., 2014; Ferreira et al., 2016; Moloney et al., 2018; Oshita and Jardim, 2014; Pang et al., 2016). The major advantage of acetonitrile over other solvents is due to its ability to separate easily from water when an appropriate mixture of salts (magnesium sulphate and sodium chloride is added during salting-out (Rejczak and Tuzimski, 2015). The other solvent (ethyl acetate, methanol and acetone) do not separate easily from water when the partitioning salts ($MgSO_4$ and NaCl) are added. This could be the probable reason why they have lower enrichment factors than that of acetonitrile. Ethyl acetate, on the other hand, formed emulsions with the fruit samples due to the presence of high sugar content. This, probably, had a negative effect on its extraction of malathion during the QuEChERS method.

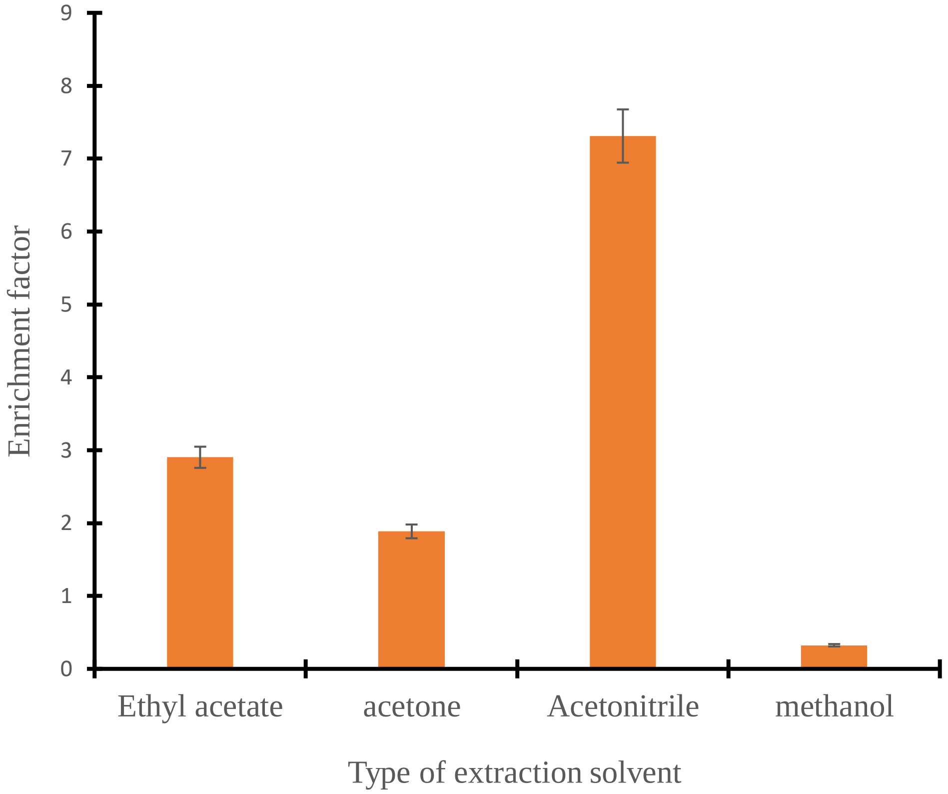

**Fig. 2:** Effect of type of extraction solvent on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: volume of extraction solvents (ethyl acetate, acetone, acetonitrile and methanol), 8 mL; mass of $MgSO_4$, 4 g; mass of homogenised fruit sample, 8 g; type of sorbent, $Zep^+$; mass of $Zep^+$, 80 mg; centrifugation rate, 5000rpm, centrifugation time, 5 min

### *3.1.2. Effect of volume of extraction solvent*

The volume of extraction solvent during pre-concentration of malathion using QuEChERS is of great importance as it has an impact on the extraction efficiency and enrichment factor. In this study, the effect of volume of extraction solvent (acetonitrile) on the enrichment factor of malathion during QuEChERS was investigated (Fig. 3). The volume of acetonitrile was varied from 2 to 12 mL. From Fig. 3, it can be seem that the maximum enrichment factor was obtained when the volume of acetonitrile was 10 mL. This volume, therefore, was taken to be the optimum and was used in the subsequent experiments. The volume of acetonitrile used must be sufficient to completely immerse the sample so as to ensure maximum solvent and malathion interaction. The acetonitrile should not get to its saturation point before extracting all the malathion in the sample. Thus, a slight excess of acetonitrile should be used in order for it to completely extract the malathion pesticide. If the volume of acetonitrile used is too low (less than 10 mL), then it will not be able to completely submerge the sample leading to low enrichment factors and poor recoveries.

The optimized volume of acetonitrile obtained in this study is consistent with the volumes used by other researchers when pre-concentrating pesticides in different matrices using QuEChERS. Prodhan et al. (2016) successfully pre-concentrated pesticides in cabbage using QuEChERS and 10 mL of acetonitrile were used as the extraction solvent. Pesticides in pears were extracted by Kemmerich et al. (2018) through the use of 10 mL of acetonitrile during the QuEChERS method. Cheng et al. (2017) also used 10 mL of acetonitrile during QuEChERS and successfully pre-concentrated pesticides in fruits and vegetables. Some researchers, however, used different volumes of acetonitrile during QuEChERS. For instance, Xiu-ping et al. (2017) used 20 mL of acetonitrile during pre-concentration of pesticides in fruits and vegetables. Thus, the amount of acetonitrile used during QuEChERS is probably matrix dependent and matrix specific. Hence, the need to optimize volume of acetonitrile during the pre-concentration of malathion using the QuEChERS method.

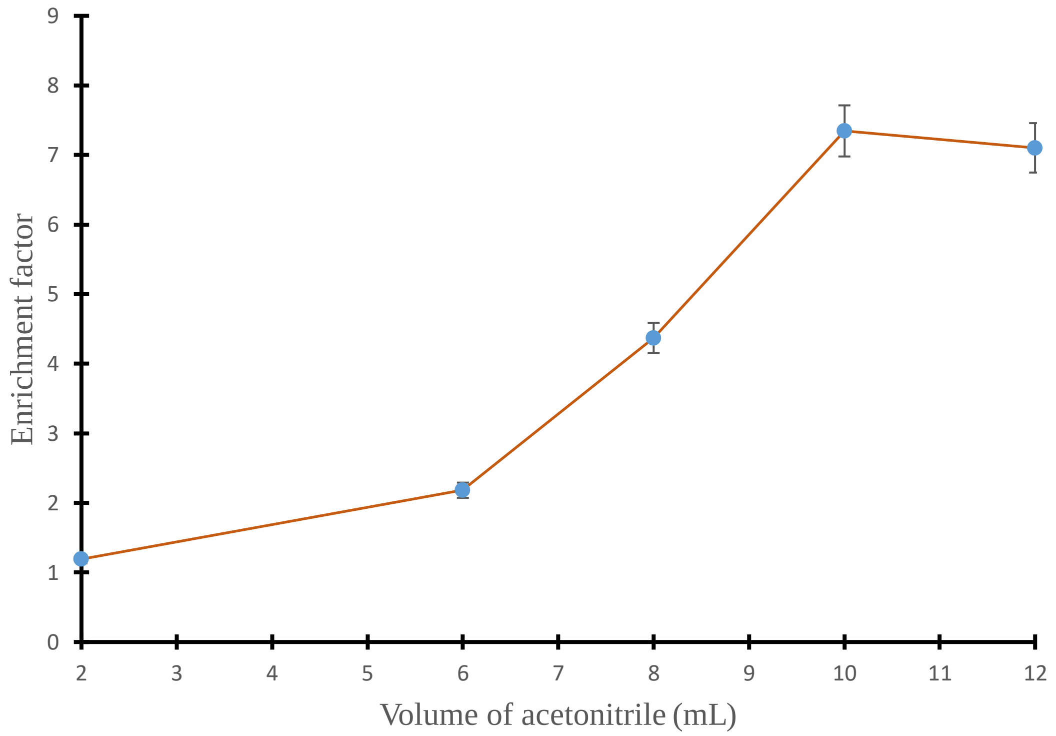

**Fig. 3.** Effect of volume of extraction solvent on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: Type of extraction solvent, acetonitrile; mass of $MgSO_4$, 4 g; mass of homogenised fruit sample, 8 g; type of sorbent, Zep$^+$; mass of Zep$^+$, 80 mg; centrifugation rate, 5000rpm; centrifugation time, 5 min

