



RHODES UNIVERSITY

Where Leaders Learn

**Pressurized hot water extraction of nutraceuticals
and organic pollutants from medicinal plants**

*A thesis submitted to Rhodes University in fulfillment of the requirements
for the degree of*

Doctor of Philosophy (Science)

by

Janes Mokgadi

Supervisor: Prof. Nelson Torto

November 2011

Dedication

To my daughter Isago and my mom with all my love

Acknowledgments

Prof. Nelson Torto: I am greatly indebted to you for playing a critical role in my personal and professional development as a mentor. I appreciate your academic direction, the small talks and the encouragement. Seemingly, you were always away but apparently your door was always open. You have left a great indelible impression on my life; *thank you for giving me the possibility...*

Prof. Charlotta Turner: Thank you, for the technical assistance, your comments and suggestions. I appreciate the support.

Dr. Veronica Obuseng: Your technical assistance is appreciated.

Shima Batlokwa: For your support and encouragement despite everything, you made life in the laboratory enjoyable for me. On a more personal note, thank you for being a good friend, a listener and very understanding. You are a rarity!!

Linda Mokgadi-Pajor: My sincerest appreciation goes to you for playing *mama-Linda* role in my absence and being dumped when *mama-Janny* comes (LOL!!). I don't know what I would have done without you big sis. Thank you for everything.

Tony: I would be horribly remiss if I did not thank you for simply being my 'North'.

To my family and friends: Thank you for your unwavering love and support.

F12 (colleagues): You are a very special group!! Your academic critics contributed a lot to my work and me as an individual. *'The presentation was good'.....*I will always remember that!.

Chemistry department (Rhodes University): Thank you for all the assistance and the support. Special thanks to Mr. Soneman, Mr. Fourie, Mr. Francis, Benita, Barbara and Nombasa. You have been of great assistance!!

Andrew Mellon Foundation, STINT (Sweden), Agilent Technologies, UNDP/SGP/GEF-Botswana: The project would not have succeeded without your financial support.

Le ka moso bagaetsho!!

Abstract

This thesis explores the robustness and the versatility of pressurized hot water extraction (PHWE) for a variety of analytes and matrices. Applications discussed include: selective extraction of alkaloids in goldenseal followed by their degradation studies; in-cell clean-up of pesticides in medicinal plants employing custom made molecularly imprinted polymers (MIPs) sorbents; in-cell pre-concentration followed by desorption of aflatoxins in plants with MIPs; desorption of pesticides from electrospun nanofiber sorbents; and removal of templates from MIPs sorbents. It was demonstrated that selective extractions could be achieved by just changing the temperature of water while adjusting the pressure. For instance, the alkaloids in goldenseal (hydrastine and berberine), were extracted at 140 °C, 50 bars, 1 mL min⁻¹ in 15 min; organochlorine pesticides from medicinal plants were extracted at 260 °C, 80 bars, 1 mL min⁻¹ in 10 min; while aflatoxins AFG2, AFG1, AFB2 and AFB1 were extracted at 180 °C, 60 bars and a flow rate of 0.5 mL min⁻¹ in 10 min. The selectivity of PHWE was further enhanced by combining it with selective MIPs sorbents at higher temperatures. In-cell clean-up of interfering chlorophyll was successfully removed from the medicinal plants during pesticides analysis while clean-up of aflatoxins AFG2, AFG1, AFB2 and AFB1 was achieved in two extraction cells connected in series. Ultrasound was also combined with PHWE for extraction of hydrastine and berberine at 80 °C and 40 bars in 30 min. PHWE was further evaluated for removal of templates from quercetin, phthalocynine and chlorophyll MIPs. The templates were thoroughly washed off their MIPs within 70 min with PHWE compared to over 8 h for Soxhlet and ultrasound assisted

extraction. Pesticides were also desorbed from electrospun nanofibers at 260 °C, 80 bars in 10 min employing only water at 0.5 mL min⁻¹. In the light of green chemistry, the decrease in the usage of organic solvents was 100%, resulting in no organic solvent waste.

Table of Contents

Dedication	ii
Acknowledgments	iii
Abstract.....	v
Table of Contents	vii
List of Abbreviations.....	xv
List of Figures	xix
List of Tables.....	xxii
Background	1
Chapter 1 Sample handling	3
1.1 Sampling.....	3
1.2 Sample preparation	4
1.2.1 Analyte release from matrix.....	10
1.2.2 Removal of interfering matrix components from liquid samples	12
1.2.2.1 Solid phase extraction (SPE)	13
1.2.2.1.1 Restricted access media (RAM)	15
1.2.2.1.2 Immunoaffinity extraction (IAE).....	16
1.2.2.1.3 Molecularly imprinted polymers (MIPs).....	17
1.2.2.1.4 Electrospinning	20

1.2.3 Derivatization	23
Chapter 2 Solid sample extraction techniques	25
2.1 Overview	25
2.2 The extraction process	25
2.3 Extraction strategy	28
2.4 Conventional extraction techniques.....	30
2.4.1 Soxhlet extraction	30
2.4.2. Ultrasound-assisted extraction.....	35
2.5 Extraction by high diffusion fluids.....	37
2.5.1 Microwave assisted extraction (MAE)	38
2.5.2 Supercritical fluid extraction (SFE).....	40
2.5.3 Pressurized liquid extraction (PLE).....	45
Chapter 3 A review on pressurized hot water extraction	49
3.1 Overview	49
3.2 Principles of PHWE	49
3.2.1 Changes in physicochemical properties of water	49
3.2.2 Solubility of analytes in pressurized hot water	52
3.3 PHWE equipment	56
3.3.1 Static set up	57
3.3.2 Dynamic extraction mode	61

3.4 In house built PHWE equipment	62
3.5 Parameters affecting extraction.....	65
3.5.1 Temperature	65
3.5.2 Pressure	68
3.5.3 Flow rate and extraction time	69
3.5.4 Selectivity	70
3.5.5 Modifiers and additives	71
3.6 Comparison with other extraction methods.....	71
3.7 Application of PHWE.....	73
3.7.1 Extraction of nutraceuticals and bioactive compounds from food and herbal materials.....	73
3.7.2 Removal of chemical contaminants from food and herbal plants.....	76
3.7.3 Environmental samples	80
3.8 Recent developments of PHWE and future perspectives.....	81
Chapter 4 Experimental.....	85
4.2 Chemicals and reagents.....	85
4.3 Instrumentation	86
4.3.1 PHWE equipment.....	86
4.3.2 HPLC conditions.....	87
4.3.3 GC Conditions.....	88

4.4 Thermal degradation studies of alkaloids in goldenseal during selective PHWE	90
4.4.1 Ultrasonication extraction	90
4.4.2 Reflux extraction.....	90
4.4.3 PHWE	91
4.4.3.1 Extraction experiment	91
4.4.3.2 Degradation experiment	92
4.4.4 Analytical parameters.....	93
4.5 Pressurized hot water extraction of alkaloids in goldenseal with in-situ ultrasonication	94
4.5.1 Ultrasound assisted and pressurized hot water extraction.....	94
4.5.2 Ultrasound assisted pressurized hot water extraction (US-PHWE).....	94
4.6 Pressurized hot water extraction as an optimal templates removal method from molecularly imprinted polymers	96
4.6.1 Preparation of chlorophyll, quercetin and phthalocynine MIP particles	96
4.6.2 Thermo-gravimetric analysis of the MIPs	97
4.6.3 Template removal methods	97
4.6.3.1 Pressurized hot water extraction	97
4.6.3.2 Soxhlet extraction and ultrasonic extraction	97
4.6.4 Determination of the absorbance of the templates in the washings using UV spectrophotometry.....	98

4.6.5 Template bleeding evaluation	98
4.6.6 Evaluation of performance of the MIPs after template removal.....	98
4.7 Pressurized hot water extraction coupled to molecularly imprinted polymers for simultaneous of extraction and clean-up of pesticides residues in edible and medicinal plants of the Okavango Delta, Botswana.....	100
4.7.1 Sampling and study area	100
4.7.2 Sample extraction and clean up procedures.....	103
4.7.2.1 Pressurized hot water extraction combined with molecularly imprinted polymer clean-up.....	103
4.7.2.2 QuEChERS AOAC Official Method	103
4.7.2.3 Dispersive SPE Clean-up	104
4.8 Simultaneous extraction, clean-up and desorption of aflatoxins in medicinal plants with pressurized hot water extraction coupled to molecularly imprinted polymers.....	106
4.8.1 Sampling and study area	106
4.8.2 PHWE and in-cell clean-up of aflatoxins from plant samples	107
4.8.3 Sample preparation and immunoaffinity column clean-up of aflatoxins from plants	109
4.8.4 Pre-column derivatization	109
4.9 Pressurized hot water extraction for desorption of pesticides from electrospun nanofiber sorbents.	110

4.9.1 Preparation of polystyrene and carbodithioate fuctionalized polystyrene nanofibers	110
4.9.2 DDE adsorption studies	111
4.9.3 Desorption of DDE from nanofibers by PHWE	111
Chapter 5 Results and Discussion.....	113
5.1 Thermal degradation studies of alkaloids in goldenseal during selective PHWE	113
5. 1.1 Optimization of extraction procedures.....	113
5.1.1.1 Effects of temperature and pressure in PHWE.....	113
5.1.2 Comparison of PHWE with conventional methods.....	114
5.1.3 Degradation behavior	115
5.1.4 Extraction/degradation curves.....	117
5.1.5 Kinetics of hydrastine thermal degradation	118
5.1.6 Peak identification	119
5.1.7 Analytical parameters	120
5.2 Pressurized hot water extraction of alkaloids in goldenseal with in-situ ultrasonication	121
5.2.1 Effect of ultrasound in extraction	121
5.2.2 Effect of ultrasonic frequency.....	122
5.2.3 Comparison with conventional methods	123

5.3 Pressurized hot water extraction as an optimal templates removal method from molecularly imprinted polymers	124
5.3.1 TGA results for the molecularly imprinted polymers	124
5.3.2 Template removal method	125
5.3.3 Template bleeding	129
5.3.4 Performance after template removal	131
5.4 Pressurized hot water extraction coupled to molecularly imprinted polymers for simultaneous of extraction and clean-up of pesticides residues in edible and medicinal plants of the Okavango Delta, Botswana.....	132
5.4.1 Pesticides detected employing PHWE-MIP	132
5.4.2 Pesticides detected employing QuEChERS method	133
5.4.3 Comparison of the proposed PHWE-MIP method with the QuEChERS method	134
5.4.4 Application of PHWE-MIP method to real samples	138
5.5 Simultaneous extraction, clean-up and desorption of aflatoxins in medicinal plants with pressurized hot water extraction coupled to molecularly imprinted polymers	144
5.5.1 PHWE procedure	144
5.5.2 Comparison of the AOAC Official Method with the PHWE method	145
5.6 Pressurized hot water extraction for desorption of pesticides from electrospun nanofiber sorbents.	150
5.6.1 Electrospinning.....	150

5.6.2 Choice of nanofiber sorbents	151
5.6.3 Effect of contact time on sorption process	152
5.6.4 Effects of sample volume on sorption process.	153
5.6.5 Optimization of temperature and time for the PHWE technique.....	153
Chapter 6 Conclusion.....	156
References	157

List of Abbreviations

µg	microgram
ADS	alkyl-diol-silica
AIBN	azobisisobutyronitrile
ASE	accelerated solvent extraction
cm	centimeter
CO ₂	carbon dioxide
CV	collecting vial
DAD	diode array detector
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenylethylene
DDT	dichlorodiphenyltrichloroethane
EC	extraction cell
EGDMA	ethylene glycol methacrylate
EPA	Environmental Protection Agency
ESE	enhanced solvent extraction
ESI	electro spray ionization
eV	electro volts
FLD	fluorescence luminescence detector
g	gram
GC-ECD	gas chromatography - electron capture detection

GIGO	garbage-in garbage-out
h	hour
HCB	hexachlorobenzene
HF-MMLLE	hollow fiber microporous membrane liquid-liquid extraction
HPLC	high performance liquid chromatography
IAE	immunoaffinity extraction
kV	kilo volts
L	liters
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
MAA	methacrylic acid
MAE	microwave assisted extraction
MDL	method detection limit
mg	milligram
MgSO ₄	magnesium sulphate
Min	minutes
MIPs	molecularly imprinted polymers
MISPE	molecularly imprinted polymers solid phase extraction
mL	milliliter
MMLLE	microporous membrane liquid-liquid extraction
MMPHWE	micelle-mediated pressurized hot water extraction

MSPD	matrix solid-phase dispersion
NIP	non imprinted polymers
OCPs	organochlorine pesticides
OKACOM	Okavango River Basin Water Commission
ORB	Okavango River Basin
PAHs	poly aromatic hydrocarbons
PBS	phosphate buffer saline
PCBs	polychlorinated biphenyls
PFE	pressurized fluid extraction
PHWE	pressurized hot water extraction
PLE	pressurized liquid extraction
POPs	persistent organic pollutants
PRV	pressure relief valve
PS	polystyrene
PSA	Primary Secondary amines
PV	pressure valve
r^2	regression
rpm	revolutions per minute
RSD	relative standard deviation
SBSE	stir bar sorptive extraction
SEM	scanning electron microscope
SFE	supercritical fluid extraction
SIM	selected ion monitoring

SPE	solid phase extraction
SPME	solid phase microextraction
SV	static valve
SWE	subcritical water extraction
TGA	thermo-gravimetric ananalysis
THF	tetrahydrofuran
USE	ultrasound assisted extraction
UV	ultra violet
v/v	volume by volume
w/v	weight by volume
WV	waste vial
α-BHC	alpha-benzenehexachloride

List of Figures

Fig. 1.1 Sample parameters to consider during sampling	4
Fig. 1.2 Stages of sample preparation	9
Fig. 1.3 General criteria used in choosing the most appropriate extraction technique	12
Fig. 1.4 Synthesis of MIPs and its selective recognition to target molecule	19
Fig. 1.5 A typical electrospinning setup	21
Fig. 1.6 Schematic representation of the Taylor cone formation	22
Fig 2.1 Positioning of the analyte in the sample matrix.....	27
Fig. 2.2 Conventional Soxhlet extractor	32
Fig. 2.3 Phase diagram for carbon dioxide	42
Fig. 2.4 Diagram of a Spe-ed™ Prime SFE by Applied Separations Inc.	44
Fig. 2.5 Factors affecting PLE and optimization parameter	47
Fig. 3.1 Phase diagram of water	50
Fig. 3.2 Changes in the permittivity of water with temperature at different pressures	52
Fig.3.3 Solubility parameter of water	54
Fig.3.4 Schematic of a PHWE system	58
Fig. 3.5 Extraction yield (%) against extraction time (min)	61
Fig. 3.6 A schematic diagram of a laboratory in house built PHWE system	63
Fig. 3.7. Dielectric constant of water and methanol/water and acetonitrile/water mixtures	66
Fig. 3.8 Vapour pressure curve of water	69
Fig. 3.9 Chemical Structures of (A) hydrastine and (B) berberine	76

Fig. 3.10 Examples of organochlorine pesticides	78
Fig. 3.11 Structures of aflatoxins.....	79
Fig. 3.12 Schematic of WEPO home-built equipment	83
Fig. 3.13 Record of papers published for PHWE	84
Fig. 4.1. Map of the Okavango Delta showing plants sampling sites	102
Fig4.2 Comparison of the extraction scheme of QuEChERS method with PHWE-MIP	105
Fig. 4.3 Modified PHWE for serial extractions and clean-up.....	108
Fig. 5.1. Effects of temperature on the extraction of hydrastine and berberine from goldenseal roots by PHWE at 50 bars, 1 mL min ⁻¹ for 15 min.	114
Fig. 5.2. Chromatograms showing degradation products of hydrastine standard	116
Fig. 5.3 Extraction curve at 140 °C (A), and degradation curves for sample (B) and a standard (C) of hydrastine at 160 °C at 50 bars, 1 mL min ⁻¹ for 15 min.....	117
Fig. 5.4 Relationship between the concentration of hydrastine standard and time	119
Fig. 5.5 Effect of time of ultrasound applied on the PHWE of berberine from goldenseal..	122
Fig. 5.6. A TGA plot for the MIPs.....	125
Fig. 5.7. Absorbance of chlorophyll in each washing	126
Fig. 5.8 Absorbance of quercetin in each washing	127
Fig. 5.9 Absorbance of phthalocynine in each washing.....	128
Fig. 5.10 Template removal by different extraction methods	130
Fig. 5.11 A chromatogram of pesticides detected from <i>N. lotus</i> when PHWE was combined with a chlorophyll MIP.....	133
Fig. 5.12 A chromatogram of pesticides detected from <i>N. Lotus</i> when QuEChERS method was employed.....	134

Fig. 5.13 Map of the Okavango Delta showing the regions of the plants sampling sites	141
Fig. 5.14 Concentration of 4,4DDE in different plants from all sampling regions.	143
Fig. 5.15. A typical chromatogram of aflatoxins B1, B2, G1, and G2	145
Fig. 5.16 SEM images of (a) Polystyrene (b) Carbodithioate-functionalized polystyrene nanofibers. (25% w/v)	151

List of Tables

Table 1.1 Typical sample preparation techniques for liquid and solid samples	8
Table 2.1 Approximate ranges of density, viscosity and diffusion coefficients of gases, supercritical fluids and liquids	37
Table 3.1. Intermolecular forces with water as a solvent	56
Table 3.2. Comparison of Soxhlet extraction, PHWE, SFE, PLE and MAE	72
Table 4.1. Parameters optimized for PHWE extraction	92
Table 4.2 Plant samples collected from the Okavango Delta	101
Table 4.3 Medicinal plants from Grahamstown	107
Table 5.1. Comparison of extraction yields (mg g^{-1}) of alkaloids in goldenseal by PHWE, reflux and sonication	115
Table 5.2 Recoveries of MIPs after extraction with different methods ($n=3$).....	131
Table 5.3 Recoveries (%) of pesticides employing both methods for all the plant samples	137
Table 5.4 Concentration of pesticides ($\mu\text{g kg}^{-1}$) detected in different plant parts.....	139
Table 5.5 Recoveries of aflatoxins by PHWE-MIP and AOAC methods.....	147
Table 5.6 Concentrations of aflatoxins by PHWE-MIP and AOAC methods.....	149
Table 5.7 Adsorption (%) of DDE ($0.25\text{-}1.00 \mu\text{g L}^{-1}$) on 10 mg electrospun PS and carbodithioate functionalized PS nanofibers.....	152

Table 5.8: Desorption (%) of DDE sorbed onto the carbodithioate nanofiber from the spiked stock solution ($0.5 \mu\text{g L}^{-1}$) by PHWE technique at 80 bars and varying temperatures.

.....154

Background

Plant extracts are widely used in the food, pharmaceutical and cosmetics industries. Their metabolites often occur in low concentrations as complex mixtures of many substances of a wide range of polarity and hydrophobicity, making their extractions very challenging [1]. Plants of medicinal value, like crops, are susceptible to insect and disease attacks both in the field and storage, so pesticides are widely used for their protection. Consequently, contamination of crude medicinal plants as well as their products has brought concern regarding the quality and safety of their use [2]. There is an increase in the use of alternative medicines and herbal supplements in western society. Consequently there is an increase in the demand for analysis of organic pollutants such as pesticides and aflatoxins in medicinal plants [3]. The complexity of plant matrices and the low concentrations, at which analytes of interest have to be identified and or quantified, make exhaustive sample preparation prior to proper separation and detection mandatory [4]. Even with the advancement of separation and detection techniques, sample preparation remains a vital part of the analytical process. Effective sample preparation is essential for achieving reliable results and for maintaining instrument performance [5].

The first step in sample preparation is usually the extraction of target analytes from the matrix. The increasing demand for faster, selective and environmentally friendly analytical methods is a major incentive to improve the classical procedures used for extraction [6]. One of the main factors to consider in an effort to change or replace the extraction method

is the choice of a solvent, which is the “hot spot” in terms of environmental impact in most extraction processes [7].

The main objective of this thesis is to evaluate the use of PHWE as an extraction technique in the analysis of various analytes. The specific aims of the thesis are

- i. To optimize extraction conditions for PHWE.
- ii. To improve or develop sample clean-up strategies in-situ with PHWE.
- iii. To evaluate the use of MIPs and electrospun nanofibers as SPE sorbents in use with PHWE as applied to neutraceuticals (alkaloids) and organic pollutants (pesticides and aflatoxins) from medicinal plants.
- iv. To explore the use of PHWE in MIP preparation for template removal.

Chapter 1 Sample handling

Analytical procedures typically involve a number of equally relevant steps for sampling, sample preparation, isolation of the target compounds, identification and quantification [6]. Most recently research has undoubtedly focused on preliminary steps, such as sampling and sample preparation (e.g. sample pretreatment, extraction, clean up and sample enrichment). These steps can be grouped together under an umbrella term, “sample handling”. Sample handling refers to any action applied to the sample before the analytical procedure [8]. Thorough sample handling ensures the integrity of samples, prevention of deterioration and cross contamination, maintains sample tracking and the chain of custody, and ensures their safe disposal [9]. Sampling and sample preparation generally account for about 80% of the whole analysis time [10].