### *3.1.3. Quantity of $MgSO_4$ used for extraction*

The quantity of $MgSO_4$ used during the pre-concentration of malathion using the QuEChERS technique has great bearing on the enrichment factor and extraction efficiency. Thus, this parameter was optimised by varying the quantity of $MgSO_4$ from 1 to 6 g. Fig. 4 shows the result obtained when mass of the $MgSO_4$ was varied with all the other QuEChERS parameters being kept constant. There was a noticeable increase in the enrichment factor of malathion when 3.5 g of $MgSO_4$ was used and this was used as the optimum mass of $MgSO_4$ during the subsequent experiments. The inclusion of $MgSO_4$ in the QuEChERS technique is done for the enhancement of phase separation but it should be noted that very high levels of the salt would negatively affect the extraction process (Coelho et al., 2008). This is due to the fact that the interaction of water and anhydrous $MgSO_4$ is an exothermic process. Excessive use of $MgSO_4$ generates a lot of heat that may weaken the interactions between acetonitrile and malathion resulting in lower enrichment factors. Thus, the use of the right amount of $MgSO_4$ in phase separation is very important as it regulates the polarity of the mixture. However, the dissolution of the right amount of $MgSO_4$ in water during QuEChERS can generate enough heat that can improve the process of extraction (Anastassiades et al., 2003). The addition of the right amount of $MgSO_4$, therefore, increases the temperature of the system, lowers activation energy and also decrease the viscosity of the solvent thereby increasing solvent matrix interaction (Richter et al., 1996). The QuEChERS method, using $MgSO_4$ for phase separation, has found widespread applications during the pre-concentration of pesticides in different matrices. Machado et al. (2017), for instance, pre-concentrated pesticides in globe artichoke leaves and fruits using QuEChERS and in the process, 4 g of $MgSO_4$ was used. Kaczyński (2017) also used 4 g of $MgSO_4$ for phase separation during the pre-concentration of herbicides in oilseeds using QuEChERS. In the same vein, Ferreira et al. (2016) used 4 g of $MgSO_4$ during the pre-concentration of pesticides in coconut. On the other hand, Bordin et al. (2017) used 3 g of $MgSO_4$ to ensure phase separation during the pre-concentration of pesticide residues in wheat flour. Thus, the optimised quantity of $MgSO_4$ (3.5 g) is within the range of the commonly used amount of $MgSO_4$ during the QuEChERS technique.

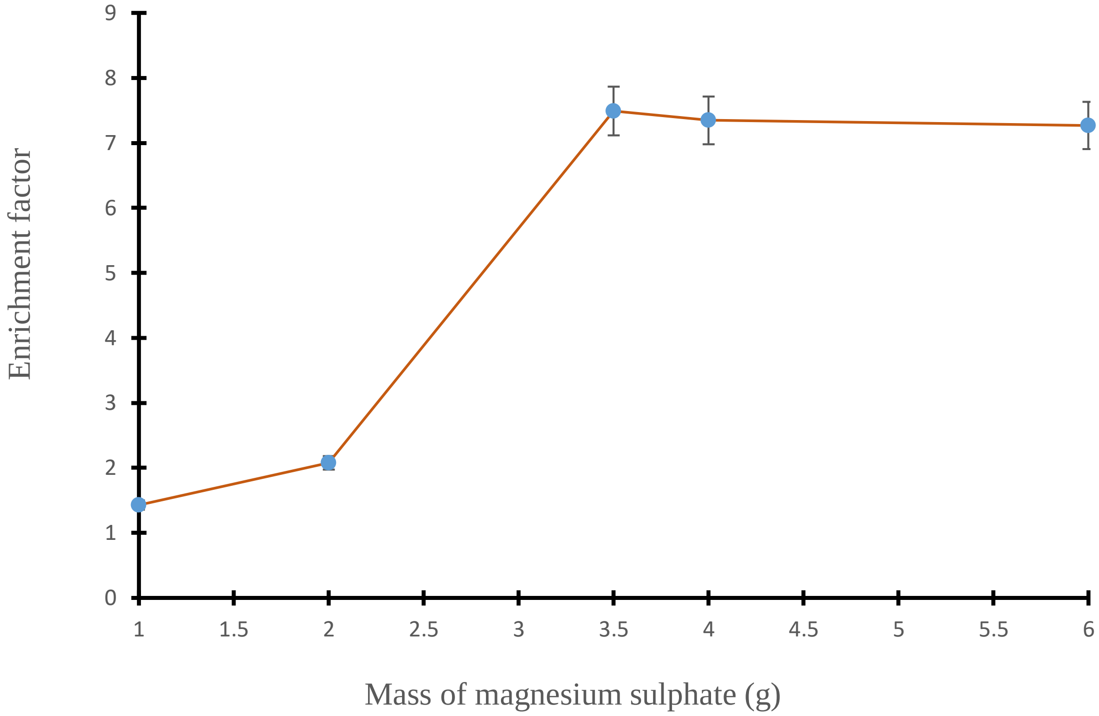

**Fig. 4:** Effect of mass of magnesium sulphate on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of homogenised fruit sample, 8 g; type of sorbent, Zep$^+$; mass of Zep$^+$, 80 mg; centrifugation rate, 5000rpm, centrifugation time, 5 min

### *3.1.4. Mass of the orange sample*

The sample should be thoroughly homogenised during the pre-concentration of malathion using QuEChERS in order to make it representative as well as to increase the surface area during extraction. The simplest way to improve efficiency during QuEChERS is to reduce the sample size to a minimum amount that will provide statistically reliable results and scale the method accordingly (Anastassiades et al., 2003). Fig. 5 shows the enrichment factors of malathion obtained when the mass of the fruit sample was varied from 1 to 12 g while keeping all the other QuEChERS parameters constant. Maximum enrichment factor was obtained when the sample mass was 10 g and this was taken as the optimum mass of the sample to be used in the subsequent experiments. When 10 g of the homogenised fruit sample was used with the volume of acetonitrile being 10 mL, then the sample mass to solvent ratio was 1: 1 (w/v). For extraction, a 1:1(w/v) sample to solvent ratio gives satisfactory recovery (Anastassiades et al., 2003).

The optimized sample mass of 10 g is in line with what other researchers used when pre-concentrating pesticides in different matrices using QuEChERS. Uclés et al. (2018), for instance, used 10 g of fruit and vegetable samples and 10 mL of acetonitrile when pre-concentrating thermally liable pesticides using QuEChERS. The same quantities were used by Chiarello and Moura (2018) when analysing pesticides in wheat flour, lettuce and apple samples. Other researchers, however, got satisfactory results by using different sample to solvent ratios during the QuEChERS process. For instance, Dubey et al. (2018) used 15 g of fruit and vegetable samples and 30 mL of acetonitrile during the QuEChERS technique. In this case, the sample to solvent ratio was 1:2 (w/v) and the pesticides in fruit and vegetable samples were successfully pre-concentrated. Tripathy et al. (2017), on the other hand, used 2 g of medicinal herbs and 15 mL of acidified acetonitrile. Thus, the sample mass to solvent ratio probably depends on the QuEChERS mode being used as well as the nature of the sample matrix.

The enrichment factors of malathion, when the mass of the sample was below 10 g, were lower than those obtained when the mass of the sample was 10 g. This could be attributed to the fact that at lower masses of the sample the heat generated during the dissolution of $MgSO_4$ in the aqueous medium during QuEChERS was distributed over fewer particles resulting in a larger increase of temperature of the sample mixture. Thus, the temperature was probably too high for the effective interaction between acetonitrile and malathion at lower sample masses resulting in lower enrichment factors. When the sample mass was greater than 10 g, there was a gradual decrease in enrichment factors. This was probably a result of the overloading of the Z-sep$^+$/PSA sorbent mixture with the interfering matrix components. At those high sample masses the carrying capacity of the sorbents were probably surpassed by the interfering matrix components resulting in the formation of unclean QuEChERS extracts and, therefore, lower enrichment factors.

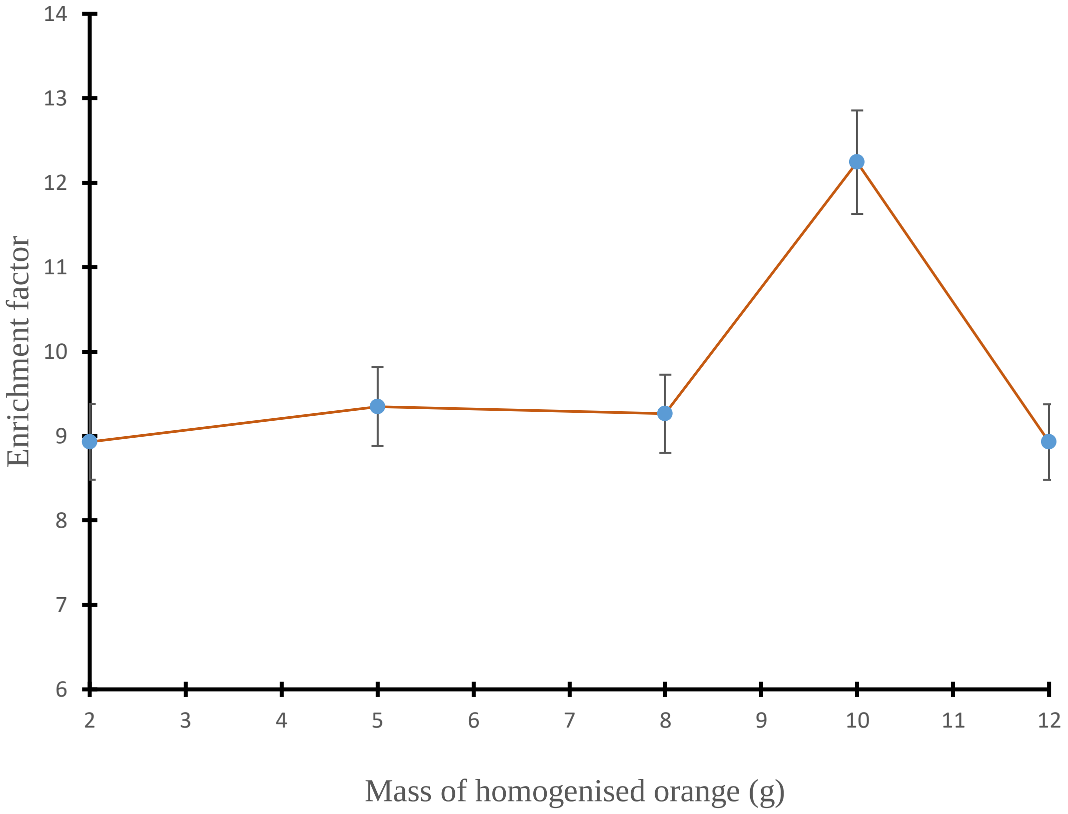

**Fig. 5:** Effect of mass of homogenised sample on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of $MgSO_4$, 3.5 g; type of sorbent, $Zep^+$; mass of $Zep^+$, 80 mg; centrifugation rate, 5000rpm, centrifugation time, 5 min

### *3.1.5. Effect of type of sorbent*

Selection of appropriate sorbent combinations plays a pivotal role during the pre-concentration of malathion using the QuEChERS technique. The cleaning sorbents, during pre-concentration of malathion by QuEChERS, remove the interfering materials from the matrix leading to the enhancement of the extraction efficiencies and enrichment factors. In this study, the effectiveness of different sorbent combinations during trace analysis of malathion were investigated (Fig. 6). The effects of sorbent type were evaluated by analysing the enrichment factors of malathion obtained when all the QuEChERS parameters were kept constant, with the exception of the type of sorbents. The results show that the $Z\text{-}sep^+$/PSA combination produced the highest enrichment factor (7.44). Thus, this was taken to be the sorbent combination of choice, and was used in the subsequent experiments.

The retention mechanism of zirconium dioxide-based sorbents (Z-Sep and $Z\text{-}Sep^+$) involves Lewis acid/base interactions, and these sorbents are known for their ability to reduce the amount of lipids from animal and plant tissue extracts (Dias et al., 2016; Moloney et al., 2018; Walorczyk et al., 2015). They are also capable of removing some pigment interferences from various matrices. Their distinct active sites make the lipid and pigment removal efficient when used during QuEChERS. The zirconia portion of the sorbent acts as a Lewis acid, attracting compounds with electron donating groups, such as the polar hydroxyl groups of lipids, whilst the hydrophobic group of the sorbent interacts with the hydrophobic chains present in the lipids (Moloney et al., 2018). Walorczyk et al. (2015) reported that Z-Sep$^+$ provided good complementary clean-up to PSA/ChloroFiltr, and resulted in the removal of matrix co-extractives to similar degree as GCB/$C_{18}$. Primary-secondary amine (PSA) is a well know clean-up sorbent employed when removal of fat content is necessary. The chemical structure of PSA provides high retention of free fatty acids and other polar matrix compounds (Dias et al., 2016). Thus, the combination of Z-sep$^+$ and PSA results in the removal of most of the co-extractives from fruit samples during the pre-concentration of malathion using QuEChERS. Consequently, in recent years, the attention of many researchers have been captured by these new d-SPE sorbents such as EMR-lipid, Z-sep and Z-sep$^+$ (Kiljanek et al., 2016; López-Blanco et al., 2016; Moloney et al., 2018; Rejczak and Tuzimski, 2016; Urban and Lesueur, 2017). Kiljanek et al. (2016) used Z-sep$^+$ and PSA for the d-SPE clean-up of honey samples during QuEChERS and obtained good recoveries on analysis. The same sorbent combination was used by Moloney et al. (2018) for the clean-up of animal fats during QuEChERS. Rejczak and Tuzimski (2016) successfully used Z-sep sorbent for the d-SPE clean-up of soya milk samples during the pre-concentration of sulfonylurea herbicides using QuEChERS.

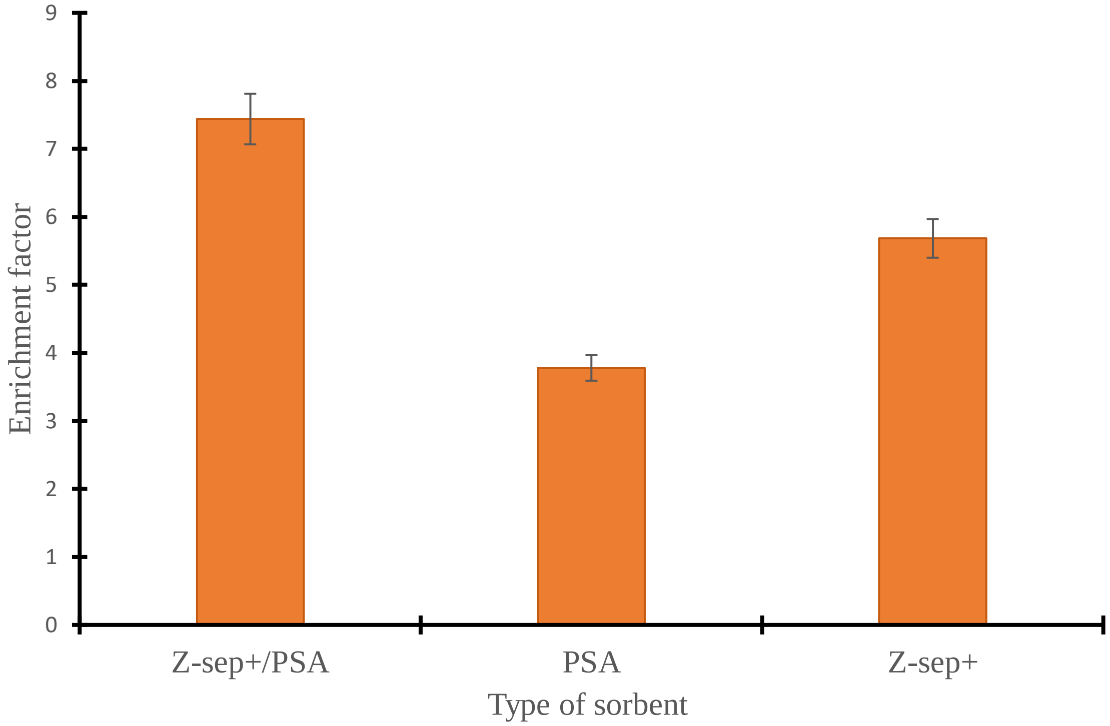

**Fig. 6:** Effect of type of sorbent on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of $MgSO_4$, 3.5 g; mass of homogenised fruit sample, 10 g; mass of sorbents (Z-sep$^+$, PSA and Z-sep$^+$/PSA), 80 mg; centrifugation rate, 5000rpm; centrifugation time, 5 min