1.1 Sampling

Sampling is an important operation within the entire analytical testing process in which only a small representative fraction of the whole sample population is taken. The move towards smaller sample sizes must be balanced by the need for a representative sample for analysis. Careful sampling strategies are required; any portion or aliquot taken for analysis must represent the original bulk sample [11].

It is often possible to use statistical methods to design a sampling plan that specifies the minimum number of sub-samples that need to be sampled to obtain an accurate representation of the population. Smaller sample sizes can be used since larger sample size results in the need for more efficient clean-up techniques and therefore possibly higher costs. However, generally, the larger the sample size that can be taken for extraction, the more precise the results [11]. Figure 1.1 shows several sample characteristics one needs to consider during sampling [12].

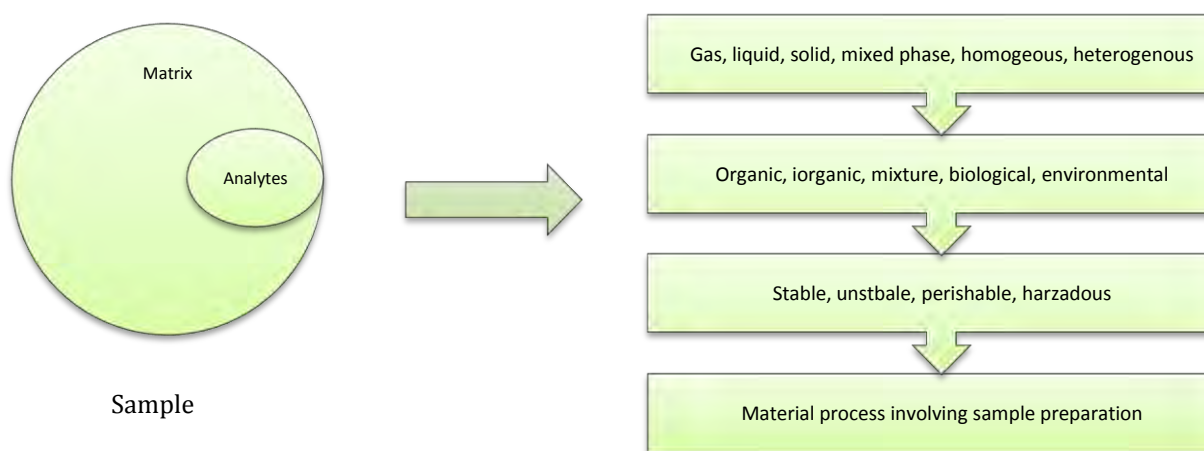


Fig. 1.1 Sample parameters to consider during sampling [12].

1.2 Sample preparation

Sample preparation is the process of extracting chemical residues from a sample with subsequent clean-up of the extract. Moreover, the analytes of interest are isolated while removing any matrix interferences that may affect the detection system [5]. It is worth

stressing that the sample preparation step largely determines the quality of the results obtained and that it is typically the primary source of errors and discrepancies between laboratories. A sample preparation procedure significantly affects assay throughput and analysis cost. Due to the need to reduce the time spent on sample preparation, there is considerable interest in finding simpler ways to combine sample preparation steps. Therefore, selecting and optimizing an appropriate sample preparation scheme is a key factor in the final success of the analysis. Additionally, the judicious choice of an appropriate procedure greatly affects the reliability and accuracy of a given analysis [13].

Most modern chromatographic instrumental techniques are sufficiently mature to enable the hyphenation of different separation techniques with each other and with detectors that provide high information density [14]. Despite the advances in instrumental analysis, sample preparation remains the weakest link and the time-determining step in the whole analytical procedure, accounting for approximately two thirds of total analysis time [15]. This is especially true in environmental analysis where sophisticated and powerful hyphenated systems for instrumental analysis of the final extracts contrast sharply with classical (although often robust and well established) large-scale and labour-intensive procedures based on, e.g., liquid-liquid extraction (LLE) or Soxhlet extraction for sample pre-treatment [16]. Like the proverbial computer rule; garbage-in garbage-out (GIGO), poor sample treatment or a badly prepared extract will invalidate the whole assay and even the most powerful separation method will not give correct results [17].

The basic concept of a sample preparation method is to convert a real matrix into a sample in a format that is suitable for analysis by a separation or other analytical techniques. This can be achieved by employing a wide range of techniques, many of which have changed little over the last 100 years. They have a common list of aims [17]:

- i. Matrix modification in order to:
 - ❖ Prepare the sample for introduction (injection) onto chromatographic column.
 - ❖ Render the solvent suitable for the analytical technique to be used.
 - ❖ Prolong the instrument's lifetime (e.g., column lifetime).
- ii. Clean-up in order to:
 - ❖ Remove impurities and obtain the required analytical performance and selectivity.
 - ❖ Reduce matrix interference.
- iii. Analyte enrichment (pre-concentration) in order to improve the method sensitivity (reduction of the limits of detection and quantification).
- iv. Analyte work-up for derivatization (addition of a chromophore, a fluorophore or to volatilize non-volatile analytes) in order to improve sensitivity.

With increasing demands on the analytical chemist to provide accurate and valid analytical measurements for regulatory requirements, poor manual reproducibility

during the sample preparation stage can be a major cause of assay variability, hence a need for automation and reduced manual sample handling. However, robots and the automation of the laboratory bring their own challenge of longer method development times and new skill requirements [18]. Although many traditional sample preparation methods are still in use, the trends in recent years have been towards:

- The ability to use smaller initial sample sizes even for trace analyses.
- Greater specificity or greater selectivity in extraction.
- Increased potential for automation or for on-line methods reducing manual operations.
- A more environmentally friendly approach (green chemistry) with less waste and the use of small volumes or no organic solvents.

Typical classical and modern approaches of sample preparation techniques for liquid and solid samples are summarized in Table 1.1 [19].

Table 1.1 Typical sample preparation techniques for liquid and solid samples [19].

Liquid sample	Solid samples
Dilution	Solid-liquid extraction (shake filter)
Evaporation	Forced flow leaching
Distillation	Soxhlet extraction
Microdialysis	Homogenization
Lyophilization	Ultrasonic assisted extraction (UAE)
Liquid-liquid extraction (LLE)	Dissolution
Solid-phase extraction (SPE)	Matrix solid-phase dispersion (MSPD)
Direct analysis by column-switching techniques (on-line techniques)	Pressurized liquid extraction (PLE)
Stir bar sorptive extraction	Supercritical fluid extraction (SFE)
	Microwave assisted extraction (MAE)
	Gas phase extraction
	Thermal desorption

The term sample preparation may refer to the various stages of the analysis procedure as outlined in Fig 1.2. [20].

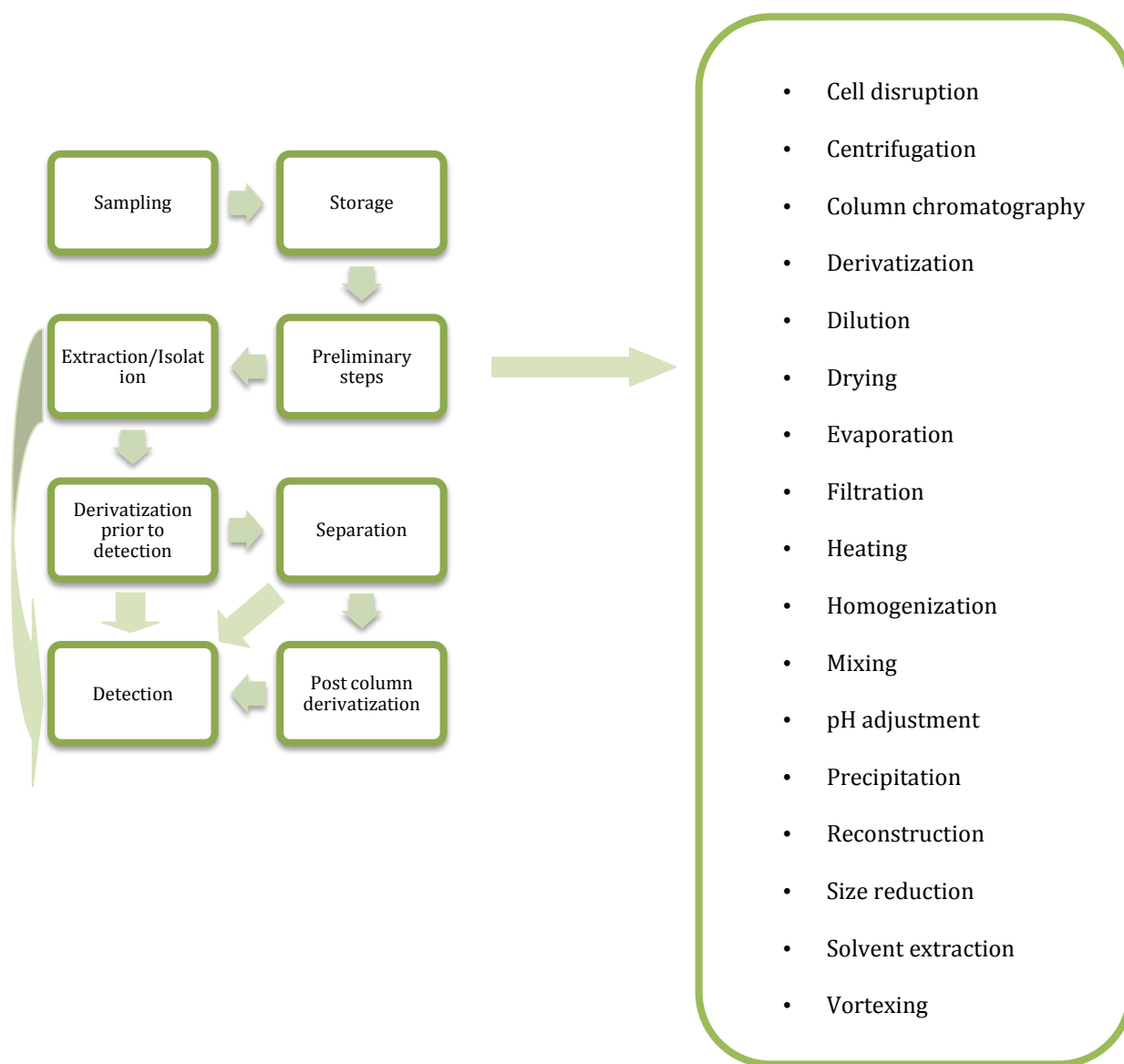


Fig. 1.2 Stages of sample preparation [20].

1.2.1 Analyte release from matrix

Extraction techniques can be classified as exhaustive or non-exhaustive. Exhaustive extraction techniques seek to liberate as much analyte as possible from the matrix. This technique is often considered to provide an evaluation of 'total' analytes concentration extracted from the matrix. Exhaustive extraction techniques are based on the release of target analytes following interactions with suitable solvents [21]. In principle, exhaustive extraction approaches do not require calibration, because most analytes are transferred to the extraction phase by employing overwhelming volumes of solvents. In practice, however, confirmation of satisfactory recoveries is implemented in the method by using surrogate standards [13]. The most commonly used exhaustive extraction techniques include Soxhlet extractions, ultrasonic assisted extractions (UAE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) [22].

On the other hand, non-exhaustive extraction techniques strive to reveal how an analyte is partitioned within a matrix. This technique is based on the principles of equilibrium, pre-equilibrium and permeation [23]. Pre-equilibrium conditions are accomplished by breaking the contact between the extraction phase and the sample matrix prior to equilibrium with the extracting phase. In permeation techniques such as membrane extraction [24], continuous steady-state transport of analytes through the extraction phase is accomplished by simultaneous re-extraction of analytes. Membrane extraction can be made exhaustive by designing appropriate membrane modules, optimizing the sample as

well as stripping flow conditions [25]. Alternatively, it can be optimized for throughput and sensitivity in non-exhaustive open-bed extraction [26].

There is a basic similarity among the extraction techniques used in sample preparation. In all approaches, the extraction phase is in contact with the sample matrix and analytes are transported between the phases. The ideal extraction method should be effective, cheap, rapid, user friendly and environmentally friendly (Fig 1.3.) [27]. Based on these criteria, none of the extraction techniques stands out as ideal in all accounts.



Fig. 1.3 General criteria used in choosing the most appropriate extraction technique [27].

Analysis of solid and semi-solids such as food and plant materials is at a disadvantage with respect to liquid samples [28]. If the whole solid sample is readily soluble, dissolution in a suitable solvent followed by liquid partitioning is the easiest. However, many matrices are largely insoluble and usually cannot be examined directly. It is necessary to extract the analyte of interest out of a matrix with 100% efficiency but also achieving as much specificity and selectivity as possible to simplify the subsequent separation steps [29].

Typical methods use exhaustive extraction in a Soxhlet system in which the solvent is continuously recycled through the sample for hours. However, the analyte must be stable in the refluxing boiling solvent. Less efficient methods include stirring the sample in hot or cold solvents for prolonged periods. The extraction process can be sped up by heating or agitating the sample (PLE and MAE). An alternative solvent, which has a higher diffusion rate as in supercritical fluid extraction and subcritical water extractions may be employed [13].

1.2.2 Removal of interfering matrix components from liquid samples

The analysis of complex matrices (e.g. environmental, biotechnological, food, biological, medical industries) requires sample handling steps aimed at the removal of unwanted matrix constituents from the sample. For chromatographic analyses, good sample

preparation is essential, because it protects the chromatographic columns and it enables greater sensitivity of detection after the removal of interfering matrix components. A selective and specific sample preparation is thus a prerequisite for reasonable, economical and sensitive analyses [30].

For example, a sorbent bed can be packed with extraction phase dispersed on a supporting material. When a sample is passed through, the analytes in the sample are retained on the bed. Large volumes of sample can be passed through a small cartridge, and the flow through the well-packed bed facilitates efficient mass transfer. The extraction procedure is followed by desorption of analytes into a small volume of solvent, resulting in substantial enrichment and concentration of the analytes. This strategy is used in sorbent trap techniques and in SPE [31].

1.2.2.1 Solid phase extraction (SPE)

SPE dates back to the 1960s and it is very popular for sample preparation of liquid samples with subsequent chromatographic analysis. In SPE, the analytes to be extracted are partitioned between a solid phase and a liquid phase. These analytes must have greater affinity for the solid phase than for the sample matrix [32]. SPE has been used extensively to remove and concentrate trace organic materials from liquid samples or solutions. It gained popularity not only because of the wide range of sorbent material, but also due to the ease of operation. A comprehensive review, covering trends, method development, coupling with liquid chromatography, and all types of SPE sorbent was published by Hennion in 1999 [33]. The main advantages of SPE are the possible integration of columns

and cartridges in on-line flow injection systems; less solvent consumption, ease of operation and possible application as analyte storage device for field sampling [34].

A wide range of sorbents have been used. These include C8 and C18 bonded phases on silica, polymeric resins (polystyrene/divinyl benzene copolymer), Florisil (activated magnesium silicate), polar sorbents such as alumina, charcoal, silica and cyano and amino-bonded [35]. Ionic functional groups, e.g. carboxylic acid or amino groups have also been bonded to silica or polymeric sorbent to create ion-exchange sorbents. Mixed-mode sorbents that use both the primary and secondary mechanisms for selective retention of analytes are available. The different phases enable interactions based on adsorption, H-bonding, polar and non-polar interactions, cation, anion exchange or size exclusion to be utilized [36].

A quick, easy, cheap, effective, rugged, and safe (QuEChERS) AOAC sample preparation approach for extraction and clean-up of pesticide residues from multiple classes, has also been reported as a dispersive form of SPE [37]. In summary, the method uses a single-step buffered acetonitrile (1% HAc) extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate (MgSO_4) to induce liquid-liquid partitioning.

In the clean-up stage of the QuEChERS method, a dispersive solid phase extraction (d-SPE) step is employed. This involves transferring a portion of the acetonitrile extract to a clean-up tube containing a combination of sorbents such as graphitized carbon black (GCB) for the removal of unwanted sample components (e.g. chlorophyll); primary secondary amine

(PSA) to remove fatty acids as well as other components; and anhydrous MgSO_4 to remove the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis. The method was formalized and adopted as AOAC Official Method 2007.01 [38].

One of the challenges of SPE is that the packing must be uniform to avoid poor efficiency due to channeling of analytes. Although pre-packed commercial cartridges are now considered reliable, automated systems can have difficulties with reproducibility for certain analytes. The sample matrix can also affect the ability of the sorbent to 'extract' the analyte due to competition for retention [36]. Many traditional sorbents are limited in terms of selectivity. Insufficient retention of very polar analytes can also be a challenge. The use of hydrophilic materials for the improved extraction of the most polar analytes by SPE was detailed by Fontanals et al [39]. More recently, a number of selective sorbents such as restricted access media (RAM), immunosorbents and MIPs have also been widely used [40].

1.2.2.1.1 Restricted access media (RAM)

One group of selective sorbents for SPE is RAM [41]. These sorbents were developed particularly for analysis of biological samples, such as plasma and serum as they are designed to exclude macromolecules, such as proteins. They combine size exclusion of proteins and other high molecular mass matrix components with the simultaneous enrichment of low molecular mass analytes at the inner pore surface [42]. Macromolecules are excluded either by a physical barrier (pore diameter) or by a chemical diffusion barrier

created by a protein network at the outer surface of the particle. The interaction sites within the pores are accessible to small molecules only and analytes are retained by conventional retention mechanisms such as hydrophobic or electrostatic interactions.

Various RAM sorbents are available with different surface chemistries [43], one of the most common being alkyl-diol-silica (ADS). Several applications are given in a review by Souverain et al. [43], including the direct analysis of pharmaceuticals in milk and tissue [44,45]. Both these applications used column-switching with LC for on-line extraction/clean-up.

Highly selective SPE sorbents allowing extraction, concentration and clean-up in a single step has been achieved by employing materials involving antigen–antibody interactions. Selective extraction methods based on molecular recognition were therefore provided [46]. Antibodies are covalently bonded onto an appropriate sorbent to form an immunosorbent, which is packed into an SPE cartridge or pre-column. Since antibodies are highly selective towards the analyte they are able to initiate the immune response with a high affinity. The corresponding immunosorbent may then extract and isolate the analyte from complex matrices in a single step, and the challenge of co-extraction of matrix interferences is therefore solved [47].

1.2.2.1.2 Immunoaffinity extraction (IAE)

IAE is based upon a molecular recognition mechanism where the high affinity and high selectivity of the antigen–antibody interactions allow specific extraction and concentration of the analytes of interest in one step [48]. IAE can efficiently eliminate the matrix

contaminations and non-target compounds to enrich the target analyte. Immunoaffinity extraction has been applied in environmental monitoring [49], pharmaceutical and biomedical analyses [50], and food analysis [51]. As a clean-up and separation technique, IAE has been successfully used to enrich analytes in biological fluid prior to CE detection [52].

One of the major disadvantages of this technique is the need to initially develop the antibody, which makes it impractical for 'one off' analyses. The analyte-antibody interaction can also be affected by the sample matrix, leading to low extraction recoveries. A review by Hennion and Pichon [52], described immuno-based extraction sorbents and the use of artificial antibodies. Most applications are for biological or environmental samples. In food analyses, it includes determination of pesticides (imazalil and phenylurea herbicides) in fruit juices [53, 54]. Methods for the analysis of mycotoxins have been developed and validated, and are now commercially available [55]. Immunosorbents have also been developed for some veterinary drugs, such as fluoroquinolones [56] and corticosteroids [57]. Rather than being dependent on antibody production, attempts have been made to mimic the specificity of immunological products with synthetic MIPs [58].

1.2.2.1.3 Molecularly imprinted polymers (MIPs)

Molecular imprinting is a method for creating specific cavities in synthetic polymer matrices with memory for the template molecules [59]. Generally, MIPs are synthesized by assembling monomers around a template to form a complex through covalent or non-

covalent interactions and joined by a cross-linking agent (Fig. 1.4). Then the template molecule is removed by chemical reactions or extraction, resulting in exposure of binding sites ('imprints') which are complementary to the template in size, shape, and position of the functional groups [60, 61]. They can consequently be used as a highly selective pre-concentration step.

Because of their particular characteristics, they may be used as analyte specific as well as reusable sorbent materials for selective pollutant clean-up purposes. The MIPs are typically characterized by very high chemical and physical stability, a fact which relieves hyphenation with other sample preparation procedures. However MIPs for particular target analytes are normally not commercially available and have to be synthesized and evaluated for every specific purpose [62].

The use of MIPs as selective sorbent materials allows performing a customized sample treatment step prior to the final determination. Thus, their use in solid-phase extraction, so-called molecularly imprinted solid-phase extraction (MISPE), is by far the most advanced technical application of MIPs [63-67]. Recent years have seen a growing interest in the combination of MIPs with other sample preparation techniques such SPME and MSPD among others [68].

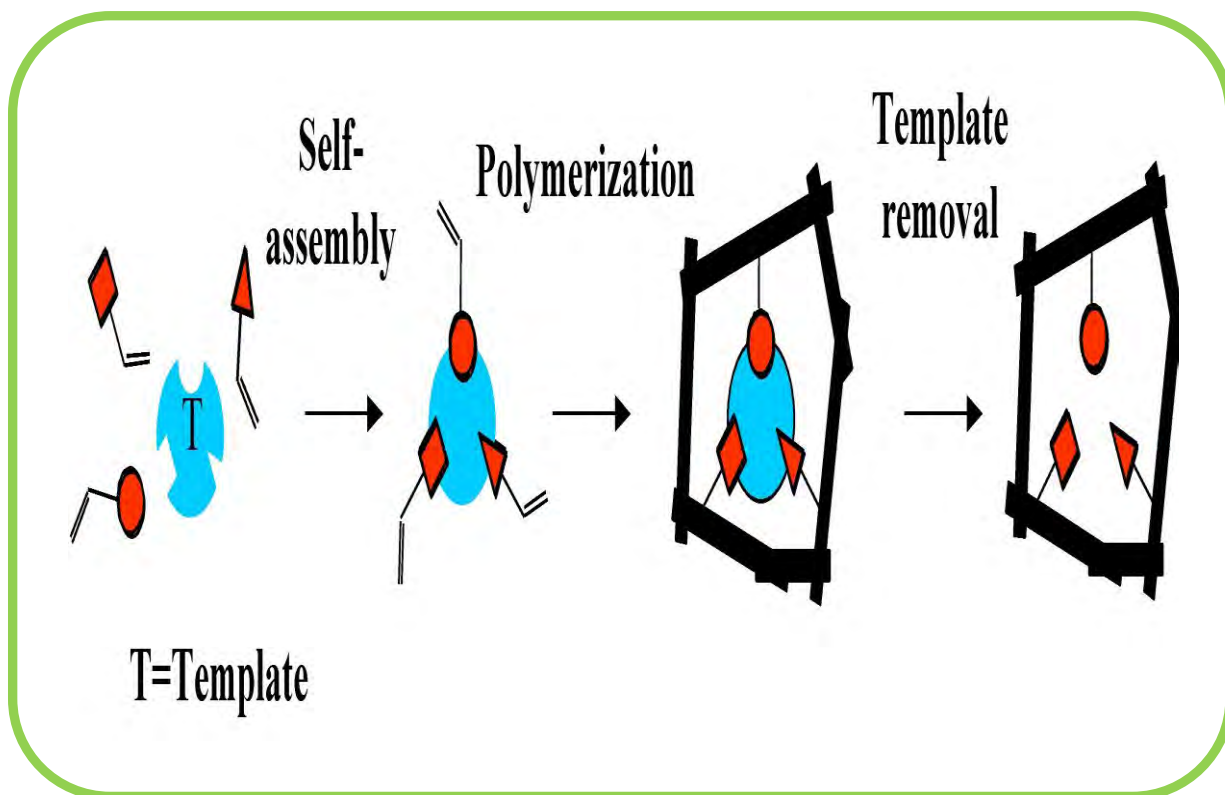


Fig. 1.4 Synthesis of MIPs and its selective recognition to target molecule [63]

The search for new materials seems to be a never-ending task as current efforts are mostly aimed at finding the optimum sorbent for specifically tailored applications. The need for further simplicity, automation and increased throughput has further stimulated interest towards the development of different SPE formats and configurations. The shape or form of sorbent material plays a vital role in the development of new SPE formats or configurations [69]. Therefore, research on alternative sorbent fabrication techniques involving several branches of science and technology is necessary. The advent of nanotechnology was a

major leap forward in the research area of sorbent based sample preparation techniques as it opened up possibilities for a new class of materials that could be used in SPE applications [70]. In recent years, increasing attention has been paid to fabricating nanofibers by a process commonly known as electrospinning (e-spinning) [71, 72].

1.2.2.1.4 Electrospinning

Electrospinning is a technique that relies on repulsive electrostatic forces to draw a viscoelastic solution into nanofibers [73]. As displayed in Fig. 1.5, the basic requirements of an electrospinning apparatus include: (1) a mode to deliver a polymer solution (capillary tube with a needle or pipette), (2) a high power voltage supply, and (3) a collector or target [74]. Electrical wires connect the high power supply to the capillary tube, which contains a polymeric solution, as well as to the target. The capillary tube and target are held at a relatively short distance from each other. Copper plates [75, 76], aluminum foil or plates [77-80], rotating drums [81-83] and human hands [84] have been utilized as targets to collect fibers during the electrospinning process. The polymer solution is forced through the syringe pump to the needle, either by gravity or by an advancement pump. Initially, as a result of surface tension, pendant droplets of the solution are held in place. A conical protrusion [85], known as a Taylor cone [86] (Fig 1.6) is formed when a critical voltage is applied to the system.

For a few centimeters, an approximately straight jet emerges from the cone. However, this straight segment cannot hold for long. The jet therefore emerges into a diaphanous and conical shape, within which exists the complicated path taken by the jet [87]. Bending

instabilities are experienced by the conically moving jet and its field is directed towards the collector, which has the opposite electrical charge. Within the time it takes the jet to reach the collector, the solvent evaporates and dry polymer fibers are deposited [88].

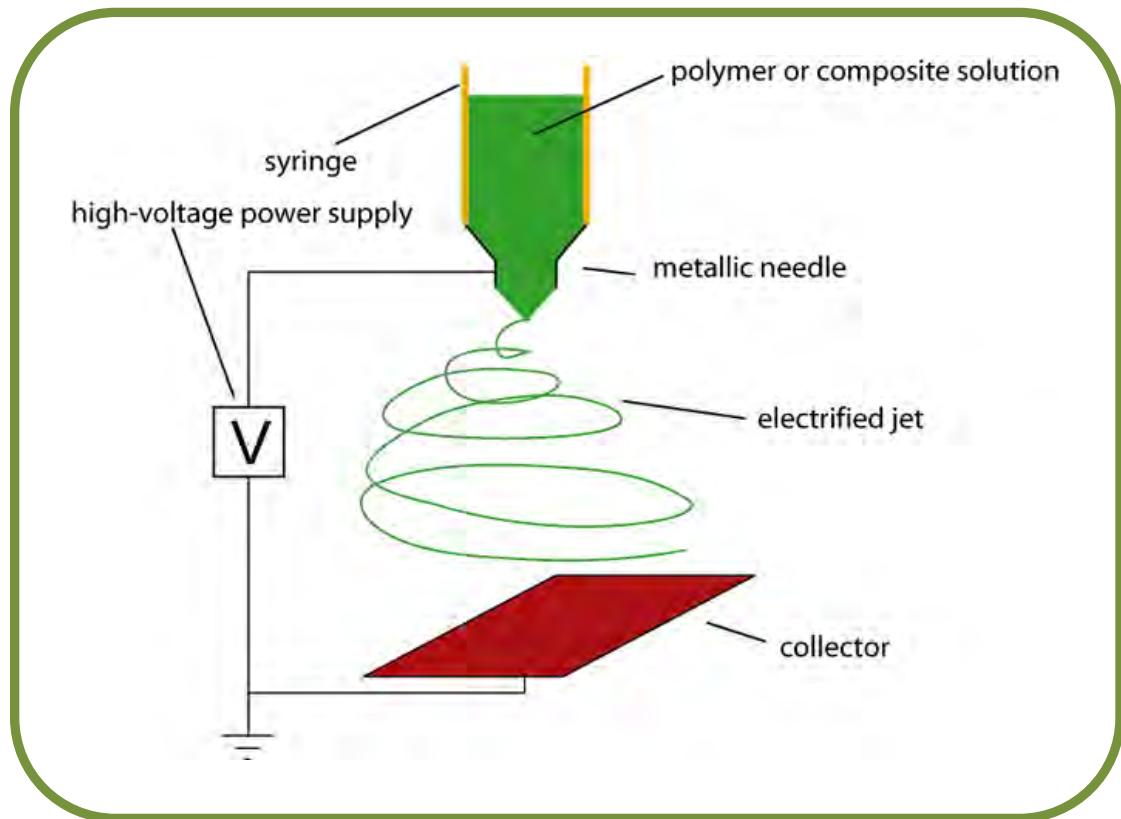


Fig. 1.5 A typical electrospinning setup [89]

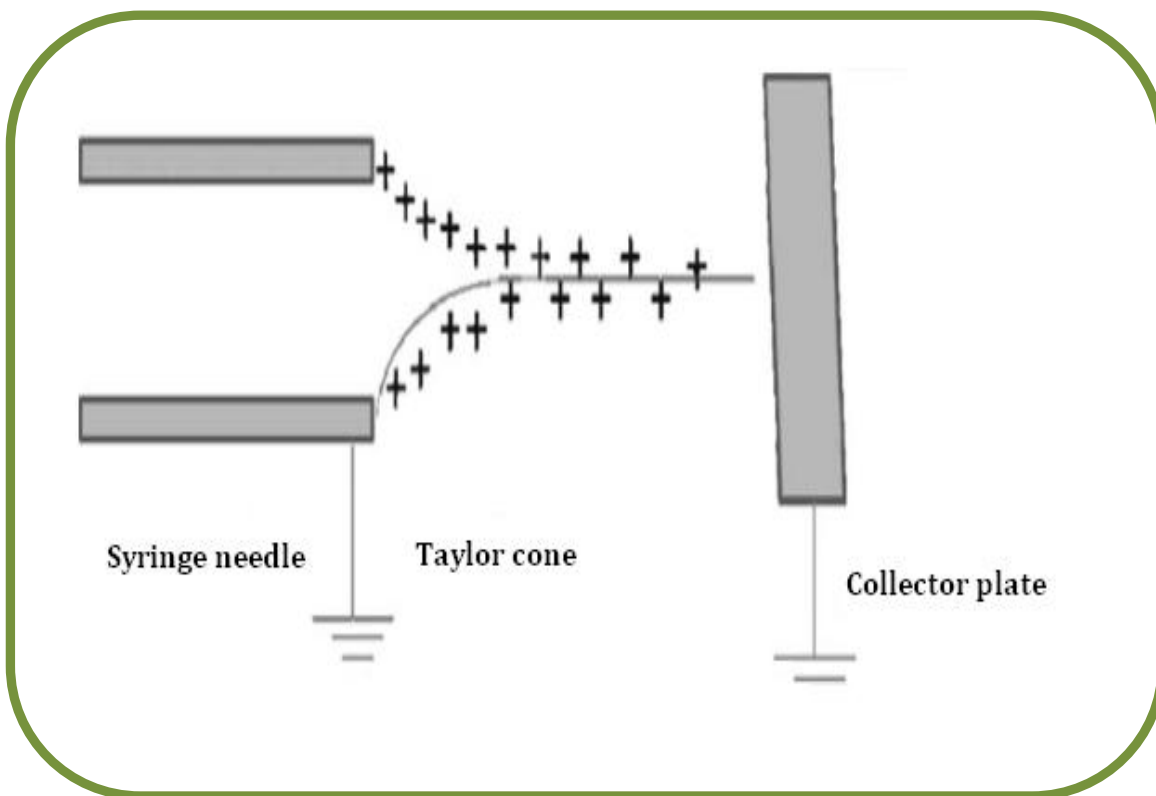


Fig. 1.6 Schematic representation of the Taylor cone formation [73]

Electrospinning offers a convenient approach to fabricate fibers with controllable diameters from the nanoscale to microscale [89,90]. It provides a method of fabricating extraction materials with an extraordinarily high surface to volume ratio, which is one of the most desirable properties for improving the sensitivity of SPE. By varying polymer architecture and processing parameters, porous fibers can also be electrospun to improve surface to- volume ratio [91,92]. Furthermore, most polymers with sufficiently high molecular weight can be electrospun and specifically surface functionalized according to the characteristics of the analyte of interest.

This does not only has a positive effect on the extraction efficiency but also improves the selectivity of SPE. Because of their nanoscale diameter, high surface to-volume ratio, controllable surface figurations, and various compositions, electrospun nanofibers are anticipated to be one of the best SPE sorbents. This could resolve challenges of commercial SPE sorbents, such as their low sensitivity and selectivity, complex process, and high cost [93]. On the basis of the hypothesis that the large surface areas of nanocomposites facilitate interaction between nanocomposites with target analytes, reducing the scale of materials used in SPE is gradually attracting interest. Several papers on electrospun nanofibers as SPE sorbents have been published recently [94-96].

1.2.3 Derivatization

Liquid and solid samples extracted into organic solvents are usually analyzed by LC or GC. LC is selected if the analytes are polar, thermally labile or have high molecular masses. Otherwise GC is preferred because of better resolution [16]. In some cases, the enhancement of detectability is required in trace analysis, when the analytes do not possess a UV-absorbing, fluorescent, or electro active functionality. Therefore derivatization is necessary. In LC analyses, UV chromophores and fluorophores are often introduced into sample molecules to increase their sensitivity to UV absorption and fluorescence detection respectively [97].

The derivatization for HPLC can be performed either “offline” (pre-column) before injection into the column, or “on-line” (post-column) by mixing the reagent with the column effluent. Pre-column derivatization offers some advantages compared to post-

column derivatization. It involves less reaction restrictions, simpler equipment and no time limitation regarding kinetics, provided that all analytes are stable. It can be performed either manually or automated. However, there are several drawbacks such as the introduction of contaminants, a possible loss of analyte as a result of side reactions, adsorption, degradation, and incomplete reactions [98].

For GC, non-volatile substances may be volatilized to render them amenable to detection [99]. Derivatization can be incorporated into analytical methods to increase the volatility of the analyte or to add functional groups to improve instrumental analysis. For example, silylating reagents are commonly used for GC derivatization. They have different functional groups (hydroxyl, carboxyl, amidic and amino groups) which render them versatile as derivatizing reagents [100].

Chapter 2 Solid sample extraction techniques

2.1 Overview

This chapter discusses the extraction process and strategies as well as various extraction techniques for solid samples. The extraction techniques discussed include the conventional Soxhlet extraction and ultrasound assisted extraction (UAE) and the modern extraction techniques (MAE, SFE and PLE) which employ environmentally friendly high diffusion fluids. Application of these techniques, including their challenges in the analysis of plants and herbal materials will also be discussed.

2.2 The extraction process

Extraction is an analytical procedure in which analytes are removed from a matrix and transferred to an extraction medium [101]. Selective extraction of analytes is based on the differences in their chemical and physical properties such as molecular weight, charge, solubility (hydrophobicity), polarity or differences in volatility [11]. Extraction always involves mass transfer from one phase to another. Principles of extraction are used to advantage in everyday life; for example in as simple a task as making a cup of tea [101]. Solvent extraction is one of the oldest methods of separation known, the science of which has evolved over a long period of time.

Much progress has been made in the understanding of solvation and properties of the solvents used in the extraction processes [102]. The process of extraction of heterogeneous

samples was illustrated by Pawliszyn in 2003 by a model that assumes that the sample particle is porous and is surrounded by an organic layer [13, 102]. The efficiency of extraction depends on the nature of the sample matrix, the analyte to be extracted and the location of the analyte within the matrix.

The extraction and recovery of the analyte from the sample matrix can be expressed in a few steps. To be able to remove the analyte from the extraction vessel, it is first desorbed from its active site in the sample matrix; then diffused through the organic part of the matrix so as to reach the matrix–solvent interface. This is the stage at which the analyte is solubilized into the extraction media. It then diffuses through the extraction media present within the pore before reaching the part of the extraction phase that is affected by convection. The final stage of the extraction process is collection of the extracted analyte [13, 101]. Quantitative analysis and selectivity are not easily obtained at the same time. Often, a compromise must be reached, especially if several analytes are to be determined simultaneously. Thus, the extraction system has to fulfill several requirements, as it depends on the analytes and the application which extraction technique is best suited for the purpose [102].

A critical step in the extraction process is the positioning and the status of the analyte within the sample matrix; five different positions have been postulated (Fig. 2.1);

1. Adsorbed to the surface of the matrix;
2. Dissolved in a solvent pore and/or adsorbed at the surface;
3. Dissolved/adsorbed in a matrix micro/nano-pore;

4. Chemically bonded to the matrix;
5. Dissolved in a bulk solution [13, 102].

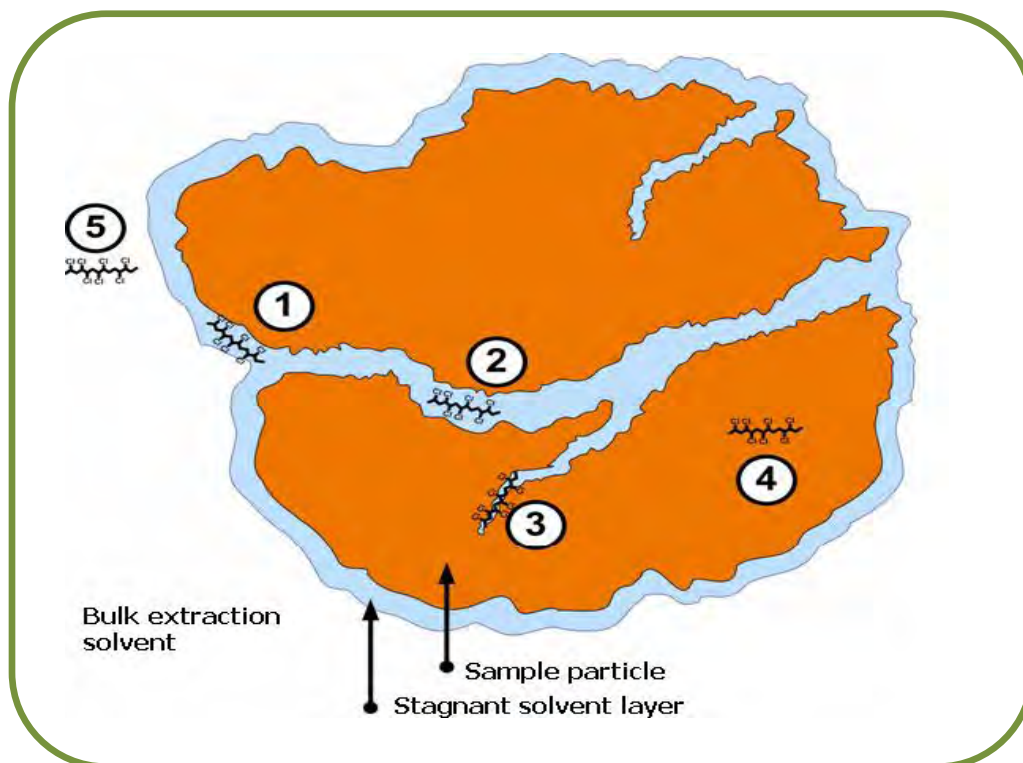


Fig 2.1 Positioning of the analyte in the sample matrix [7]

In practical environmental applications (*e.g.* extraction of pollutants from soils and sediments), the first step is usually the rate-limiting step, as solute–matrix interactions are very difficult to overcome and to predict. However, for plant materials, the rate may be limited by either the solubilization or diffusion step. As a result, the optimization strategy will strongly depend on the nature of the matrix to be extracted [103, 104].

2.3 Extraction strategy

Knowledge about distribution coefficients and distribution ratios as well as solubility parameters is useful in order to select an appropriate solvent as well as extraction conditions. The fundamental thermodynamic principle common to all chemical extraction techniques involves the distribution of analyte between the sample matrix and the extraction media. When a liquid is used as the extraction medium, the distribution constant, K_{es}

$$K_{es} = \frac{a_e}{a_s} = \frac{C_e}{C_s} \quad \text{equation} \quad (2.1)$$

defines the equilibrium conditions and ultimate enrichment factors achievable by use of the technique; where a_e and a_s are the activities of analytes in the extraction phase and matrix, respectively, and can be approximated by the appropriate concentrations C_e and C_s [13].

Ideally, an extract of high purity and selectivity should be achieved. This implies that the analyte of interest must have high solubility in the solvent while other compounds have no or minimal solubility. The rule of thumb in solvent choice is “like dissolves like”. This indicates the use of polar solvents for polar analytes and likewise non-polar solvents for non-polar analytes [101]. The fundamental aspect in the choice of solvent is the solubility characteristic of the desired analyte, their diffusivity in the solvent and the characteristics of the sample [105,106].

The theory of solubility was proposed by Hildebrand [7] by combining the correlation between vaporization and inter-molecular forces, van der Waal's forces and hydrogen bonding, and the correlation between vaporization and solubility behavior. The inter-molecular attractive forces have to be overcome to vaporize a liquid so as to dissolve the analyte [7]. The 'solubility parameter' (δ), was defined as the square root of the cohesion energy density (c) of a solvent or solute, giving a numerical value indicating the analyte behavior in a specific solvent (see equation 2.2) [7], where ΔH is heat of vaporization (J mol^{-1}), R is the gas constant ($\text{J K}^{-1} \text{mol}^{-1}$), T is the temperature (K) and V is the molar volume of the analyte.

$$\delta = \sqrt{c} = \sqrt{\frac{\Delta H - RT}{V}} \quad \text{equation} \quad (2.2)$$

Hansen [107] worked further and assumed that the total cohesion energy density is a linear addition of three components; hydrogen bonding ability contribution (δ_H), dispersion coefficient contribution (δ_D) and polarity contribution (δ_P). They are linked by equation 2.3, where δ_t is the total solubility parameter.

$$\delta_t^2 = \delta_H^2 + \delta_P^2 + \delta_D^2 \quad \text{equation} \quad (2.3)$$

It should be noted however that the choice of solvent in particular situations involves other factors besides the solvent power. The process of solute analyte transfer across an interface between two liquid phases may be rate controlled by molecular diffusion, motion of eddies,

irregular surface disturbances or even by chemical reactions in the bulk of a phase or an interface region. Partitioning of processes has a central role of concern in the extraction procedure. These involve partitioning of the analyte between the surface of the matrix and the solvents as well as chemisorption of the analyte on the active sites and within the solvent. Different matrices behave differently therefore a variety of equilibria take place [108].