### *3.1.6. Effect of the mass of PSA*

PSA is the base sorbent used for QuECHERS d-SPE clean-up of fruits and vegetables because it removes many organic acids and sugars that might act as instrumental interferences. The mass of PSA should be carefully optimised during QuEChERS pre-concentration to ensure effective extraction of the target analyte (malathion) by acetonitrile. The effect of mass of PSA on the enrichment factor of malathion was investigated by varying its amount from 50 to 600 mg, with all the other QuEChERS parameters being constant (Fig. 7). The optimum mass of PSA during the pre-concentration of malathion by QuEChERS was found to be 400 mg since it produced the highest enrichment factor (7.65), and this optimal mass of PSA was used in subsequent experiments. PSA retains acidic components in the fruit matrices during the pre-concentration of malathion using QuEChERS. The retention of the acidic interfering matrix components by PSA is based on acid-base interactions. PSA is basic and hence, it can adsorb acidic matrix components from the fruit samples. Thus, optimum mass of the PSA sorbent should be used to avoid overloading it with acidic interfering components. The use of masses below the optimum (400 mg of PSA), would result in incomplete removal of the interfering matrix components as the amount of PSA would be inadequate for their effective removal. Thus, unclean acetonitrile extracts would be formed and the enrichment factors of malathion would be very low. The use of the optimum mass of PSA, on the other hand, would ensure the formation of cleaner acetonitrile extracts and higher enrichment factors. However, there was no significant change in the enrichment factors of malathion, as compared to its enrichment factor at optimum PSA mass, when masses above the optimum were used. Additional amount of PSA would not make the acetonitrile extract any more cleaner as the right amount of PSA, the optimum mass, would have effectively removed all the interfering materials. Thus, the use of excessive amounts of PSA during pre-concentration of malathion using QuEChERS, on the other hand, is not cost effective.

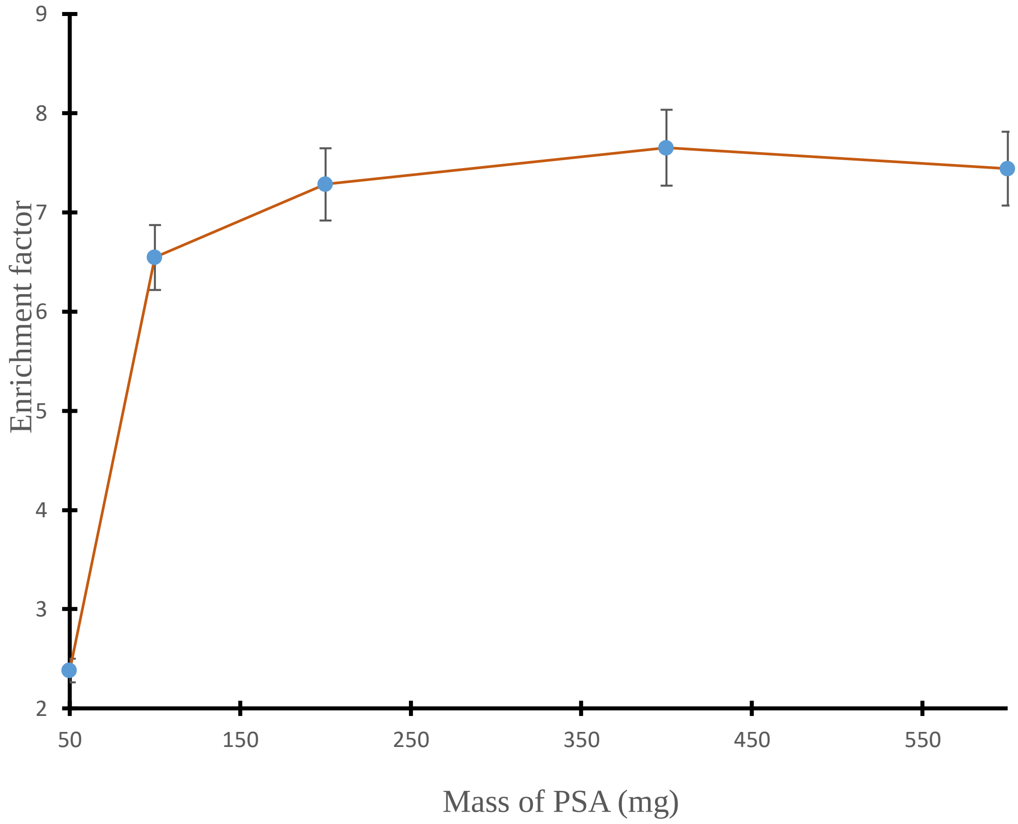

**Fig. 7:** Effect of mass of PSA on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of $MgSO_4$, 3.5 g; mass of homogenised fruit sample, 10 g; type of sorbent, Z-sep$^+$/PSA; mass of Z-sep$^+$ sorbent, 80 mg; centrifugation rate, 5000rpm, centrifugation time, 5 min

### 3.1.7. *Effect of the mass of Z-sep$^+$*

Z-sep$^+$ is a commercial name of a mixture of two sorbents, $C_{18}$ and silica coated with zirconium dioxide, in the ratio of 2:5 (w/w). This sorbent has proved to be effective at removing hydrophobic components of the matrix during QuEChERS (Kiljanek et al., 2016; Moloney et al., 2018). The effect of the mass of the Z-sep$^+$ sorbent, during pre-concentration of malathion using QuEChERS, was evaluated by varying its mass from 20 to 150 mg while keeping the mass of PSA (400 mg) and other QuEChERS parameters constant (Fig. 8). The highest enrichment factor (7.94) was found when the mass of Z-sep$^+$ was 60 mg and this was taken as its optimum mass during the subsequent experiment. There was a general increase in enrichment factors of malathion from 20 to 60 mg. An increase in mass of Z-sep$^+$, from 20 to 60 mg, resulted in an increase in the number of active sites of the sorbent exposed to malathion and also an increase in surface area of the sorbent causing a more effective removal of the hydrophobic matrix components. Hence, the increase in the enrichment factors of malathion from 20 mg to 60 mg. When 60 mg of Z-sep$^+$ were used, probably there was enough active sites to interact with all the hydrophobic interfering components. This probably explains why there was no marked change in the enrichment factors when the mass of Z-sep$^+$ was increased beyond 60 mg. Moloney et al. (2018) used 500 mg of Z-sep$^+$ when pre-concentrating pesticides in animal fats using the QuECHERS technique. On the other hand, López-Blanco et al. (2016) pre-concentrated pesticides in fatty vegetable samples using 250 mg of Z-sep$^+$. The masses of Z-sep$^+$ used by these researchers are larger than the optimised mass (60 mg) in this study. The optimum mass of Z-sep$^+$ is dependent on the quantity of hydrophobic components in the sample matrix. In animal fat and fatty vegetable samples there are large quantities of hydrophobic components. Thus, a large amount of Z-sep$^+$ is required for the clean-up of such matrices. On the other hand, the fruit samples, used in this research, does not contain a lot of hydrophobic components and therefore, a small quantity is required for their effective removal.

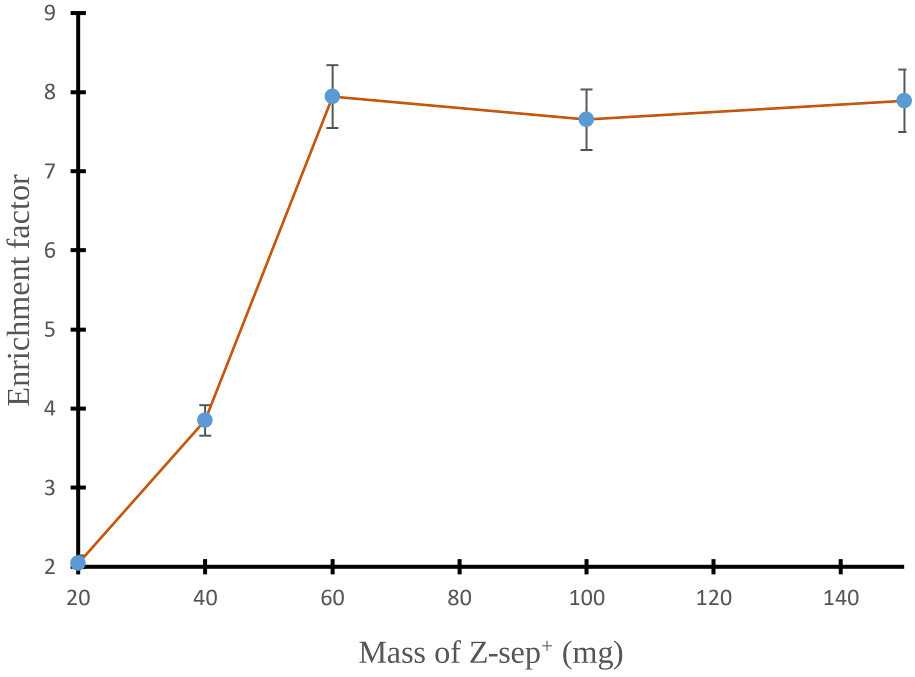

**Fig. 8:** Effect of mass of Z-sep+ sorbent on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of $MgSO_4$, 3.5 g; mass of homogenised fruit sample, 10 g; mass of PSA sorbent, 400 mg; centrifugation rate, 5000rpm, centrifugation time, 5 min