2.4 Conventional extraction techniques

The conventional extraction techniques for solid samples rely on extraction with organic solvents and these include; Soxhlet extraction, USE, blending and solid-liquid extraction. The broad polarity range of solvents and their general applicability made these techniques popular. Environmentally, however, the use of large volumes of organic and often chlorinated solvents is unfavorable. Furthermore, they often require complex time-consuming multi-step procedures which can lead to low accuracy, contamination and losses of analytes [109].

2.4.1 Soxhlet extraction

Soxhlet extraction is a general and well-established technique developed in 1879. The technique is based on exhaustive extraction of organic analytes in a Soxhlet system by an organic solvent, which is continuously refluxed through the sample contained in a porous thimble [101,110]. The extracted analytes accumulate in a heated flask and so they must be stable in the refluxing boiling solvent. Soxhlet extraction is the oldest technique used for

the isolation of non-polar and semi-polar organic pollutants from different types of solid matrices, including biota samples [111]. It has been used for decades and has been adopted by the U.S. Environmental Protection Agency (EPA) as method 3540C [112].

In a conventional Soxhlet system as shown in Fig. 2.2, the sample is placed in a thimble-holder, and filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. In the solvent flask, solute is separated from the solvent using distillation. The solute is left in the flask and fresh solvent passes back into the plant solid bed. The operation is repeated until complete extraction is achieved [113].

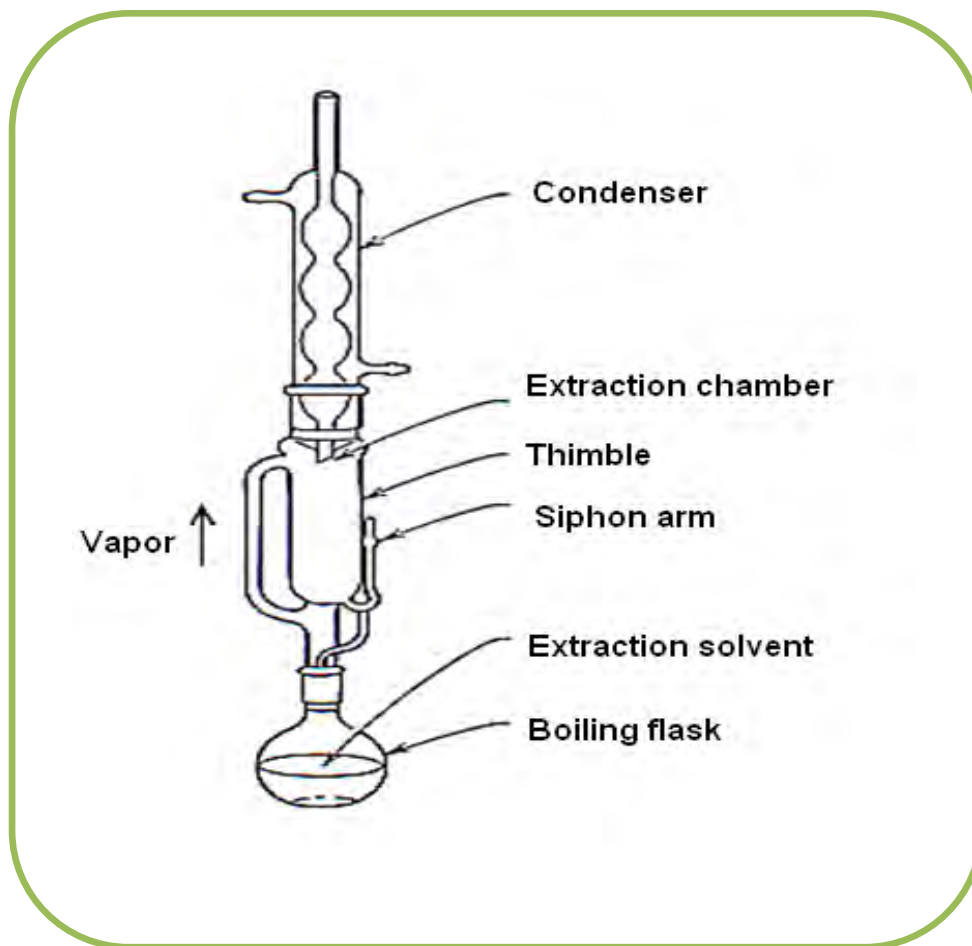


Fig. 2.2 Conventional Soxhlet extractor [114].

A suitable extracting solvent should be selected for the extraction of targeted analytes using the Soxhlet extraction method. Different solvents yield different extracts composition [115]. Typical solvents to extract persistent organic pollutants (POPs) from animal and plant tissues are *n*-hexane, dichloromethane, and mixtures of toluene-methanol, *n*-hexane-acetone and dichloromethane-acetone. Usually, animal and plant fresh tissues should be cut, shredded and then ground with sodium sulphate in order to reduce their water content. This also helps to open up the tissue structure which enables good

penetration of solvent into the sample matrix [110]. Hexane is the most widely-used solvent to extract edible oils from plant materials due to its fairly narrow boiling point range. However, n-hexane, the main component of commercial hexane, is the top listed of 189 hazardous air pollutants by the US EPA [116].

The use of alternative solvents such as isopropanol, ethanol, hydrocarbons, and even water, has increased due to environmental, health, and safety concerns. However, alternative solvents often result in less recovery due to a decreased molecular affinity between solvent and solute. The costs of alternative solvents could be higher. A co-solvent is sometimes added in order to increase the polarity of the liquid phase. A mixture of solvents such as isopropanol and hexane has been reported to increase the yield and kinetics of extraction [117].

Soxhlet extraction strongly depends on matrix characteristics and particle size as the internal diffusion may be the limiting step during extraction. During Soxhlet extraction, the solvent is usually recovered by evaporation. The extraction and evaporation temperatures have a significant effect on the quality of final products. The high boiling temperature for solvent recovery can be decreased by using vacuum or membrane separation to recover the solvent [118].

The advantages of conventional Soxhlet extraction include; the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix; maintaining a relatively high extraction temperature with heat from the distillation flask;

and that no filtration is required after extraction [113]. In relation to modern extraction techniques, it is a low-cost method. The Soxhlet extraction glassware is rather inexpensive. However, the cost is elevated by the large solvent volumes employed. It requires little operator involvement after the sample is loaded and refluxing proceeds until the termination of the extraction. Attempts to automate the technique were somewhat successful, and a few commercial systems are available in which several samples can be extracted in parallel with much shorter extraction times and less organic solvent than using conventional Soxhlet [119-121]. Wide industrial applications, better reproducibility and efficiency, and less extract manipulation are other advantages of Soxhlet extraction.

The main disadvantages of conventional Soxhlet extraction include; the extraction time is long; (up to a few days); large volumes of organic solvents are used (300-500 mL); agitation cannot be provided in the Soxhlet device to accelerate the process. The large volumes of solvent used require an evaporation/concentration procedure; and the possibility of thermal decomposition of the target compounds cannot be ignored as the extraction usually occurs at the boiling point of the solvent for a long time [119]. Therefore Soxhlet is an old-fashioned, time and solvent consuming extraction technique. Some solvents used in the conventional Soxhlet have recently been questioned because of their toxicity.

Many applications of Soxhlet extraction are for environmental samples, such as soils, but it has been used for analysis of food particularly fat content or as a preliminary extraction

technique for fat soluble analytes followed by further clean-up [120]. It has also been used for the extraction of antioxidants from herbs and spices [121].

2.4.2. Ultrasound-assisted extraction

The simplest solid-liquid extraction technique is to blend the solid sample with an appropriate organic solvent and ultrasonicate them. The process is carried out in discrete systems using an ultrasonic bath or a closed extractor fitted with a sonic probe. Sonication involves the use of sound waves to stir the sample immersed in the organic solvent [122]. Briefly, energy in the form of acoustic sound waves in the ultrasound region above 20 kHz, is used to accelerate mass transport and mechanical removal of analytes from the solid matrix surface by a process called “cavitation”. This consists of the formation and implosion of vacuum bubbles through the solvent, thus creating microenvironments with high temperatures and pressures [123]. The mechanical effect of ultrasound induces a greater penetration of solvent into solid materials and improves mass transfer leading to an enhancement of sample extraction efficiency.

Sonication-assisted extraction is faster (5–30 min for sample) than the Soxhlet method and allows extraction of a large quantity of sample at a relatively low cost [124]. Unfortunately, it still uses about as much organic solvent as the Soxhlet extraction and also filtration is required after extraction. Moreover, it is labour intensive. The efficiency of the extraction is dependent upon the nature and homogeneity of the sample matrix, the ultrasound frequency and the sonication time used [125].

UAE extraction has been approved by EPA as method 3550B [112]. It is an inexpensive, simple and efficient alternative to conventional extraction techniques. The main benefits of the use of ultrasound in solid–liquid extraction include the increase of extraction yield and faster kinetics [126]. Ultrasound can also reduce the operating temperature allowing the extraction of thermo-labile compounds. Compared with other modern extraction techniques such as MAE, the ultrasound apparatus is cheaper and its operation is easier. Furthermore, USE like Soxhlet extraction, can be used with any solvent for extracting a wide variety of natural compounds [127]. Rodríguez-Sanmartín et al. reported USE of PAHs from mussel soft [128], and from pine needles [129]. Ultrasound-assisted extraction has also been used to extract bioactive compounds from plants such as essential oils and lipids [130] and pesticides in honey [131].

UAE has recently been carried out using a dynamic extraction set-up (a flow system) which continuously supplies fresh extraction solvent to the extraction vessel. A considerable reduction of extraction time, solvent consumption and sample handling, with respect to the extraction in static way, was reported [123]. Another feature of such dynamic arrangement is that the analytes are transferred out of the extraction vessel system as soon as they are extracted. This can be especially important to avoid degradation of the analytes due to sonication or if thermo-labile analytes are extracted at higher temperatures and pressures. Domeño et al. [132] used a dynamic sonication-assisted extraction procedure for extracting PAHs from lichens using hexane. The reported total extraction time was only 10 min compared to 2 h in the static extraction mode and 6 h in Soxhlet extraction, while the PAHs relative recoveries obtained by the three methods were similar.

2.5 Extraction by high diffusion fluids

High diffusion fluids are defined as fluids created by raising and or controlling the temperature and pressure [7]. They provide different physico-chemical properties compared to those obtained at ambient temperature and pressure. These properties are intermediate to those of liquids and gases making them attractive as extraction solvents. Essential parameters for extraction media are compared and these include density, viscosity and diffusion coefficients, Table 2.1 [7].

Table 2.1 Approximate ranges of density, viscosity and diffusion coefficients of gases, supercritical fluids and liquids [7].

Fluid	Density (g cm ⁻³)	Viscosity (g cm ⁻¹ s ⁻¹)	Diffusion coefficient (cm ² s ⁻¹)
Gas	(0.6-2)10 ⁻³	(1.3)10 ⁻⁵	0.1-1.0
Supercritical fluid	0.2-0.9	(1.3)10 ⁻⁴	(0.1-5)10 ⁻⁴
Liquid	0.6-1.6	(0.2-3)10 ⁻³	(0.2-3)10 ⁻⁵

The most important characteristic for the utilization of any solvent in an extraction procedure is its capability to dissolve solutes (solvation capability). Below the critical temperature, the gas and liquid phases co-exists, the solvent power for the gases being minimal and only the liquid being used as a solvent [109]. For supercritical fluids, the solvation capability is highly related to the density of the fluid, showing a significant increase as the density increases towards the critical density. Very little solutes dissolve in gas like supercritical fluids. Only when the properties of the fluid become liquid like does the solubility begin to increase [108, 109].

The viscosity values of high diffusion fluids are one order of magnitude lower than those of liquids solvents and the values of diffusion coefficients are one order of magnitude higher than those of liquid solvents as seen in Table 2.1. This explains why these fluids render high diffusivities, resulting in significantly faster extractions. Due to these properties, high diffusion fluids have attracted attention in sample preparation techniques especially in the analysis of plant materials [23].

2.5.1 Microwave assisted extraction (MAE)

MAE uses microwave radiation (0.3–300 GHz) as the source of heating a solid sample–solvent mixture [133]. Due to the particular effects of microwaves on matter (namely dipole rotation and ionic conductance) heating with microwaves is instantaneous and occurs in the core of the sample, leading to very fast extraction. Heat generation in the sample by the microwaves field requires the presence of a dielectric compound. The

greater the dielectric constant, the more thermal energy is released and the more rapid is the heating for a given frequency [134].

Consequently, high diffusion fluids can be obtained using microwave energy in combination with high temperature and controlled pressures. The absorption of microwave radiation results in the disruption of weak hydrogen bonds which improves solvent penetration and enhances analyte solvation. Usually, the extraction solvent has a high dielectric constant, so that it strongly absorbs the microwave energy. However, in some cases (for thermo-labile compounds), the microwaves may be absorbed only by the matrix, resulting in heating of the sample and release of the solutes into the cold solvent [135].

The nature of the solvent is of great importance in MAE. It should selectively and efficiently solubilize the analytes in the sample at the same time absorbing the microwaves without leading to a strong heating (so as to avoid eventual degradation of the analyte [135, 136]. Thus, it is common practice to use a binary mixture (e.g. hexane–acetone, 1:1) where only one of the solvents absorbs the microwaves. Other important parameters affecting the extraction process are the applied power, the temperature and the extraction time. Moreover, the water content of the sample needs to be carefully controlled to avoid excessive heating [137].

The application of microwave energy to the samples may be performed either in closed vessels with pressure and temperature control (pressurized MAE) or in open vessels at

atmospheric pressure (focused MAE) [136]. Whereas in focused MAE, the temperature is limited by the boiling point of the solvent at atmospheric pressure, in pressurized MAE the temperature may be elevated by simply applying adequate pressures [138]. MAE is considered a good alternative to traditional Soxhlet extraction of solid samples because it reduces extraction time, employs small volumes of organic solvents and improves extraction yields. An official EPA method 3546 (MAE) has been approved for the extraction of organic analytes from solid environmental samples [112].

MAE has been used to extract pesticides and herbicides from soil [139], fungal metabolites [140] essential oils from plant materials [141], and PAH in sediments [142]. Comparisons have been made with other extraction techniques such as SFE [143] and Soxhlet extraction [144,145]. However, MAE has several drawbacks; the extract must be filtered after extraction; polar solvents are needed; clean-up of extracts is almost always needed and the equipment is moderately expensive [146].

2.5.2 Supercritical fluid extraction (SFE)

SFE is an extraction technique that utilizes a solvent in its supercritical state. Supercritical fluids have similar densities to liquids; have gas like viscosities (Table 2.1) and so analytes exhibit higher diffusion coefficients [147]. This combination of properties results in a fluid that is more penetrating, has a higher solvating power and may extract solutes faster and more efficiently than liquids [148]. In addition, the density (and therefore the solvent

power of the fluid) may be adjusted by varying both temperature and pressure, affording the opportunity of theoretically performing highly selective extractions [149].

Supercritical state is achieved when the temperature and the pressure of a substance are raised above its critical value. The supercritical fluid has characteristics of both gases and liquids. Compared with liquid solvents, supercritical fluids have several major advantages; the dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure or temperature. Supercritical fluid has a higher diffusion coefficient, lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer [150]. Several fluids have been tested in SFE. By far, the most widely used extraction fluids has been carbon dioxide (CO_2) with critical values of 31.1 °C and 73 atm as shown in Fig 2.3. The popularity of CO_2 is due to the fact that it is chemically inert, easy to clean, environmentally friendly, 'solvent free' and inexpensive. Other supercritical solvents that have been used include ammonia, dinitrogen oxide and water [7].

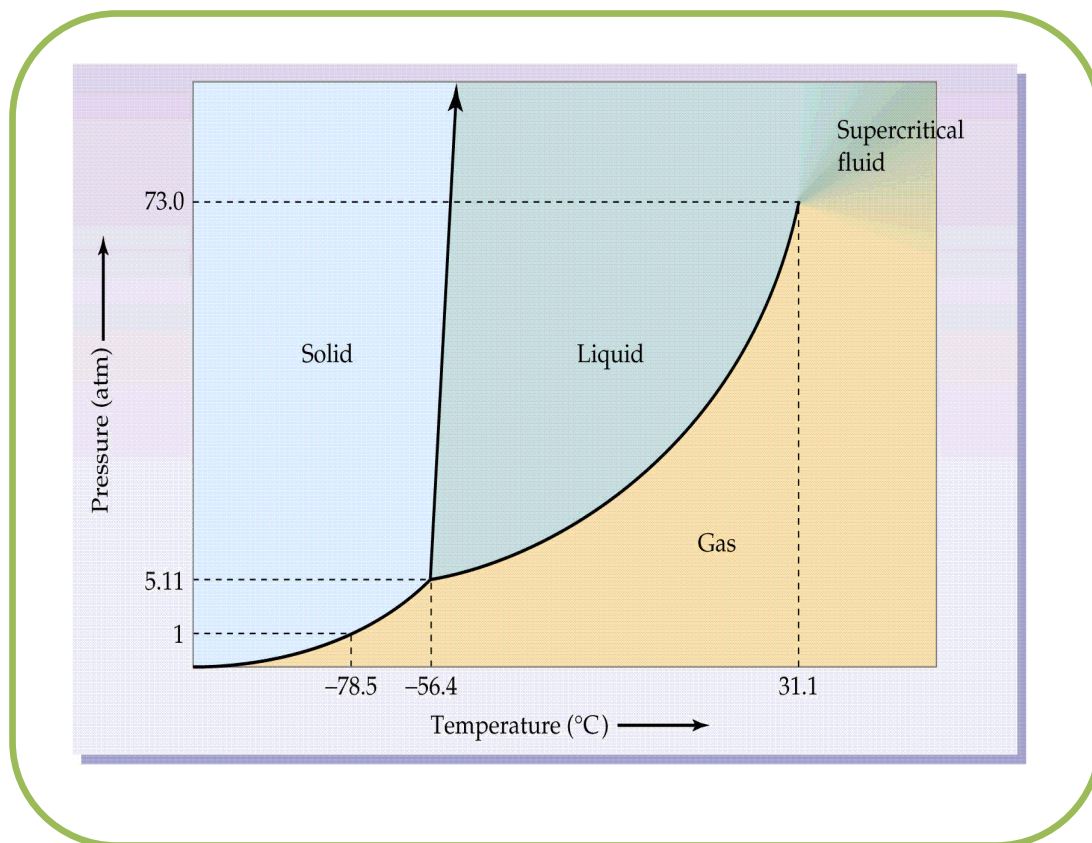


Fig. 2.3 Phase diagram for carbon dioxide [151].

The main advantage of the SFE technique is that it is analyte selective and the extracts are relatively clean and therefore the clean-up step is usually not necessary. Extraction times are often relatively short [152]. Since the density of the fluid is a function of its temperature and pressure, precise control of these parameters allows a solvent with a narrow window of solvating strength to be obtained. It is possible therefore, to substitute a variety of conventional solvents with a single supercritical fluid [153]. Furthermore, supercritical fluids have a density of a liquid and can solubilize a solid like a liquid solvent. The solubility of a solid in a supercritical fluid increases with the density of the fluid, which can be achieved at high pressures [154]. Supercritical carbon dioxide (SC CO₂) extraction uses a

moderate extraction temperature as low as 30 °C. The low supercritical temperature of CO₂ makes it attractive for the extraction of heat sensitive analytes [155]. As SFE uses no or only minimal organic solvent (organic modifiers) in extraction, it is a more environmentally friendly extraction process than conventional solvent-solid extraction. SFE can be directly coupled with a chromatographic method for simultaneous extraction and separation of highly volatile analytes [156].

However, the polarity of supercritical CO₂ is poor at low pressures and high temperatures. To overcome the limited solubility of polar analytes in SC CO₂, addition of polar co-solvents as modifiers to the SC CO₂ is known to significantly increase the solubility of polar compounds [152]. Methanol is the most commonly used solvent because it is an effective polar modifier and is up to 20% miscible with CO₂. However, the use of methanol as a modifier requires a slightly higher temperature to reach the supercritical state. This could be disadvantageous for thermo-labile analytes. The other disadvantage of using a modifier is that it can cause poor selectivity. The limited sample size and the high cost of the equipment is one other disadvantage of SFE [157]. Fig 2.4 shows simple SFE equipment.

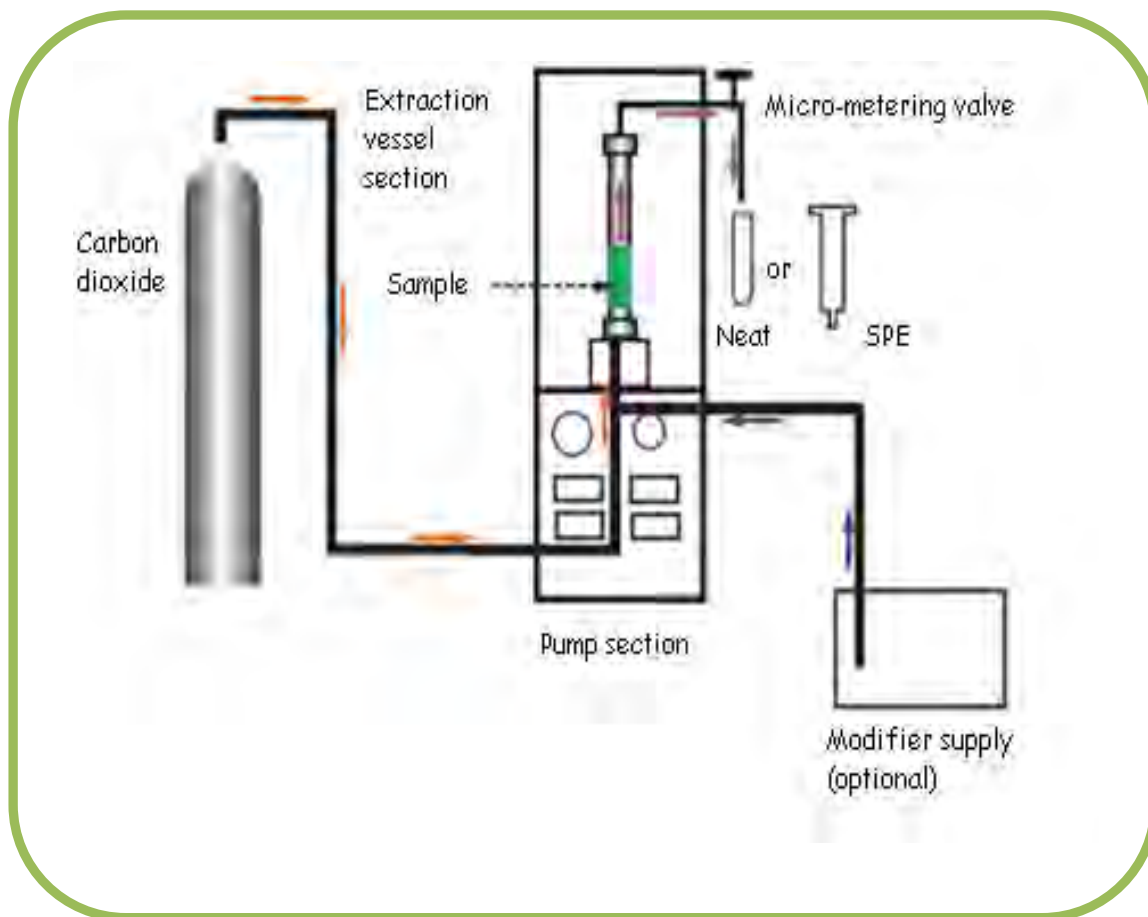


Fig. 2.4 Diagram of a Spe-ed™ Prime SFE by Applied Separations Inc. [158]

Several food applications have been reviewed by Zougagh et al. [159]. Examples include the extraction of pesticides from plants [160] and honey [161], and the determination of PAHs in vegetable oil [162]. Chuang et al. [163] investigated the use of SFE for analysis of pesticides in baby food. A review on SFE was recently detailed by Herero et al. [164].

2.5.3 Pressurized liquid extraction (PLE)

In PLE high diffusion fluids are generated at elevated temperatures and pressures, which enhance the extraction performance as compared to those techniques carried out at or near room temperature and atmospheric pressure [165]. The merits of the use of solvents at temperatures above their atmospheric boiling point are the enhanced solubility and mass transfer properties. Dionex Corporation first introduced PLE in 1995 at the Pittcon Conference, as Accelerated Solvent Extraction Technology (ASE®). PLE is also known as pressurized solvent extraction (PSE) and enhanced solvent extraction (ESE) [102].

Due to the growing interest in the extraction of bioactive compounds and nutraceuticals from plants and herbs; as well as the parallel concern of using technologies that are more “green”, PLE is a more promising extraction technology to fulfill these demands. It has many advantages that make it an excellent substitute to traditional methods such as Soxhlet and solid–liquid extractions. Using elevated temperature and pressure during the extraction does not only improve the extraction yield, but it also decreases time and solvent consumption. The set-up in the PLE equipment provides protection for oxygen and light sensitive compounds [166].

Special caution should be given to thermo-labile compounds, as these may be degraded unless a careful optimization of the extraction parameters is conducted. More research needs to be carried out in the area of using combinatory and hyphenated sample preparation and analytical techniques. These combined procedures result in a substantial decrease in the quantity of sample used as well as volumes of extraction solvents, with

better efficiency and selectivity. So far, PLE has been combined with ultrasound-assisted-extraction and solid phase extraction [165].

PLE is suitable for a wide range of analytes, ranging from polar to non polar. Factors affecting PLE include; the type of solvent, extraction time, particle size and water content of the sample. Optimization strategies involving these factors have been discussed and are illustrated in Fig 2.5. Although the solvent used in PLE is usually organic in nature, pressurized hot water or subcritical water can also be employed [102]. In cases where water is used as the extraction solvent, the technique is referred to as PHWE, sub-critical water extraction or superheated water extraction. In this thesis this technique will be referred to as PHWE.

PLE is usually used for the extraction of high-temperature stable organic pollutants from environmental matrices. Kaufmann and Christen [167] reviewed developments in PLE for natural products. A review of fundamentals and practical use of PHWE was reported by Smith [29]. Very few applications of PLE have been published in the field of bioactive compounds [102]. To obtain those bioactive compounds and natural food ingredients in an environmentally friendly approach, a green extraction approach is required. PHWE is an emerging greener technology compared to conventional extraction techniques (Chapter 3).

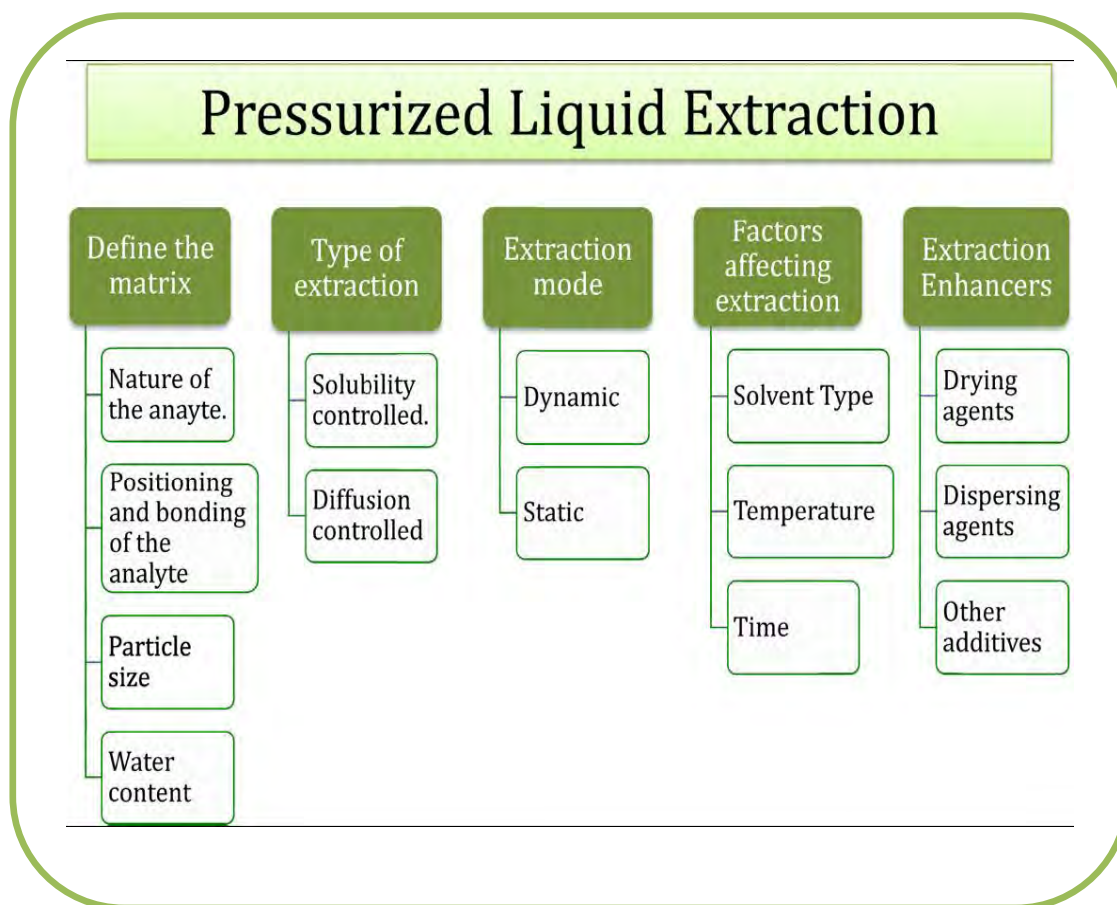


Fig. 2.5 Factors affecting PLE and optimization parameter [102].

Chapter 3 A review on pressurized hot water extraction

3.1 Overview

This chapter presents a detailed literature review of PHWE. It will focus on the following topics;

- Principles of PHWE
- Commercial and in house built PHWE equipment
- Factors that affect extraction with PHWE
- Application of PHWE especially with respect to analytes of interest will be discussed.

3.2 Principles of PHWE

3.2.1 Changes in physicochemical properties of water

The term “pressurized hot water” is used to denote the region of condensed phase of water between the temperature ranges of 100 °C (boiling point of water) to 374 °C (critical temperature of water). Other common terms such as “superheated water”, “near critical water”, “subcritical water”, “high temperature extraction” and “extraction using hot compressed water” have also been used [168]. Figure 3.1 depicts the phase diagram of water.

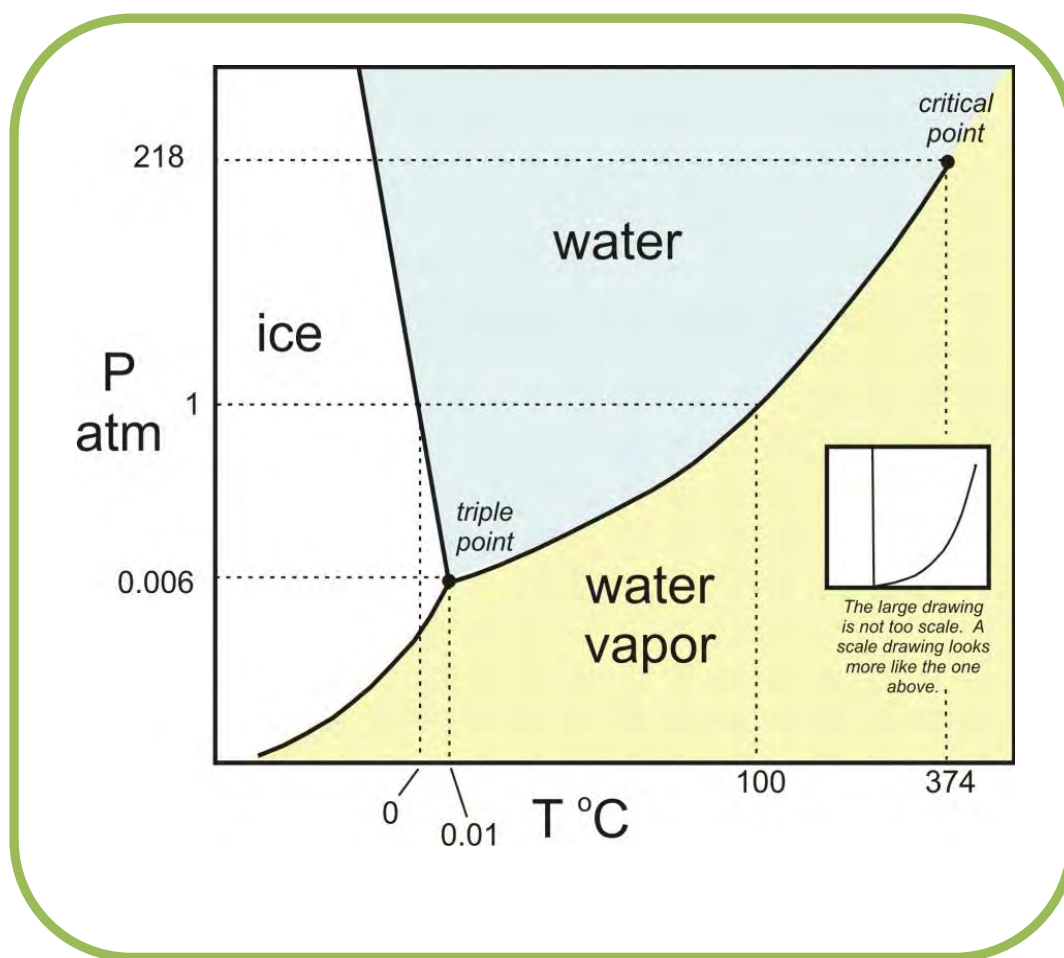


Fig. 3.1 Phase diagram of water [169]

Water is a unique solvent because of its highly hydrogen-bonded structure. At room temperature it has a disproportionately high boiling point for its mass, a high dielectric constant and high polarity. Hence, traditionally water is not considered as a suitable extraction solvent for non-polar or organic compounds at room temperature. However, when the temperature of water is raised above its atmospheric boiling point while maintaining it as a liquid by applying pressure, its physical and chemical properties change quite dramatically [168].

For instance, there is a steady decrease in its permittivity (Fig 3.2), viscosity and surface tension but an increase in its diffusivity characteristics. With enough pressure to maintain water in the liquid phase at elevated temperature, the initial value of the dielectric constant (ϵ) of 80 at 25 °C decreases to 27 at 250 °C and 50 bars. This lies between those of methanol ($\epsilon = 33$) and ethanol ($\epsilon = 24$) at 25 °C. Under these conditions, water behaves like certain organic solvents which can dissolve a wide range of medium to low polarity analytes [170]. In addition to being tunable in terms of properties merely by changing its temperature, water is environmentally friendly and non-toxic and it can easily be obtained and disposed off.

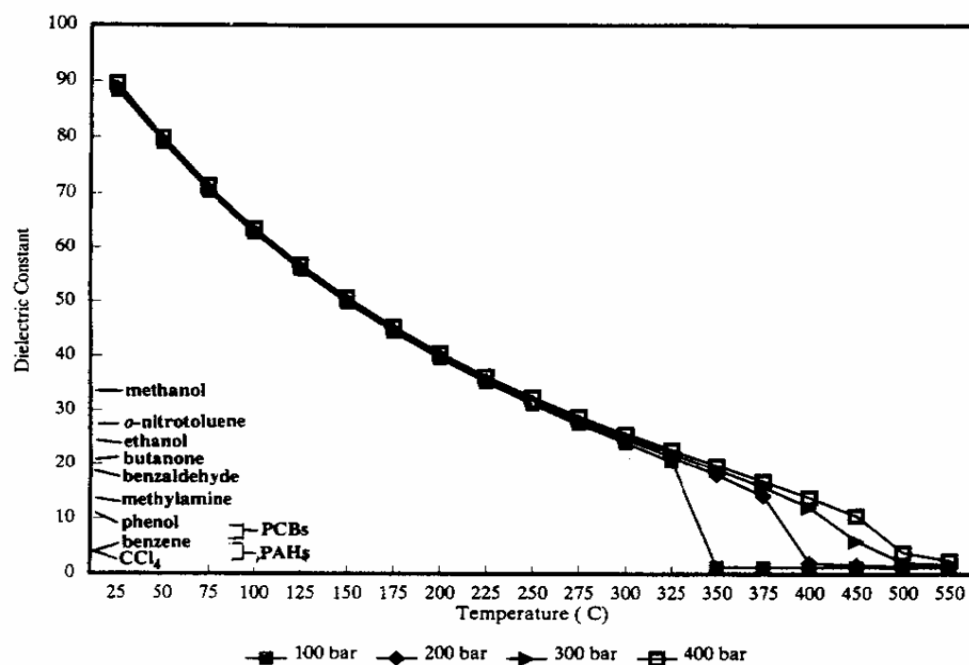


Fig. 3.2 Changes in the permittivity of water with temperature at four different pressures. The drop to low values corresponds to the formation of superheated steam [168].

3.2.2 Solubility of analytes in pressurized hot water

Hawthorne and co-workers studied the changes in the water solubility of typical analytes with temperature. They also studied the interactions between a wide range of polar and non-polar analytes with different potential sorbents [170]. They found that although low temperature water could break inert or dipole bonding between analytes and matrices, higher temperatures were required to break van der Waals forces. The highest

temperatures were needed to break π - π electronic interactions. They demonstrated that increases in solubility of 10^4 - 10^5 are typical for moderately polar and non-polar organic analytes by raising water temperature from 25 to 200 °C [171].

For example, the solubility of the PAH anthracene increases by $\sim 20\,000$ -fold when the temperature is raised from 25 to 200 °C while that of the pesticide chlorothalonil increases by $130\,000$ -fold over the same temperature range. Srinivas and others used the Hansen solubility parameter, (equation 2.2), to assess the solubility of flavonoids in pressurized hot water and water/ethanol mixtures [172]. It is very useful in predicting the solubility of an analyte and its chemical behavior in subcritical water at different conditions. For example, the calculated Hansen solubility parameter of water between 25 °C and 325 °C is shown in Fig. 3.3. The solubility parameter, which is a measure of the intermolecular forces in a pure substance, decreases as a function of temperature. Solvents with similar solubility parameters are miscible [173, 174].

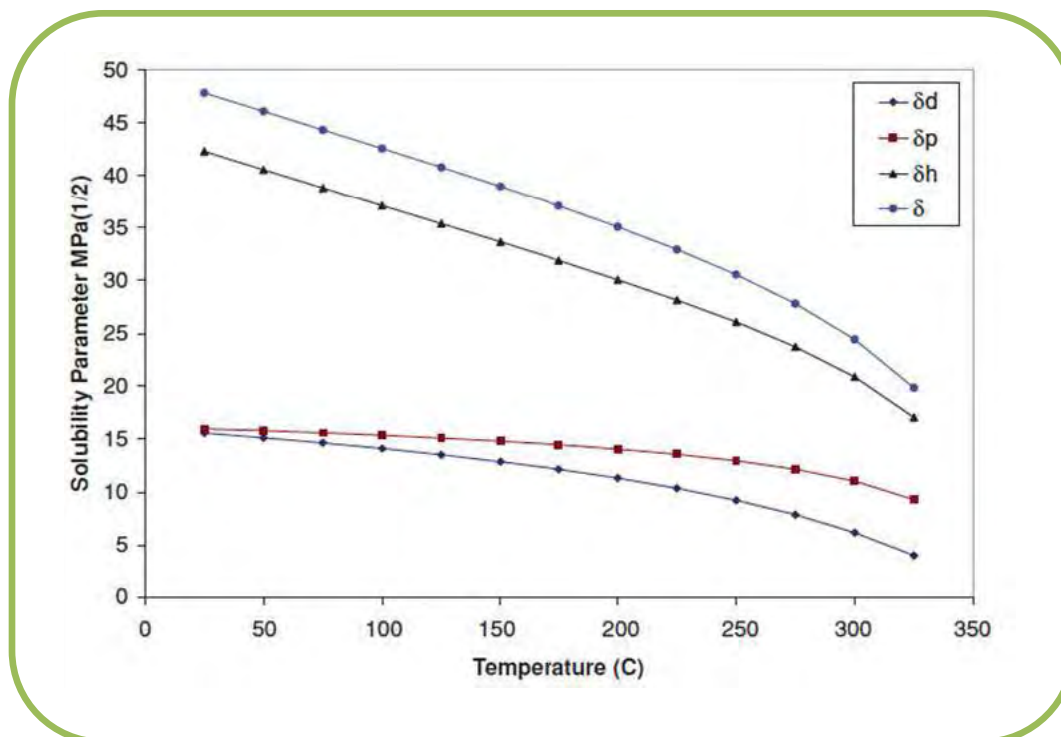


Fig.3.3 Solubility parameter of water [107]

The “hot ball” model originally described for SFE by Clifford and his colleagues [174] and Vandenburg et al. [175] for the extraction of additives from polymeric samples using PLE have been proposed. It was demonstrated that by plotting $\ln(m_1/m_0)$ where (m_1) is the mass of analyte remaining in the particle (in the model: sphere) of radius (r) at time (t) , (m_0) is the initial quantity of analyte and (D) the diffusion coefficient of the analyte in the solvent, a linear portion is given in equation 3.1 [175]. A plot of $\ln(m_1/m_0)$ against time falls steeply initially, after which it becomes linear, and according to equation 3.1.

$$\ln(m_1/m_0) = -0.4977 - (\pi^2 Dt/r^2) \quad (3.1)$$

The physical explanation of the shape of the curve is that the analyte near the surface is rapidly extracted until a smooth falling concentration gradient is established across the particle. The extraction rate is then completely controlled by the rate at which the analyte diffuses to the surface. By plotting the quantity of extracted analyte versus the extraction time for different solvents at different temperatures, the resulting curves showed a good fit to the “hot ball” model [176]. However, the “hot ball” model only takes diffusion into account, which is really a limitation. The most challenging extractions occur when a solid is present as part of the matrix due to the heterogeneous nature of the matrix [13]. The models described can at most be considered as a useful guide when developing new extraction methods based on PHWE. Ionic, hydrogen bonding, dipole-dipole, induction and dispersion forces are important when water is used as an extractant. The presence of these forces between different types of molecules is noted in Table 3.1 [176].