### *3.1.8. Effect of centrifugation rate*

Centrifugation was done to facilitate and expedite phase separation between the malathion-impregnated acetonitrile and the matrix components during the QuEChERS technique. The rate of centrifugation should be carefully controlled to ensure that all the malathion in the sample is extracted. The effect of centrifugation rate was evaluated by varying it from 3000 to 6000 rpm while keeping all the other QuEChERS parameters constant (Fig. 9). From Fig. 9, it was observed that the optimum enrichment factor for malathion was obtained when the centrifugation rate was 4500 rpm, and this was the optimum centrifugation rate used in the subsequent experiments. The centripetal force from the centrifuge causes the sedimentation of particles during centrifugation (Romanò, 2018). Below 4500 rpm, the centripetal force was probably not large enough to cause the sedimentation of all the interfering materials thereby causing unclean acetonitrile extracts and reduced enrichment factors. The sedimentation of particles by centrifugation is, in effect, a method of concentrating them. Therefore, one of the major physical forces opposing such concentration is diffusion (Antonopoulou et al., 2018). Thus, at higher centrifugation rate (above the optimum 4500 rpm) probably the diffusive forces become more pronounced resulting in poor sedimentation of smaller interfering materials and, therefore, poor extraction efficiency. There was no significant variations between the optimised centrifugation rate in this study and what was used by other researchers. For instance, Kaczyński (2017) pre-concentrated pesticides in fish muscle and liver using QuEChERS, and the homogenised samples were centrifuged at 4000 rpm. López-Blanco et al. (2016) centrifuged homogenised fatty vegetable samples at 5000 rpm during the QuEChERS technique while Walorczyk et al. (2015) centrifuged chlorophyll-containing samples at a rate of 5000 rpm. Thus, the optimised centrifugation rate is within the commonly used speeds of centrifugation.

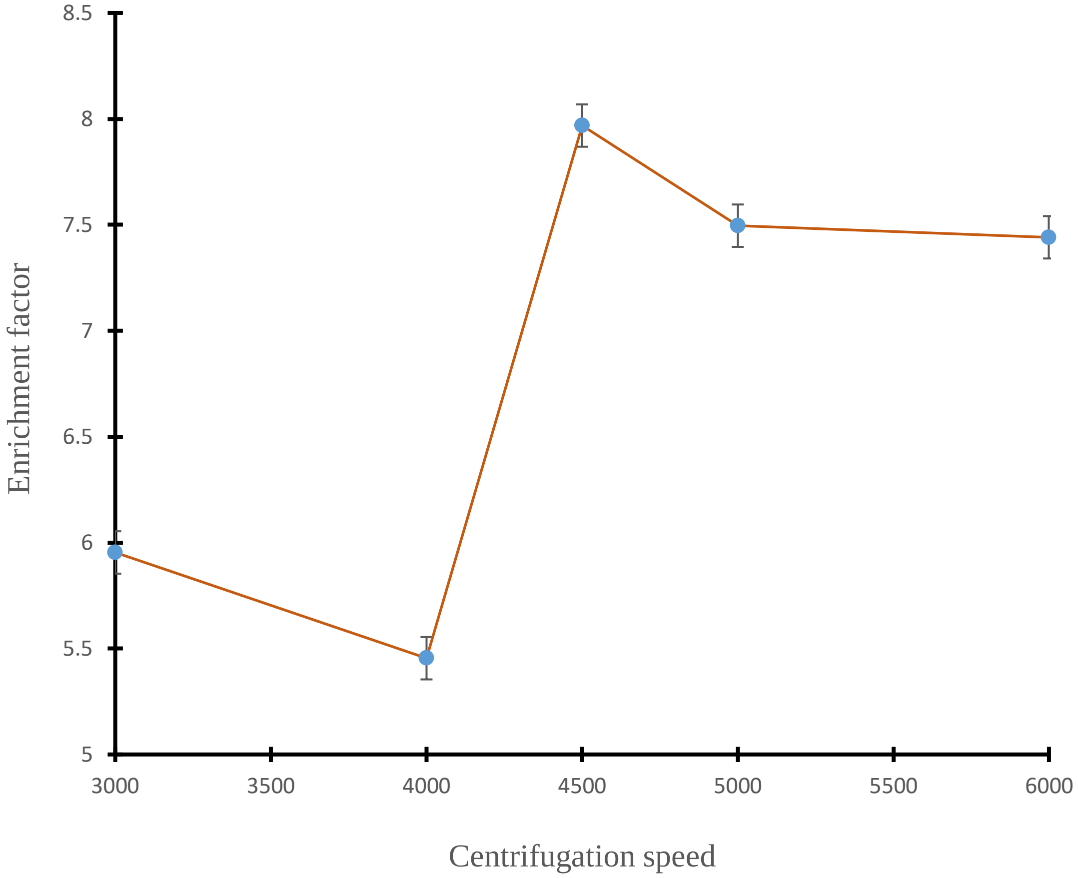

**Fig. 9:** Effect of centrifugation speed on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of $MgSO_4$, 3.5 g; mass of homogenised fruit sample, 10 g; type of sorbent, Z-sep$^+$/PSA; mass of PSA sorbent, 400 mg; mass of Z-sep$^+$, 60 mg; centrifugation time, 5 min

### *3.1.9. Effect of centrifugation time*

The enrichment factor of malathion during its pre-concentration using QuEChERS depends, among other factors, on the centrifugation time. The influence of centrifugation time on the enrichment factor of malathion during QuEChERS was evaluated by varying the time from 2 to 10 min while keeping the other QuEChERS parameters constant (Fig 10). The results in Fig. 10 show that the highest enrichment factor of malathion was obtained when the centrifugation time was 3 min and this was used as the optimum centrifugation time in this study. During QuEChERS, the interfering particles should be afforded enough time to undergo sedimentation when the centrifugation process is performed. The sedimentation time depends on particle size and density as well as the nature of the matrix. The centrifugation time, therefore, should be optimized to ensure complete sedimentation of the interfering matrix components. Less centrifugation time, less than the optimum of 3 min in this case, would result in incomplete sedimentation of interfering components in the matrix and unclean acetonitrile extracts which would, therefore, entail lower enrichment factors of malathion. The sedimentation process is opposed by diffusion of the sedimented particles during centrifugation (Antonopoulou et al., 2018). The centrifugation time should be just enough to promote sedimentation but not adequate to make the diffusion rate surpass the sedimentation rate. Thus, the lower enrichment factors encountered after 3 min were probably due to the increase in the diffusion rate of the interfering materials from the sedimented phase into the acetonitrile layer at times beyond the optimum centrifugation time.