Table 3.1. Intermolecular forces with water as a solvent [176]

Force	Type of atom/molecule/compound	Temperature dependence of the interaction
Ionic	Ionic compounds	Small
Hydrogen bonding	Polar molecule, where nitrogen, oxygen or fluorine atoms are linked by a hydrogen atom	Great
Dipole-dipole	Polar molecules with permanent dipole moments	Great
Induction	Non polar compounds	Small
Dispersion (London force)	All atoms and molecules	Small

These physical parameters and intermolecular forces have an effect on solvation, a process in which the solvent molecules form a layer around the solute molecules. The chemical similarity between solute and solvent facilitates the solvation process. The factors that are most significant in solvation are dipole moments, polarities, capability to form hydrogen bonds, and sizes of the molecules. The solvation of an analyte is essential in all extractions. If the analyte is not solvated in the extraction solvent it will not be extracted [177].

3.3 PHWE equipment

There are two main set-ups for PHWE, static and dynamic instruments. They both require pressure to maintain water in the liquid state and temperature for selective extractions.

3.3.1 Static set up

Static PHWE is a batch process with one or several extraction cycles that allows replacement of solvent in-between. During extraction, the solvent is kept in the extraction cell as the extraction takes place. The basic instrumentation for commercial static PHWE equipment is shown in Fig. 3.4.

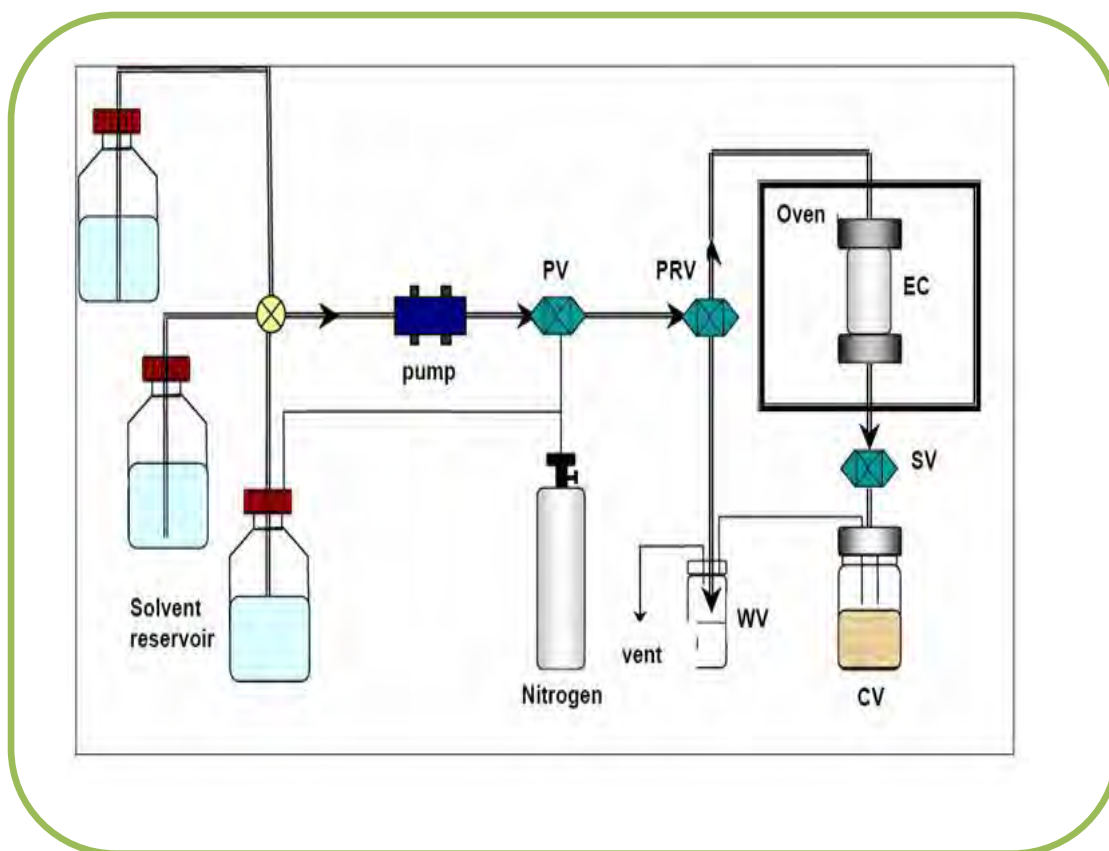


Fig.3.4 Schematic of a PHWE system [105].

One or several solvent reservoirs can be used to work with the selected solvent (water) or mixture of solvents (water/ethanol, for example). When different reservoirs are available, a solvent controller is also provided. A high-pressure pump is used to pump the solvent into the extraction cell. A filter paper is inserted into a stainless steel extraction cell (EC) followed by the sample, sometimes mixed with a dispersing or drying agent, if needed. However, an advantage of PHWE is that drying of the sample is usually not necessary. The cell is either loaded on a carousel or automatically placed in the oven. For simpler equipment, the cell is manually placed into the oven. Commonly, a static valve (SV) in combination with a pressure valve (PV) and a pressure relief valve (PRV) controls the

pressure in the sample vessel during the static extraction by adding more solvent to the cell or by opening the static valve as appropriate [105].

The operating procedure consists of several steps:

1. Pre-heating step to reach thermal equilibrium:- during this heating, thermal expansion of the solvent occurs and causes an increase in pressure within the cell.
2. Static extraction:- once the target values are achieved, extraction is performed during a selected time, typically 5-30 min.
3. Solvent replacement when extraction cycles are used:- after static time, part of the solvent in the extraction cell is replaced with fresh solvent, to start the next extraction cycle.
4. System purge:- after the last cycle, the sample cell is purged with an inert gas such as nitrogen to remove the remaining solvent from the cell and the lines to a collecting vial, (CV).
5. Pressure release:- system is depressurized at atmospheric conditions (through the vent) to the waste vial (WV).

By using this procedure, individual or sequential extractions can easily be repeated with new solvent or temperature conditions while purging in the same or a different CV.

In the static extraction mode, extraction efficiency strongly depends on the partition-equilibrium constant and solubility of analytes at elevated temperatures. Thus, highly

concentrated samples or low solubility analytes may lead to incomplete extraction due to the limited volume of water used [178]. The extraction process could be explained in two stages as in SFE. It starts as solubility-controlled followed by a desorption-controlled phase [179]. In desorption-controlled sample matrices, there are usually strong interactions between matrix and analytes, or long diffusion paths for the analytes to pass through the sample matrix. In this case the temperature of the solvent and particle size might be critical factors to enhance the extraction efficiency. Increasing the temperature and or reducing the particle size will most likely decrease extraordinarily long extraction times. For the solubility-controlled sample matrices, the analyte–matrix interactions are quite weak. The extraction rate mainly depends on the partitioning of the analyte between the matrix and the extraction solvent [180,181]. In this case the extraction yield is enhanced by replacement with fresh extraction solvent frequently (Fig. 3.5) [102].

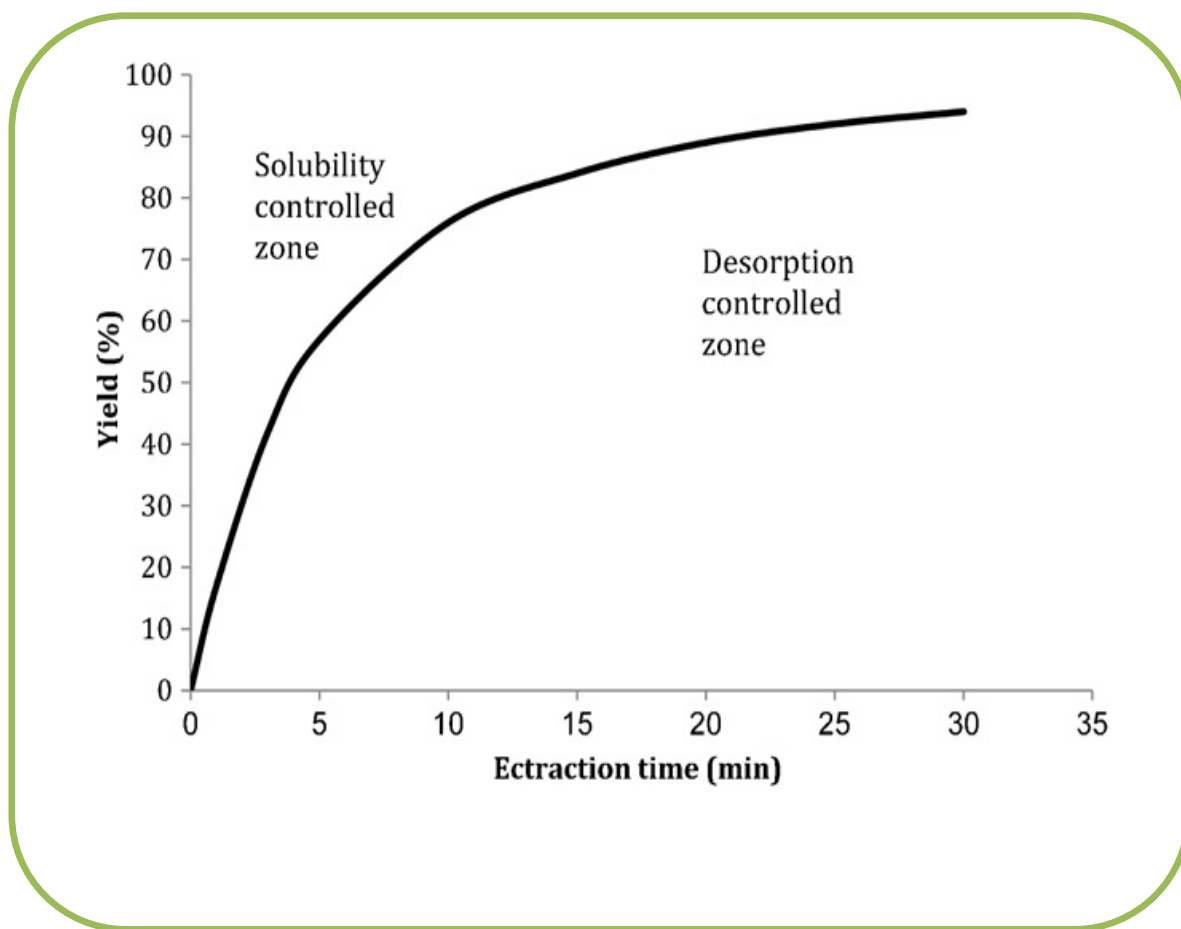


Fig. 3.5 Extraction yield (%) against extraction time (min) [102].

3.3.2 Dynamic extraction mode

In the dynamic setup, water is continuously pumped through the sample cell with high-pressure pumps. Compared to static extraction, the dynamic PHWE enhances the extraction by forcing water through a narrow sample cell at high pressure. The equilibrium is displaced completely as fresh solvent is continuously pumped through the sample thus achieving complete extractions. This generally enhances extraction yields while degradation of temperature sensitive analytes may be avoided [182,183]. Both extraction

time and flow rate are important parameters for the optimization of dynamic PHWE. The extraction time strongly depends on the extraction temperature, nature of matrix and analytes. Using dynamic PHWE, it was observed that an extraction time of 20 min at 100 °C gave higher yields of stevioside and rebaudioside A from *Stevia rebaudiana* as compared to heating under reflux for 60 min [184, 185].

There are currently no commercially available dynamic PHWE systems in the market, even though the ASE-350® provides both static and dynamic modes in the same run. This is featured by the ability of the instrument to introduce fresh solvents during the extraction process. In the static mode, the flow rate is constant so as to deliver water. A wide range of extraction temperatures (from room temperature to 200 °C) and the pressure (35–200 bars) can be applied in the dynamic PHWE [177].

3.4 In house built PHWE equipment

Typically, an in house built system consists of a water supply; a pump for delivering the solvent; a heater for heating the solvent; a pressure vessel which serves as an extraction cell; a means to control the pressure in the system and a collection vessel for the extract. Fig. 3.6 depicts a laboratory assembled PHWE system [170].

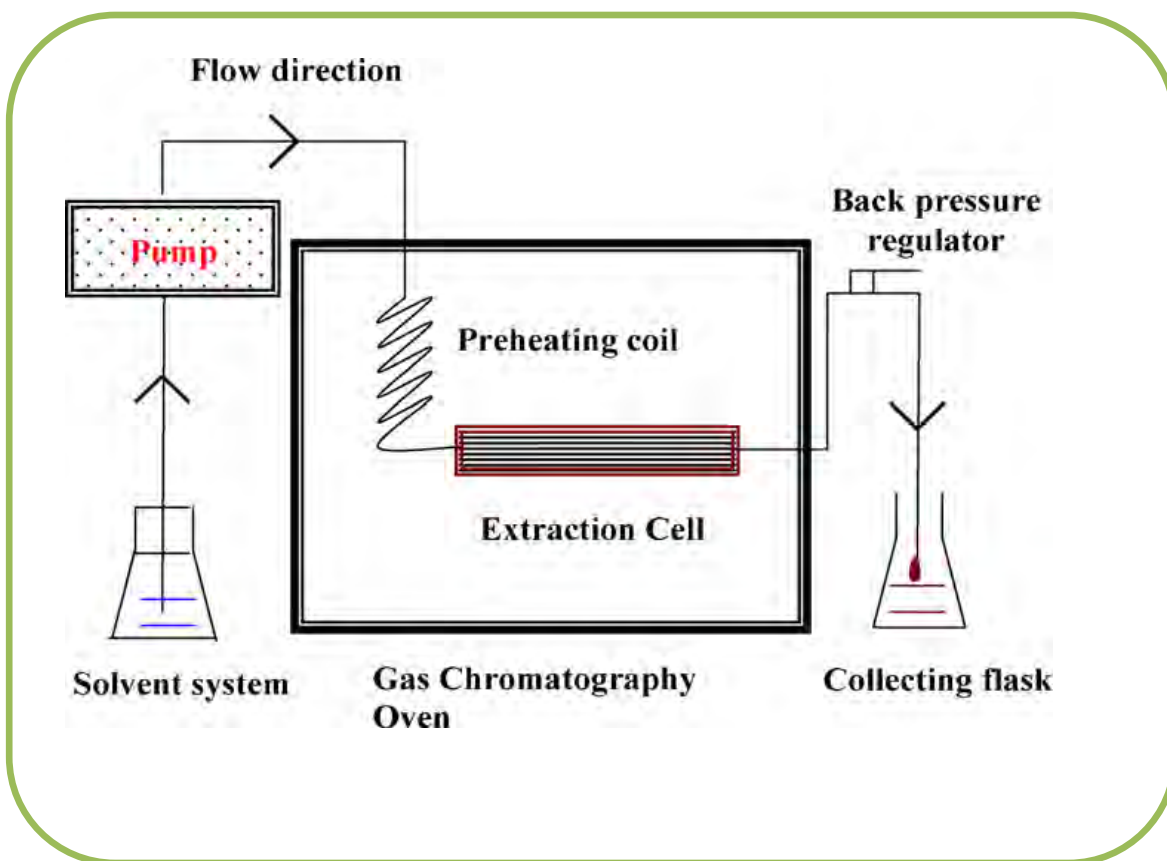


Fig. 3.6 A schematic diagram of a laboratory in house built PHWE system [181].

The set-up consists of a stainless steel preheating coil to ensure that water is at its operating temperature before entering the stainless extraction cell. The extraction is carried out at an elevated temperature maintained by a gas chromatograph oven. The extraction processes is conducted at pressures between 10 and 60 bars. The outlet flow is controlled by a back pressure regulator. For certain set-ups, a second pump is used to deliver chloroform or dichloromethane into a fused silica lined tee placed in the oven between the extraction cell and collection valve to prevent deposition of analytes when

water cools during collection. A cooling trap may be used to cool the solvent coming out of the extraction cell to room temperature [186].

There are several ways to collect the analytes from PHWE. Solid-phase trapping [187] and collection in organic solvent [188] are applied in most cases. Analytes can also be collected by microporous membrane liquid-liquid extraction (MMLLE) [189], hollow fibre microporous membrane liquid-liquid extraction (HF-MMLLE) [190] or anion exchange discs [191]. A study of the suitability of SPE, LLE, flat sheet membranes and hollow fibre membranes for trapping in PHWE showed that the choice of system depends on the application [192].

Solid-phase microextraction (SPME) [193] and stir bar sorptive extraction (SBSE) [194] have also been applied with PHWE. If PHWE is coupled to LC, GC or LC-GC [195,196], trapping of the analytes is done on-line with one of the techniques mentioned above (except LLE). For fast screening purposes with high sample throughput, PHWE has been coupled with enzyme immunoassay [197]. Also, capillary electrophoresis coupled to mass spectrometric detection has been applied in the analysis of PHWE extracts. If destruction of analytes is desired, as in soil remediation, it is possible to couple PHWE on-line with supercritical water oxidation [198].

3.5 Parameters affecting extraction

The main parameters that influence the selectivity and extraction efficiency of PHWE include temperature, pressure, extraction time, flow rates and modifiers/additives. The geometry of the extraction cell and flow direction has little effect on the recovery of the analytes from sample materials. Although matrix and other effects play a role, many of these are less critical in PHWE due to the harsh extraction conditions (high temperature), particularly for non-polar analytes [199].

3.5.1 Temperature

Of the factors affecting PHWE, temperature has the greatest effect. The extraction speed, efficiency and selectivity are affected simply by adjusting the temperature. The ionic, hydrogen bonding and dipole–dipole interactions between the water molecules decrease with increasing temperature thus lowering the dielectric constant (Fig 3.7) [200], although hydrogen bonding of some degree is still present in supercritical water. Increase in temperature disrupts the solute-matrix interactions and increases the capacity of solvents to solubilize the analytes.

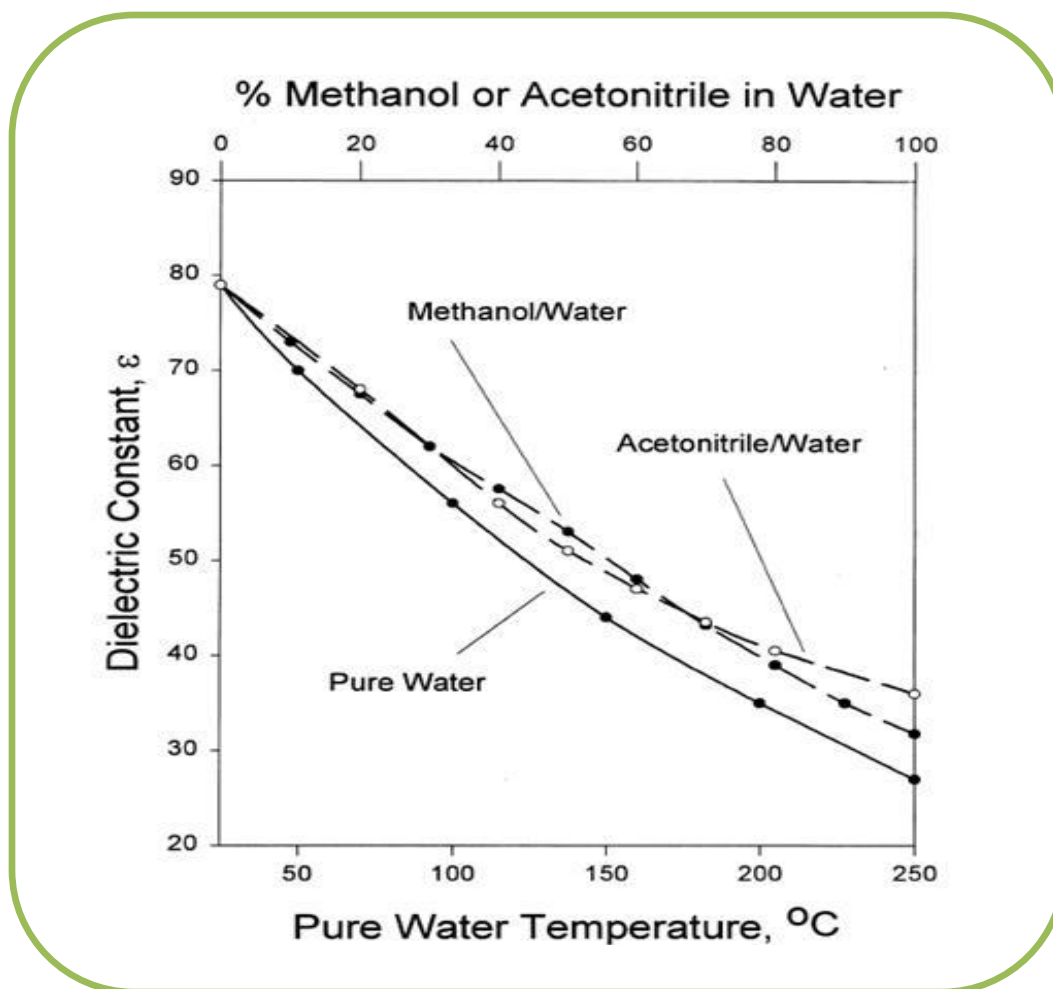


Fig. 3.7. Dielectric constant of water and methanol/water and acetonitrile/water mixtures [200].