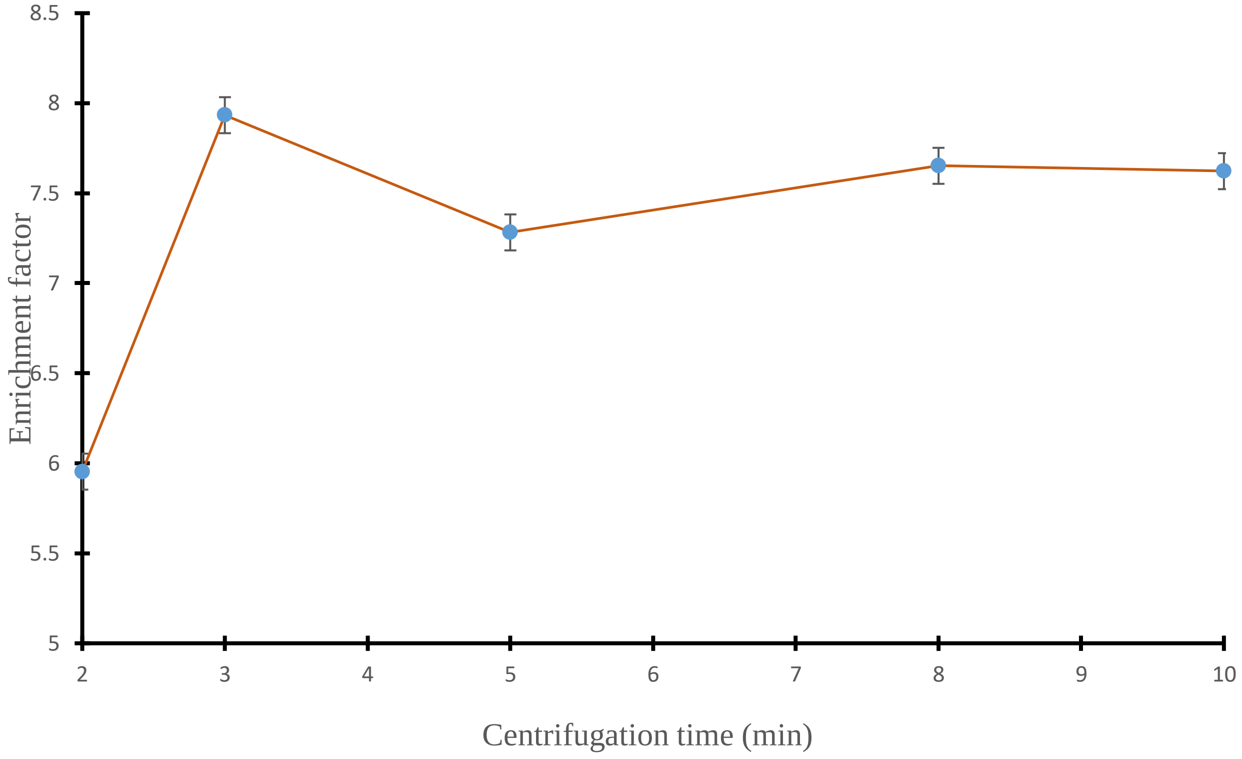

**Fig. 10:** Effect of centrifugation time on enrichment factor of malathion during QuEChERS (n= 3). Experimental conditions: type of extraction solvent, acetonitrile; volume of extraction solvents (acetonitrile), 10 mL; mass of $MgSO_4$, 3.5 g; mass of homogenised fruit sample, 10 g; type of sorbent, Z-sep+/PSA; mass of PSA sorbent, 400 mg; mass of Z-sep+, 60 mg; centrifugation rate, 4500 rpm

### *3.2. Validation and application of the developed method*

To validate the developed method the following parameters were considered: linearity, limit of detection and limit of quantification. Linearity was assessed from the determination coefficient ($R^2$) value of the calibration curve, which was 0.9999. Thus, the calibration curve of the malathion showed a satisfactory linearity and a strong correlation between concentration and absorbance over the studied range. The linear range of the developed method was from 0.1 to 0.9 mg $kg^{-1}$. The limit of detection and quantification were 0.017 and 0.05 mg $kg^{-1}$ respectively. The developed method, therefore, showed high sensitivity to malathion.

The developed and validated method was applied to the detection of malathion in orange, apple and pear samples (Table 1). The fruit samples were cut into small pieces and were then homogenized using a blender. An aliquot of each homogenised fruit sample was weighed (10

g) for analysis in accordance with the developed procedure. The collected orange samples did not show any residue of malathion (Table 1), its concentration was below the detection limit of UV-Vis spectrophotometer used. However, malathion was detected in apple and pear samples. The concentrations of malathion in apple and pear samples were 0.07 and 0.09 mg kg$^{-1}$ respectively (Table 1). The concentrations of malathion in the fruit samples used in this study were higher than those obtained using other analytical techniques (Table 1). However, these concentrations of malathion, obtained using the developed method, were lower than the maximum allowable limits in oranges, apples and pears. Thus, these fruits can be safely consumed by humans without the risk of malathion poisoning.

**Table 1**

Comparative study of the concentration of malathion in fruit samples

| Matrix | Mean concentration (mg kg$^{-1}$) | Maximum allowable limit (mg kg$^{-1}$) | Analytical technique | Ref. |
|---|---|---|---|---|
| Orange | 0.0300 | 4.00 | GC-FPD | Nakano et al., 2016 |
| Apple | 0.0260* | 0.50 | MIMSPD-GC | Wang et al., 2013 |
| Pear | 0.0085* | 0.50 | MIMSPD-GC | Wang et al., 2013 |
| Orange | nd | 4.00 | QuEChERS-UV-Vis spectroscopy | This study |
| Apple | 0.07 | 0.50 | QuEChERS-UV-Vis spectroscopy | This study |
| Pear | 0.09 | 0.50 | QuEChERS-UV-Vis spectroscopy | This study |

**Note**: nd- not detected; MIMSPD- molecularly imprinted matrix solid-phase dispersion; *- converted from µg kg$^{-1}$ to mg kg$^{-1}$

## 4. Conclusion

Malathion is one of the organophosphate pesticide widely used in agriculture to control pests. However, it is very poisonous also to non-target organisms even at trace levels. It is, therefore, imperative that efficient and cheap methods are developed to monitor its concentration levels in the environment in general and food matrices in particular. In this work, a cheap and reliable QuEChERS method was successfully developed that is used in conjunction with UV-Vis spectrophotometry. The developed method is fairly sensitive to malathion as evidenced by the low LOD and LOQ values of 0.07 mg kg$^{-1}$ and 0.09 mg kg$^{-1}$ respectively. In the developed QuEChERS method, malathion extraction in acetonitrile using a sample to solvent ratio of 1:1 (w/v) and clean-up using a sorbent mixture of Z-sep+ /PSA were found to be the optimum conditions for the orange, apple and pear matrices. This sorbent combination resulted in cleaner acetonitrile QuEChERS extract.

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## Conflict of interests

There is no conflict of interests

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# Chapter 5

# General conclusions and future work

This chapter gives general conclusions based on the research findings of this work. The recommended future work is outlined also in this chapter.

## 5.1. Conclusions

Many techniques can be used to pre-concentrate pesticides and heavy metals when found in trace amounts in different matrices. These techniques can be categorised into classic and modern pre-concentration methods. Recent trends show an increase in the popularity of the modern pre-concentration techniques that conform to the principles of green chemistry (Paper III). The extent of the green aspect of a pre-concentration technique during trace analysis of pesticides and heavy metals depends largely on miniaturisation and the use of environmentally friendly solvents such as ionic liquids and natural deep eutectic solvents. Researchers are taking a conscious step to move away from the use of pre-concentration techniques that choke the environment with large volumes of toxic organic solvents. Thus, they are gravitating towards miniaturized pre-concentration techniques that are more effective and environmentally friendly, that is green pre-concentration techniques (Paper III).

The DLLME technique is a miniaturised, green sample pre-concentration method that is based on a ternary solvent system: water, extraction solvent and dispersion solvent. It has been widely used to pre-concentrate pesticides (Paper I) and heavy metals (Papers IV and V). During pre-concentration of heavy metals using DLLME, chemical modification is mandatory to enable their extraction with organic solvents. Often, chelating agents such as dithizone (Paper IV) and diphenylcarbazide (Paper V) are used to effect the derivatization of heavy metals to organometallic compounds before pre-concentration by DLLME. The chemical form of the chelate depends, among other things, on the pH of the sample. Thus, all the parameters that affect DLLME should be optimised with special attention being given to pH.

QuEChERS is a versatile pre-treatment technique that has been successfully applied during the analysis of pesticides in different complex matrices such as biological, environmental and agricultural samples (Papers II and VI). The versatility of the QuEChERS technique is derived from, among other things, the possibility of introducing modifications based on the application of different solvents, salts, buffers and sorbents (Paper II). This allows the QuEChERS methodology to be implemented in the analysis of a broad spectrum of analytes and matrices. The QuEChERS extraction method is evolving with new, effective sorbents, such as Z-sep, being developed. The Z-sep sorbent enhances sample clean-up for complex matrices by effectively removing more fats and pigment from sample matrix (Paper VI).

## 5.2. Future work

The major thrust in future endeavours should be to reduce further the amount of toxic organic solvents used in pre-concentration techniques as well as cost reduction. Thus, the researchers should direct their energies towards enhancement of the "greenness" of pre-concentrations techniques.

QuEChERS is a versatile technique that has found widespread applications in the pre-concentration of organic pollutants. The commercial sorbents that are used during the QuEChERS technique are expensive and this hampers its application in some situations. The cost of performing the QuEChERS technique can be reduced by using biological materials as sorbents. Thus, future work is going to be based on the development of QuEChERS techniques that involve the use of biosorbents that are equally efficient but cheap as compared to the commercial sorbents.