The change in viscosity which is much pronounced during the first 100 °C increase in temperature from ambient conditions enables better penetration of the matrix particles. Also, the decrease in surface tension due to high temperatures allows the water to better 'wet' the sample matrix. These factors, and the improved mass transfer, enhance the recoveries. For polar analytes relatively low extraction temperatures are recommended (100–150 °C), whereas for moderately and low-polar analytes such as pesticides

temperatures of 200–300 °C are preferred. Class selective extraction can be obtained with temperature programming [201].

The increase in temperature always results in the increase of the rate of reaction. Since the temperature is a measure of the kinetic energy of the system, a higher temperature implies higher kinetic energy of the molecules and therefore more collision per unit time. A rule of thumb for most chemical reactions; for each 10 °C increase in temperature the reaction rate will double. Therefore, in theory, very high extraction temperatures could be recommended for the extraction of non-polar analytes. In practice, however, several factors limit the maximum temperature. It is not practical to apply very high extraction temperatures (much over 300 °C) due to instrumental challenges like corrosion and leakage [202].

The analytes may also degrade or otherwise react at higher temperatures. The selectivity in the extraction is often lost at high temperatures. Substantial quantities of matrix and interfering compounds are extracted along with the target analytes. This may lead to blockages and pressure increase inside the equipment. Additional clean-up of the extract may also be needed. Degradation of the analytes and co-elution of matrix compounds in analysis is a particular challenge when food and plant materials are extracted, and it is often then advisable to use the lowest possible extraction temperature [203].

3.5.2 Pressure

The main advantage of applying pressure during the extraction is that a temperature above the boiling point can be used while the solvent maintains its liquid state. The use of elevated pressure at high temperature and reduced solvent surface tension helps to force the solvent within the matrix pore to contact the analyte and extract them. This results in disruption, which could enhance the mass transfer of the analyte from the sample to the solvent. High pressure during the extraction controls challenges related to air bubbles found within the matrix that hinder the solvent from reaching the analyte. These conditions boost the analyte solubility and desorption kinetics from the sample matrix [204]. However, the effect of pressure on recovery of most substances is usually negligible as long as the physical state of water is not changed [205]. The recovery of organic pollutants from solid environmental samples was suggested to have little dependence on pressure [206]. Similarly, varying pressure did not improve the recovery of essential oils from medicinal plants and ginsenosides from American ginseng [207].

If the extraction with steam is preferred, then the pressure should be kept low. In practice, the back pressure of the equipment places a lower limit on the pressure in PHWE. Since the relative permittivity of water increases with pressure, a large pressure increase may decrease the recoveries of non-polar analytes. Steam has proven to be more effective than liquid water in the extraction of non-polar organic analytes, for two reasons [208]. Firstly, the relative permittivity of steam is lower than that of liquid water at the same temperature. Lower relative permittivity favours the extraction of non-polar analytes. Secondly, steam spreads more uniformly through the sample in the extraction vessel

because of its lower viscosity, and it diffuses more effectively than liquid water. Thus undesired channelling leading to lower recoveries is avoided. On the other hand, the capacity of steam to dissolve analytes is lower due to its lower density. Thermal desorption is dominant with steam. Figure. 3.8 shows the vapour pressure curve of water [209].

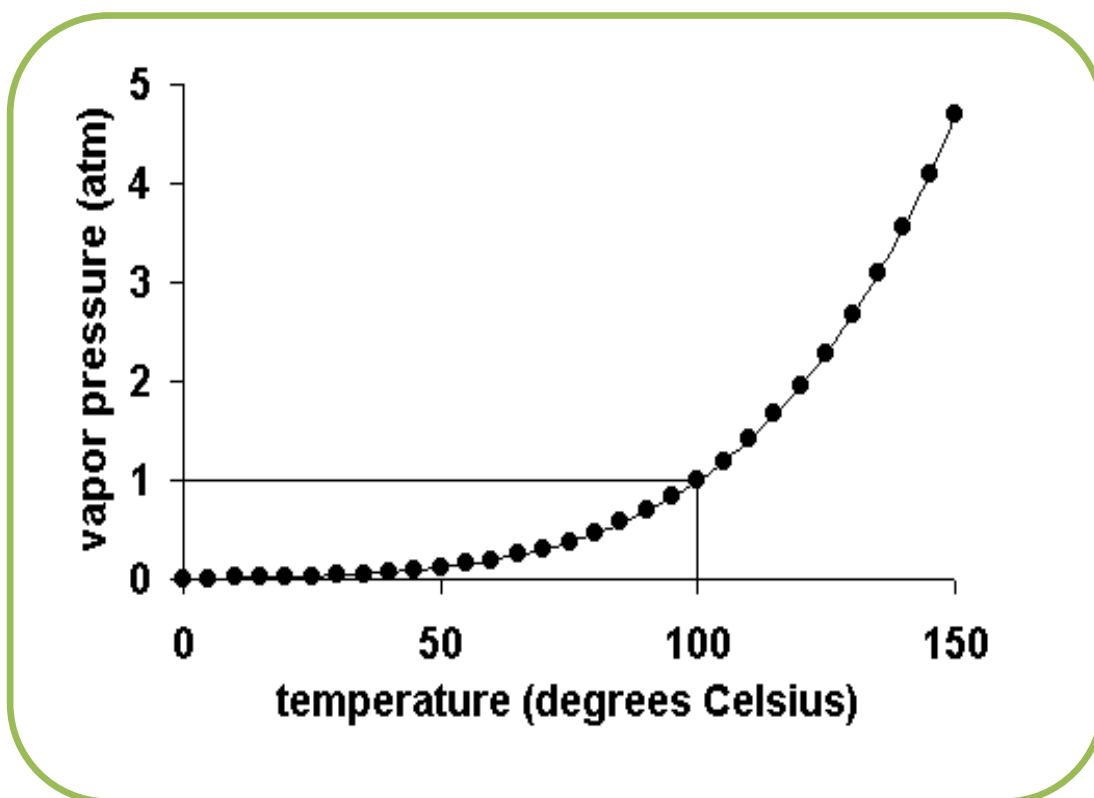


Fig. 3.8 Vapour pressure curve of water [209].

3.5.3 Flow rate and extraction time

Flow-rate affects the recovery in PHWE if the extraction is solubility restricted, as it is when analytes have low aqueous solubilities. It determines the extraction yield obtainable

per unit and residence time for extracted analytes in the extracting solvent. With respect to thermally labile analytes, it is necessary to thoroughly optimize the flow rate in order to assure quantitative extraction yields without causing degradation of the analytes and excessive dilution of the sample extract [210]. Generally, fast flow rate results in a shorter extraction and residence time for the extracted analytes. However the dilution of the sample extract may lead to decreased sensitivity of detection of analytes [211].

3.5.4 Selectivity

The solvating properties of water and accordingly also the selectivity of pressurised hot water extraction are mainly controlled by temperature. For example, the solubility of alkanes in water increases rapidly with temperature, whereas the solubility of inorganic analytes decreases. Analytes of different polarity can therefore be selectively extracted through use of different extraction temperatures [212]. If the target analytes are thermally stabile, class-selective extraction can be carried out by increasing the temperature and letting the more thermo-labile analytes in the sample degrade. Selectivity can also be enhanced by using special solid phase sorbents for the trapping of the analytes or through choice of suitable trapping solvent. In PHWE, selectivity is mainly achieved by carefully optimizing the temperature, as previously discussed [212].

3.5.5 Modifiers and additives

The addition of some organic, inorganic modifiers and additives may enhance the solubility of analytes in water as well as increase the interactions of target analytes with water. They can also alter the physicochemical properties of water at elevated temperature. A higher quantity of natural sweetener from licorice (*Glycyrrhiza glabra*) roots was achieved by PHWE with dissolved ammonia (0.01%, w/v) [213]. PHWE with extraction fluids containing 5% ethanol was also reported to enhance the extraction of anthocyanins in red cabbage. The degradation of compounds were reduced by micelle-mediated extraction (MMPHWE) with Triton X-100 compared with PHWE without the use of surfactant [214].

3.6 Comparison with other extraction methods

The benefits and disadvantages of each technique discussed in this thesis are also listed in Table 3.2. Among the benefits of PHWE are that no harmful organic solvents are needed and selectivity for the analytes is high. Although PHWE equipment is not commercially available, this is not particularly serious because the equipment can easily be constructed in the laboratory.

Table 3.2. Comparison of Soxhlet extraction, PHWE, SFE, PLE and MAE [215, 216, 217].

		Traditional technique	Instrumental extraction techniques			
Extraction technique		Soxhlet	PHWE	SFE	PLE	MAE
Typical extraction time		4-48 h	5-30 min	30-90 min	12-20 min	30-60 min
Typical solvent		Acetone-hexane, acetone-dichloromethane, dichloromethane, toluene, methanol	Water	CO ₂ /CO ₂ +modifier	Acetone-hexane, acetone-dichloromethane	Acetone-hexane
Typical solvent consumption (mL)		300	A few mL for elution of analytes	8-50/no solvent needed in SFE-GC	15-40	25-50
Selectivity for analytes classes		Non-selective	Selective	Slightly selective	Non-selective	Non-selective
Selectivity for sample matrix		Some selectivity	Selective	Selective	Non-selective	Non-selective
Benefits		Simple well known procedure, easy to carry out, cheap equipment, also automated	No organic solvent needed, wet samples can be extracted without drying	No or little organic solvent needed, also automated	Fully automated	Generally 14 vessels extracted simultaneously, also automated
Disadvantages		Time consuming, a lot of manual work, large consumption of organic solvent	No commercial equipment available (ASE, SFE and MAE)	Expensive equipment, need for modifiers when CO ₂ the extractant	Expensive equipment, blockages, frequent need for additional clean-up	Need for additional clean-up to remove the matrix

3.7 Application of PHWE

In recent years, PHWE has gradually become a useful option for the isolation of bioactive and nutritional compounds from plants and food materials. In addition, it has been employed in the extraction of organic contaminants from foodstuff for food safety analysis. [218]. This increasing interest is mainly due to the fact that PHWE can be automated, results in reduced extraction time and solvent consumption and its set-up suits analytes that are oxygen and light sensitive. PHWE requires minimal sample pre-treatment especially for non-fatty sample; only homogenization or drying [219].

3.7.1 Extraction of nutraceuticals and bioactive compounds from food and herbal materials

The term “nutraceutical” is described as “any substance that may be considered a food or part of a food, and provides medical or health benefits, including the prevention and treatment of disease”. Nutraceuticals are pharmaceuticals with physiological or metabolic function [220]. They may include dietary fibers, different types of phenolic compounds and antioxidants, polyunsaturated fatty acids, amino acids, proteins and minerals.

Diets rich in nutraceutical compounds have been attributed to many health promoting effects, such as the reduction of the risk of developing coronary heart diseases [221], cancer and hypertension [222], diabetes and inflammatory processes [223]. Determination of nutraceutical compounds in plants is important to facilitate research and development in

relation to nutrition and health effects. Extraction process represents a critical factor in this development [102].

The use of pure water for extraction mimics the tradition herbal preparations which usually involve sequential steps with boiling in water. The extraction efficiencies of the marker compounds from *Gastrodia elata* and *Stevia rebaudiana* using PHWE were found comparable or higher than heating under reflux using water [224]. Both marker compounds GA and VA present in *Gastrodia elata* were extracted by PHWE as an alternative extraction method to the traditional heating under reflux. PHWE was also employed for the extraction of volatile components from botanicals at optimized conditions. The extraction of volatile essential oil from *Cuminum cyminum* L. at a temperature of 150 °C by PHWE gave comparable yields with reference to Soxhlet extraction and steam distillation (hydrodistillation) [225]. Comparable results were also reported for PHWE, hydrodistillation and Soxhlet in the extractions of *Borneol* [226] and *Pulegone* [227] in plant materials. Thus, PHWE is offered as a fast, clean and high efficiency extraction method for volatile components present in plants.

A large group of phenolic compounds are flavonoids which possess antioxidative properties. A number of methods employing PHWE for extraction of flavonoids has been published. For instance, Ollanketo and others [228] have extracted rosmarinic and carnosinic acids as well as carnosol and methyl carnosate sage employing PHWE at 100 °C [229]. Ibanez et al. [230] also extracted carnosol, rosmanol, carnosic acid, methyl carnosate, cirsimaritin and genkwanin from rosemary leaves employing subcritical water

at temperatures between 25 and 200 °C. The results demonstrated that it was possible to tune the selectivity of the extraction by varying the temperature.

PHWE has been employed for the extraction of anthocyanins from berries [231], red onions [232] proanthocyanidins from grapes and grape pomace [233-237]. Generally, anthocyanins extractions were performed at temperatures between 120 and 160°C.

The total sugar present in defatted rice bean was determined to be the highest using PHWE at 200 °C [238]. In the study of the extraction of catechins and proanthocyanidins from dried grape seeds, the results were found to be comparable to conventional extraction with 75% methanol [239]. Using PHWE, five different capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, an isomer of dihydrocapsaicin, and homodihydrocapsaicin) present in peppers were successfully isolated at 200 °C and quantified by HPLC before the extraction yield decreased at higher applied temperatures [240]. Other applications of PHWE include alkaloids [241], terpenes from Ginkgo biloba leaves [242], lignins [243], manitol from olive leaves [244] and essential oils from traditional Chinese medicines [245].

In this thesis, both the extraction and degradation of the thermo-labile alkaloids present in goldenseal; hydrastine and berberine (Fig. 3.9), employing PHWE were investigated. In another study PHWE was combined with sonication for the extraction of alkaloids in goldenseal.

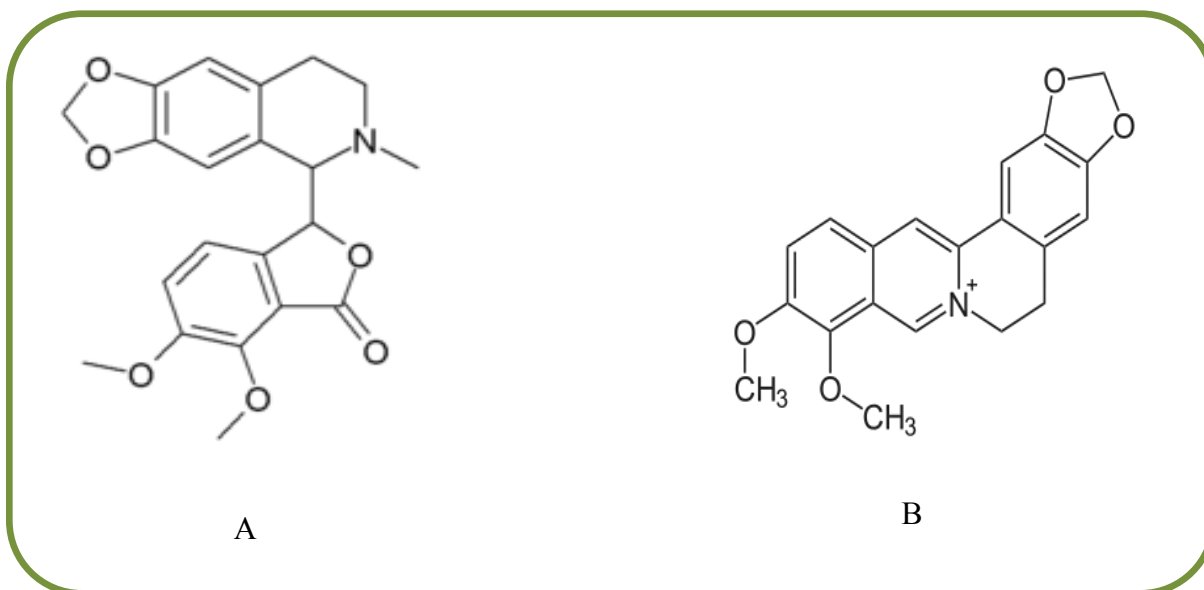


Fig. 3.9 Chemical Structures of (A) hydrastine and (B) berberine [246].

3.7.2 Removal of chemical contaminants from food and herbal plants

Chemical contaminants are chemicals or compounds that can potentially harm the health of humans, wildlife and aquatic life. These include pesticides and naturally occurring toxicants such as aflatoxins [247]. They display a high acute toxicity since they are mutagenic, carcinogenic, and are also endocrine disruptors [248]. The International Agency for Research on Cancer (IARC) defined aflatoxins as Group 1 carcinogens to humans [249] while the Stockholm Convention described pesticides as persistent organic pollutants (POPs) [250].

Pesticides include many chemically diverse groups, such as organophosphorus compounds,

organochlorinated compounds, carbamates, pyrethrins, pyrethrinoids, and phenoxyacids [251]. On the other hand the most important members of aflatoxins are AFB₁, AFB₂, AFG₁ and AFG₂, as well as two metabolic products, M₁ and M₂ [252]. Chemical contamination in medicinal herbs has become a worldwide concern as the consumption of herbal products significantly increased in recent years. To minimize exposure of humans to many of the chemicals mentioned above, MRLs of contaminants in food have been set by the EU and Public Health Agencies across the world. The European commission (EC) has established the maximum acceptable level of aflatoxins in corn, groundnut, dried fruit and cereals for direct human consumption as: 4 ng g⁻¹ for total aflatoxins (AFB₁, AFG₁, AFB₂, AFG₂) and 2 ng g⁻¹ for AFB₁ alone [253]. The monitoring of aflatoxins and pesticide residues demands the development of fast and effective methods with minimal sample preparation.

PHWE has proven to be feasible to extract pesticides residues from the skin of grapes [189] and in wheat flours and their products [254]. Seven pesticides (flutolanil, simazine, haloxyfop, acifluorfen, dinoseb, picloram, and ioxynil) were extracted from four Mediterranean summer fruits (peaches, melon, watermelon, and apricot) employing pressurized hot water at 60 °C and 1500 psi, followed by an SPE clean-up step [255]. Luthje et al. 2005 [256] also developed a new approach for pesticides determination in grapes. In the study, an on-line coupling PHWE MMLLE-GC-MS for the analysis of pesticides (procymidone and tetradifon) in grape skin was utilized. The introduction of MMLLE as a trapping step after PHWE helped to clean and concentrate the extract before on-line transfer to the GC.

In this thesis, a MIP designed to target the interfering chlorophyll was coupled to PHWE to perform an in-cell clean-up during selective extraction of organochlorine pesticides residues in various edible and medicinal plants of the Okavango Delta, Botswana. Examples of pesticides in question include α -Benzenhexachloride (α -BHC), heptachlor, Aldrin, trans-chlordane, 4,4'-DDD, 4,4'-DDE, 2,4'-DDD, dichlorvos, and hexachlorobenzene (HCB) (see structures in Fig. 3.10).

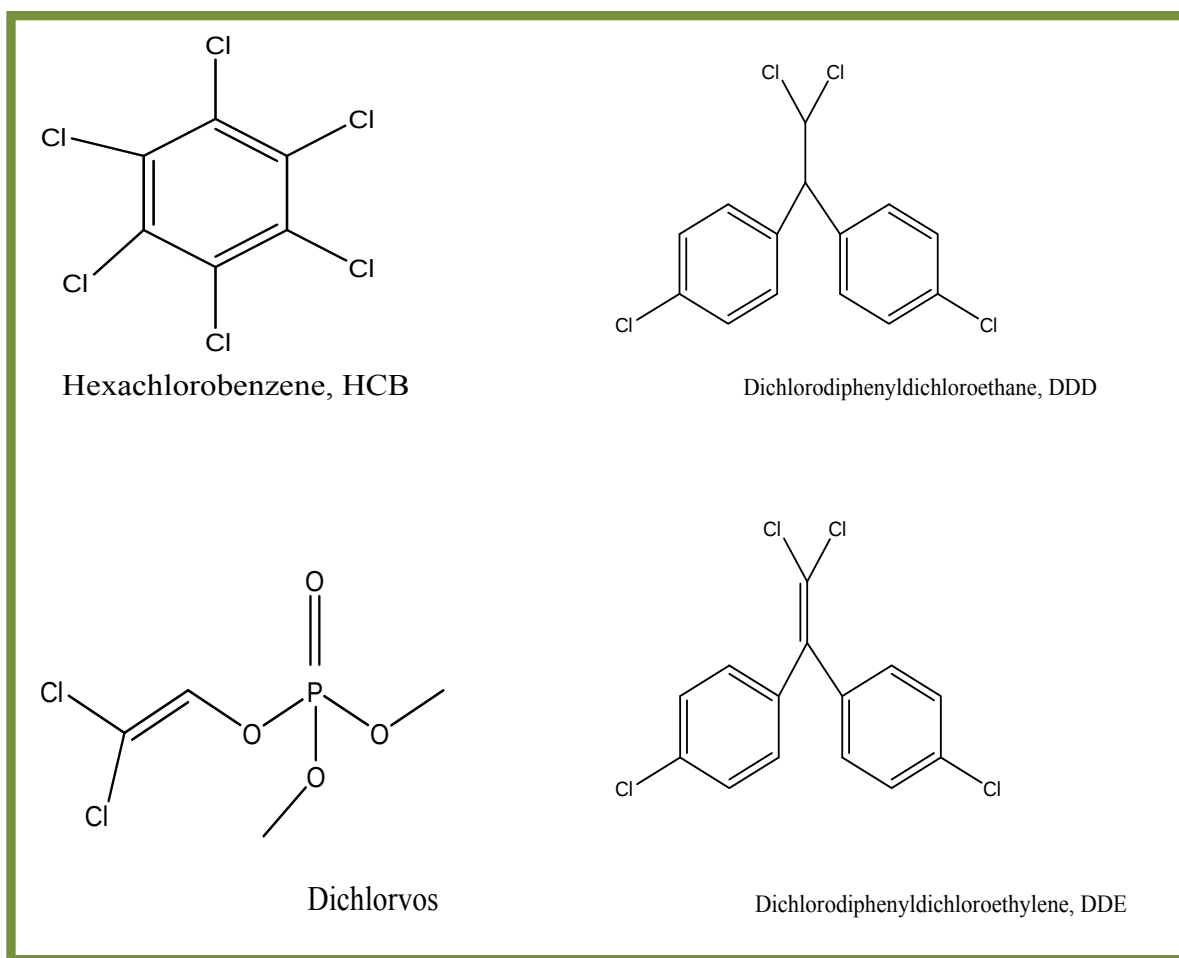


Fig. 3.10 Examples of organochlorine pesticides

In another study, a class selective aflatoxin MIP was custom synthesized for in-cell clean-up and pre-concentration of aflatoxins AFB1, AFB2, AFG1 and AFG2 (see structures in Fig. 3.11) during extractions with PHWE in various medicinal plants of the Eastern Cape, South Africa. PHWE was modified to perform extraction and in-cell clean-up followed by desorption in serially connected extraction cells.