DLLME, on the other hand, has been widely applied to pre-concentrate both organic and inorganic pollutants in various complex matrices. The technique uses small volumes of toxic organic solvents as extraction and disperser solvents. These organic solvents, although in small amounts, contribute to pollution of the environment. Thus, the focal point of future work is the identification of alternative solvents to further minimize or eliminate the release of organic solvents into the environment when performing the DLLME technique. More research is going to be directed towards the use of solvents like ionic liquids, deep eutectic solvents and solvents with switchable hydrophilicity in the DLLME technique.

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# Appendix

## Supplementary data

This section gives supplementary data for the papers in this dissertation.

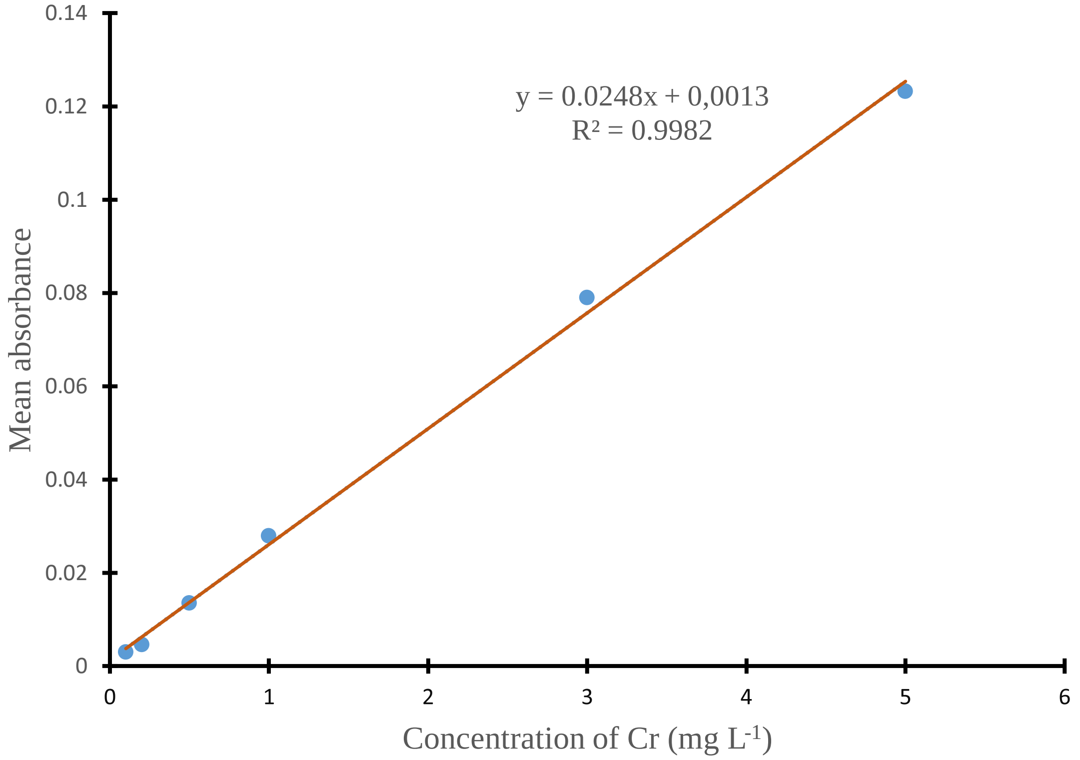

**Fig. A1:** Calibration curves for chromium

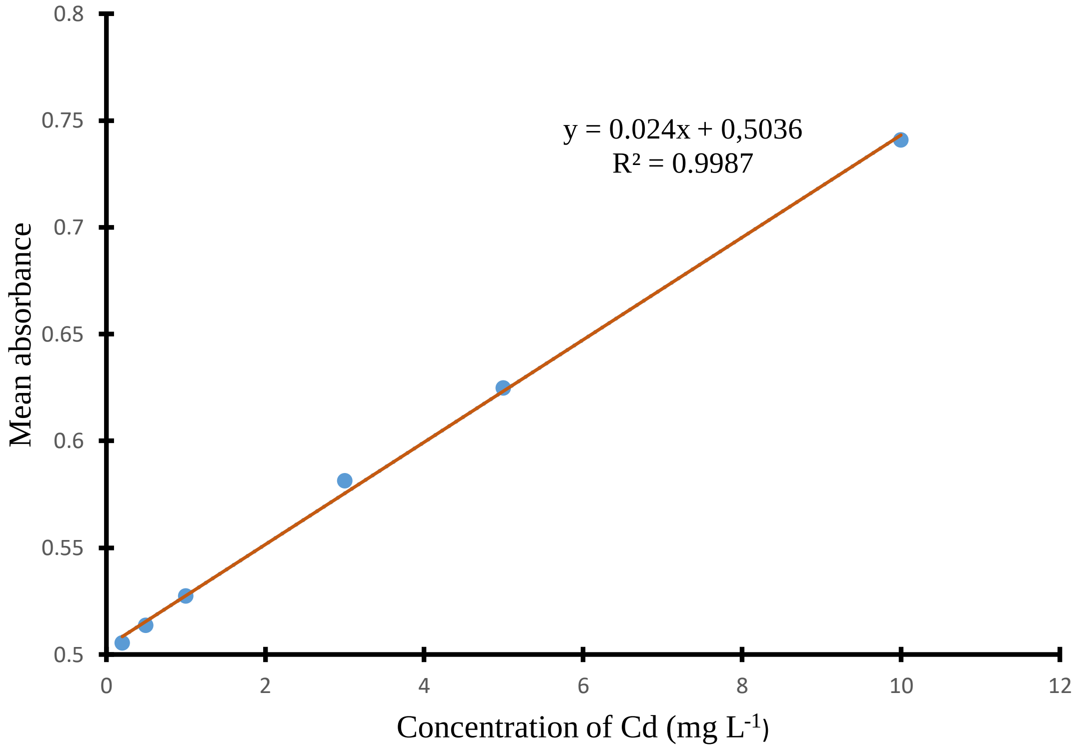

**Fig. A2:** Calibration curves for cadmium

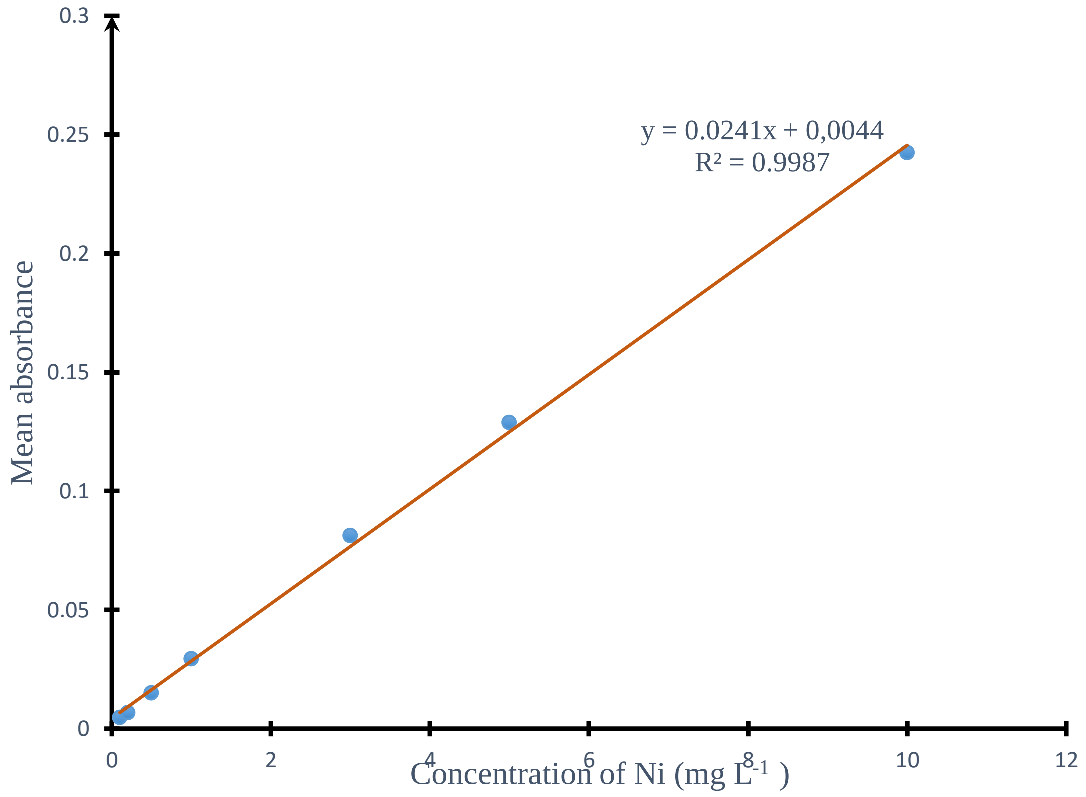

**Fig. A3:** Calibration curves for nickel

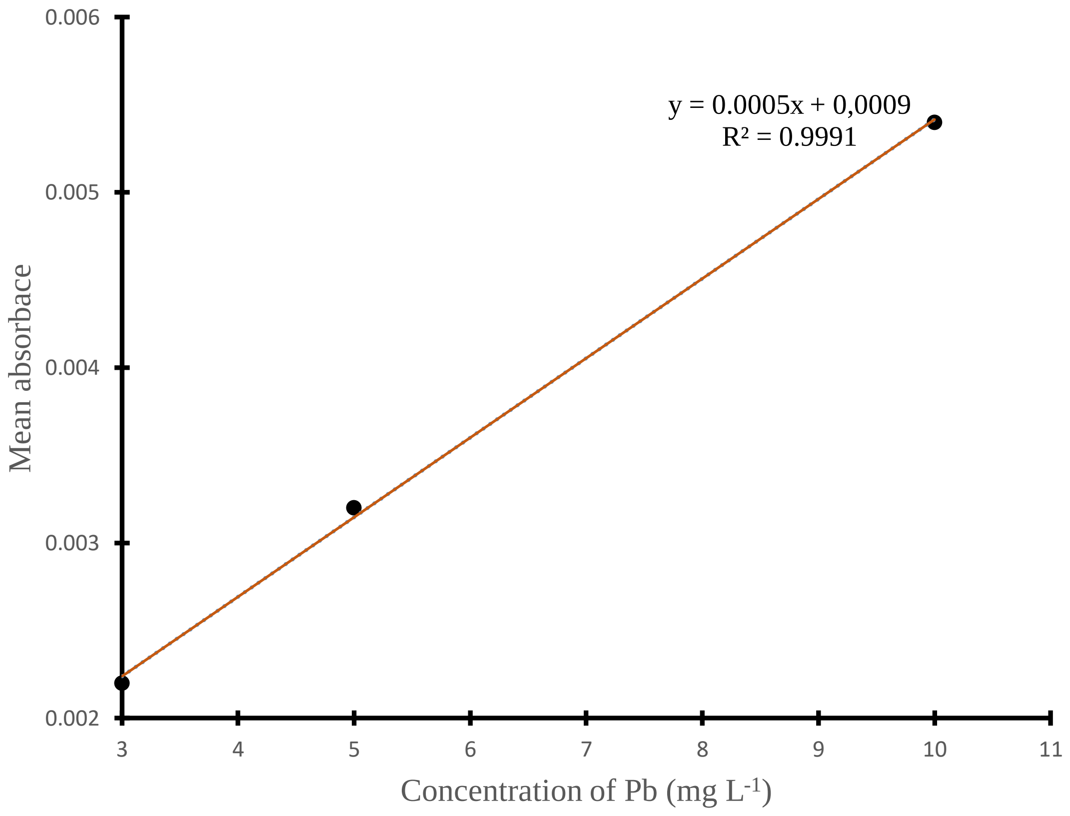

**Fig. A4:** Calibration curves for lead

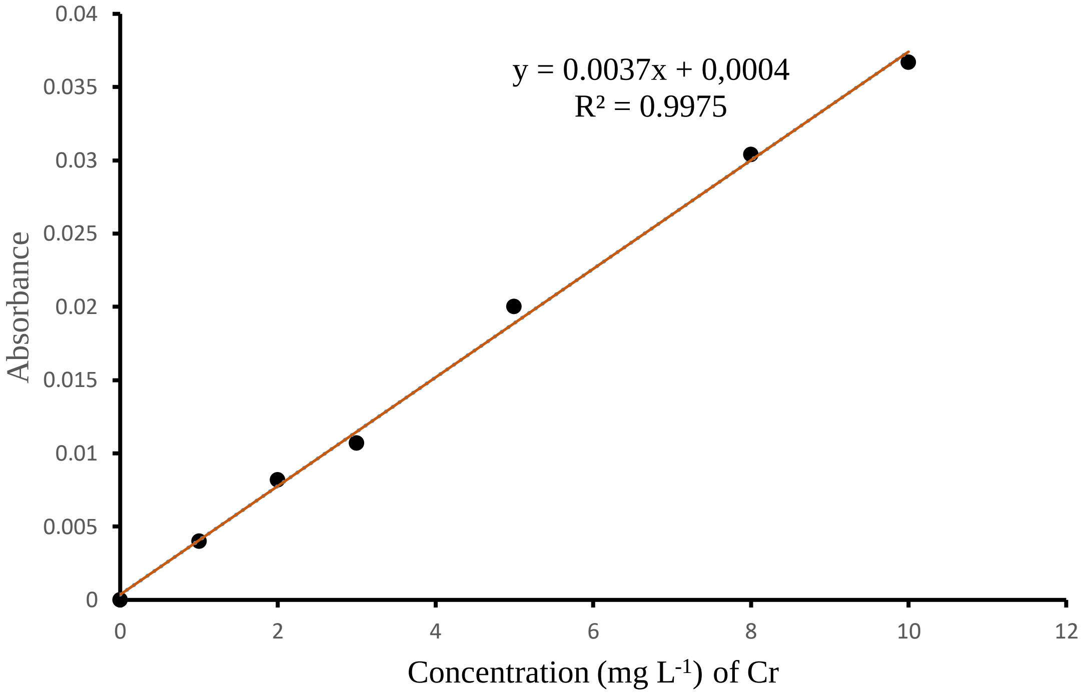

**Fig. A5:** Calibration curves for chromium during speciation

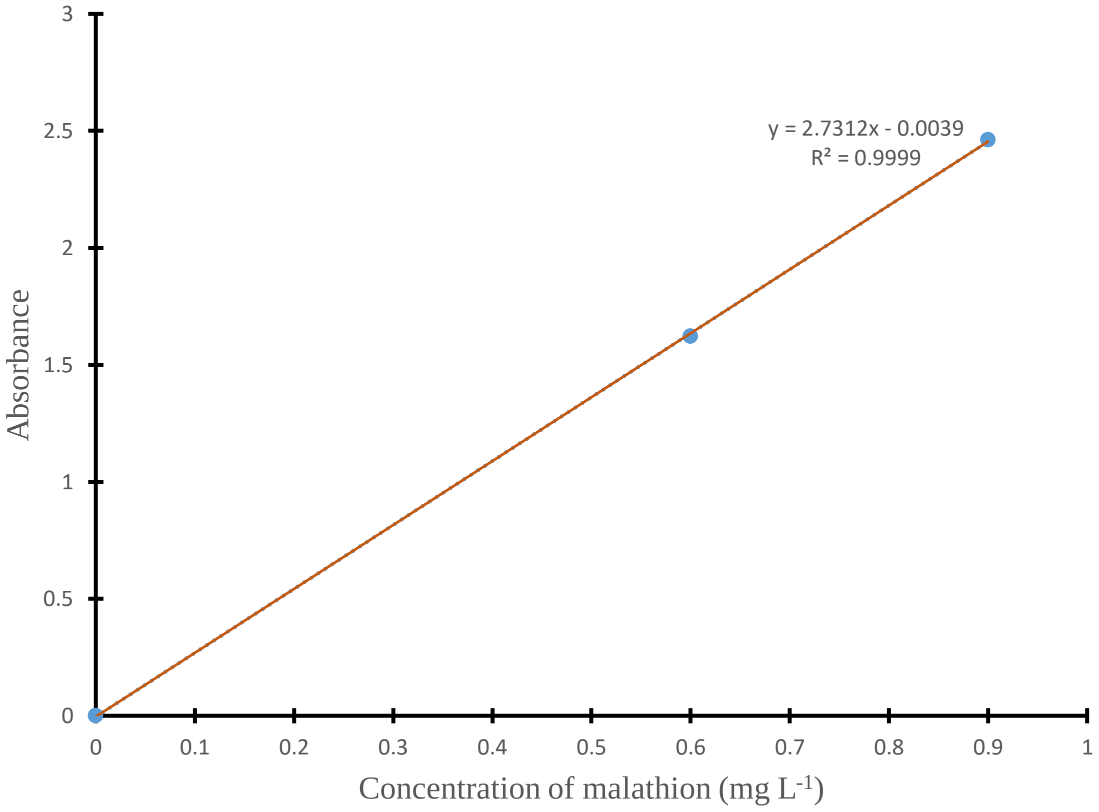

**Fig. A6:** Calibration curves for malathion