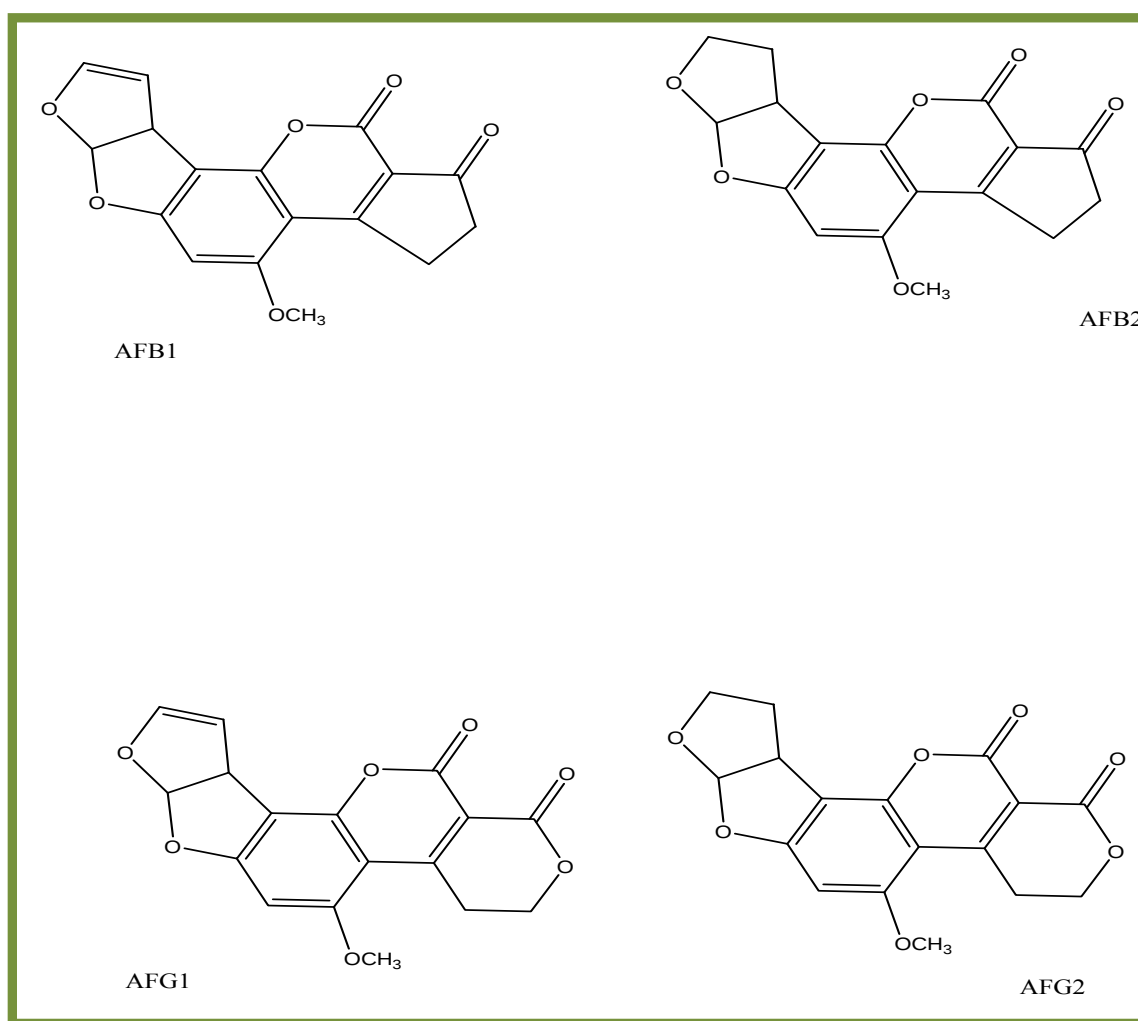


Fig. 3.11 Structures of aflatoxins

The other applications of PHWE in this thesis were;

- ❖ Desorption of pesticides adsorbed on the nanofibers sorbents for quantification employing PHWE.
- ❖ The efficiency of water under subcritical conditions as the sole leaching solvent in the removal of templates (chlorophyll, quercetin and phthalocynine) from MIPs was evaluated for the first time.

3.7.3 Environmental samples

Application of PHWE by Hawthorne and co-workers [257] to extract some PAHs from the soil under appropriate controlled experimental conditions has demonstrated the feasibility of using polar solvent such as water as an extraction medium. A comparison of the recoveries of PAHs by the conventional Soxhlet extraction, PHWE, SFE and PFE methods showed that the qualities of the extracts were rather different. The colour of the PHWE extracts was lighter than the extracts obtained from the other methods. This observation was due to n-alkanes which were more readily extracted by other methods as compared to PHWE [258]. The solubility behaviour of three PAHs, namely the acenaphthene, anthracene, and pyrene, in superheated water was studied at temperatures from 50 to 300 °C in order to understand the mechanisms of extraction in PHWE. The extraction yields of PAHs were also found to be comparable to the other reference methods such as Soxhlet extraction [259].

A review on the usage of high temperature pressurized water (both in sub- and supercritical conditions) in the presence of oxidants such as hydrogen peroxide, oxygen,

persulfate was reported for the extraction, destruction and oxidation of PAHs from soil samples [260]. The subcritical fluid extraction method was also highlighted as one of the remediation technologies specifically for PAH-contaminated soils in a recent review [261]. PHWE was also demonstrated as a feasible option for other classes of compounds such as nitrogen-based pollutants, dioxins [262], brominated based compounds [263], chlorinated organic pollutants [264], organic liquid products [265] and surfactants [276] present in environmental samples. At 200–400 °C, PHWE extracted these analytes which are usually bound tightly to the sample matrix in either dynamic or static mode. PHWE was optimized for the extraction of alanine, aspartic acid, glutamic acid, glycine, serine and valine in soil samples over the temperature range of 30–325 °C at pressures of 17.2 or 20.0MPa [267]. At 30 °C (at 17.2 MPa) no amino acids were extracted as they might be too strongly bounded by the soil matrix to be extracted at such a low temperature. The extraction efficiencies for glycine, alanine, and valine increased with increasing extraction temperatures from 150 to 250 °C (at 17.2 MPa). However, amino acids were not detected in extracts collected at 325 °C (at 20.0 MPa) due to amino acid decomposition at this temperature [268].

3.8 Recent developments of PHWE and future perspectives

One limitation of PHWE is that the extracts end up in large volumes, especially with the dynamic mode which is preferred over static. This possess a challenge to analysis of analytes present at very low concentrations as they become very dilute hence pre-concentration is required. Furthermore, if GC is employed for analysis, the water must be removed post-extraction, as it may not be compatible with the system. This is usually

achieved by blow drying with nitrogen or freeze drying. One interesting approach is the building of a multi-unit system that combines sub- and supercritical techniques for extraction and drying in one step. Ibáñez and coworkers developed a new process combining PHWE with particle formation on-line using SC CO₂ as a dispersant and hot nitrogen for drying the extractant. This is a novel way of obtaining dried complex extracts from natural sources in one step. This process has been patented as WEPO[®] (Water Extraction and Particle formation On-line) [269], and it is based on CAN-BD (Carbon Dioxide assisted Nebulization with a Bubble Dyer[®]) particle formation process [270]. With the WEPO[®] process, complex dried extracts that preserve their intact antioxidant activity were obtained from oregano and rosemary plants, as fine powders with particle sizes lower than 140 µm length.

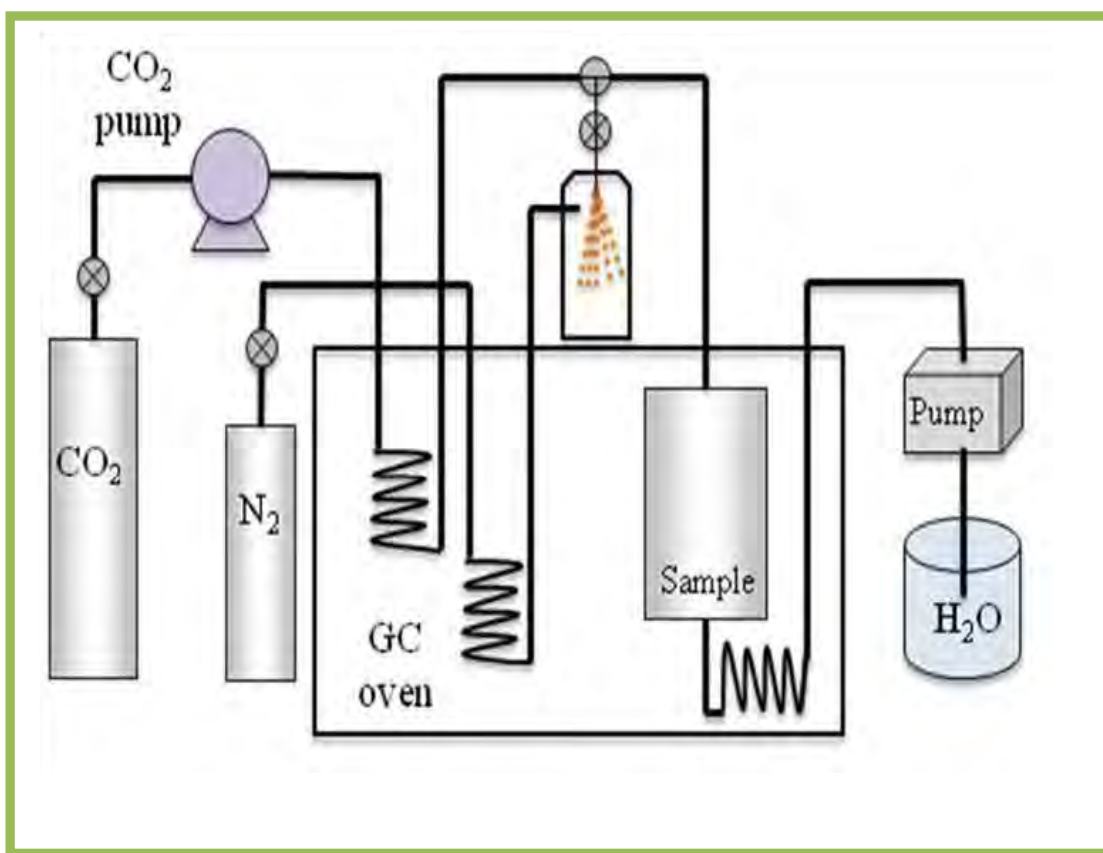


Fig. 3.12 Schematic of WEPO home-built equipment [269].

Another interesting approach is the coupling of PHWE with enzymatic hydrolysis. For example, thermo stable β -glycosidase was employed with subcritical water to catalyze hydrolysis of quercetin glycosides in onion waste [271]. The enzyme β -glycosidase was shown to maintain its activity at 95 °C. Compared to the conventional hydrolysis which is based on methanol and HCl at 80 °C, enzyme hydrolysis was preferred [272]. Combining SWE with sonication was used for the extraction of volatile oils from *Lithospermum erythrorhizon*. This combination gave better extraction yields compared to SWE alone [273].

There has been a new breakthrough in the development and the improvement of the extraction and analytical instrumentation as well as the integration of different steps in one step. This approach has added a new level of diversity by multiple on-line coupling of different analysis and detection techniques (hyphenation) [274, 275]. The prospect of hyphenation of PHWE with analytical instruments such as GC and HPLC, as well as in-cell clean-up in one step may be future strategies in analytical chemistry.

Generally, there is an increase in the number of published articles (Fig. 3.13) where PHWE was employed for the extraction of food and plant materials in the last few years.

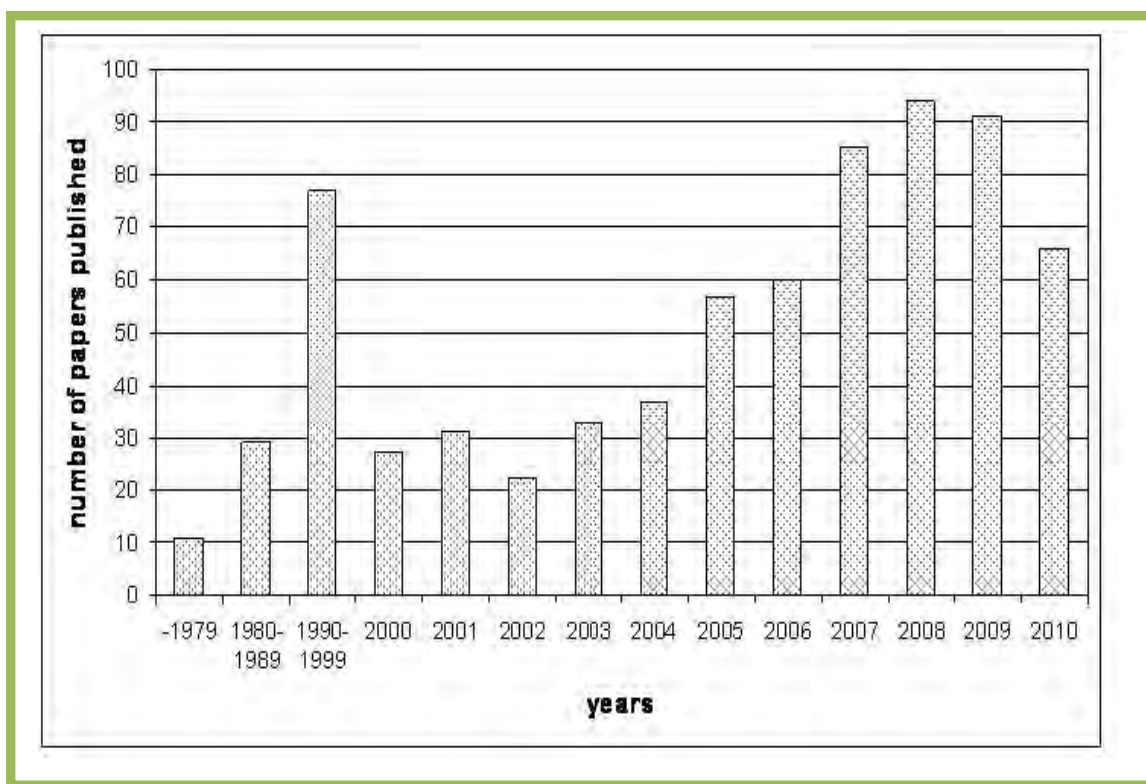


Fig. 3.13 Record of papers published for PHWE [274].

Chapter 4 Experimental

4.1 Chemicals and reagents

Berberine chloride, hydrastine chloride and hydrastinine chloride standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Formic acid and sea sand (acid purified) were purchased from Merck Chemicals (Gauteng, South Africa) while HPLC grade methanol was purchased from Merck KGaA (Darmstadt, Germany). Potassium hydroxide (KOH) and sodium chloride (NaCl) were from Saarchem Analytic (Krugersdorp, South Africa). Goldenseal roots, Willow Products, (Port Elizabeth, South Africa) and capsules, Solgar Corporation, (Leonia, NJ, USA) were purchased from a local herbal store in Grahamstown, South Africa.

α -BHC (97.9%) and heptachlor (98.5%) were obtained from Supelco (Bellafonte, PA, USA). Aldrin (98.1%), trans-chlordane (98.4%), 4, 4'-DDD (98.9%), 4, 4'-DDE (99.5%), 2, 4'-DDE (99.6%), dichlorvos (99.7%), and HCB (99.6%) were obtained from Riedel-de-Haën (Seelze, Germany). Individual stock solutions ($1000 \mu\text{g mL}^{-1}$) of each pesticide were prepared in acetone and stored in a freezer at -20°C . Tetrahydrofuran (THF), HPLC/UV grade acetone and n-hexane were obtained from Ultrafine Limited (London, England). Aflatoxin AFB1, AFB2, AFG1, AFG2 standards ($3 \mu\text{g mL}^{-1}$ in methanol) were from Sigma-Aldrich. Agilent SampliQ Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755 and

SampliQ QuEChERS AOAC Dispersive SPE kit, p/n 5982-5058 were supplied by Agilent Technologies Inc. (Santa Clara, CA, USA).

Methacrylic acid (MAA), ethylene glycol methacrylate (EGDMA), tetrahydrofuran (THF), ethanol, methanol (MeOH), chlorophyll, and azobisisobutyronitrile (AIBN) were also supplied by Sigma-Aldrich (Saint Louis, MO, USA). Copper(II) phthalocynine was a gift from the DST/MINTEK Nanotechnology Innovation Centre at Rhodes University (Grahamstown, South Africa). All other reagents used in this study were of analytical grade. Ultra high purity (UHP) water was generated from a Millipore Alpha-Q system supplied by Millipore (Molsheim, France). All the MIPs utilized in this thesis were custom made in our lab [275].

4.2 Instrumentation

4.2.1 PHWE equipment

All extraction and degradation experiments were performed using an in house built PHWE equipment (Fig. 3.6), featuring a gas chromatographic oven with a maximum temperature of 350 °C. Inside the oven, a pre-heater stainless steel coil was present to maintain the programmed temperature, followed by the extraction cell (3 cm in length and 10 mm i.d.) closed with screw caps at both ends, which permitted a continuous flow of water. The screw caps contained stainless steel frits, to ensure that the sample remained inside the extraction cell. A cooler system (made from coiled stainless steel tubing) was used to cool the water from the oven temperature to about 25 °C. A restrictor controlled the pressure in the system in order to maintain the extracting water in liquid state. The sample was

collected in a glass vial. Ultrapure water was pumped using a Bio LC pump Dionex Model GS50 Gradient Pump, Dionex Corporation (Sunnyvale, CA, USA).

An ultrasonic extractor from Integral Systems (Randburg, South Africa) was used for the extraction of alkaloids to compare with PHWE while the conventional reflux apparatus were used.

4.2.2 HPLC conditions

For all experiments, an Agilent HPLC 1200 series (Santa Clara, CA, USA) equipped with a binary pump, an autosampler, column oven and diode array detector (DAD) were used. Separation was achieved on an Agilent ZORBAX Eclipse Plus C₁₈ column (4.6 mm x 75 mm x 3.5 µm) and detection at 242 nm. The mobile phases used consisted of (A) 0.1% formic acid (pH 2.7) and (B) methanol, with an isocratic program of (A/B, 40:60, v/v) and a 6 min run time. The column temperature was set at 35 °C with a flow rate of 1 mL min⁻¹ and injection volume of 5 µL. A Finnigan MAT LCQ ion trap mass spectrometer (MS) equipped with an electro spray ionization (ESI) source was used for mass analysis of the degradation products. The spectra were acquired in the positive ion mode, with the capillary temperature set at 200 °C and sheath gas set at 80 arbitrary units, with the capillary and tube lens voltages set at -20 and -5V respectively.

An Agilent 1200 series HPLC coupled with a fluorescence luminescence detector (FLD), with an Agilent ZORBAX Eclipse Plus C₁₈ column (4.6 x 150 mm x 5 µm) analytical column by Agilent Technologies, (Santa Clara, CA, USA) was used to separate and detect the aflatoxins at 333 and 460 nm excitation and emission wavelengths respectively.

Commercial cartridges (Easi-Extract® Aflatoxin) were from R-Biopharm Rhone Ltd (Glasgow, Scotland). The pHs of all solutions were adjusted with a Jenway 3510 pH meter by Bibby Scientific Ltd, (Dunmow, United Kingdom). An MSE Mistral 1000 by Sanyo Gallenkamp, (Loughborough, England), was employed for centrifugation.

4.2.3 GC Conditions

The determination of OCPs was performed on an Agilent 6890 gas chromatograph equipped with ⁶³Ni micro-electron capture detector (GC-ECD). Separation was performed on a DB-1 column, (30 m × 0.25 mm i.d., and 0.25 µm film thickness). Helium was used as carrier gas at a constant flow of 1.0 mL min⁻¹ and high purity nitrogen was used as a make-up gas (60 mL min⁻¹). The temperature program was as follows: initial temperature of 60 °C was held for 2 min, increased to 190 °C at a rate of 5 °C min⁻¹, and then increased to 280 °C at a rate of 10 °C min⁻¹. The injector and detector temperatures were set at 250 °C and 300 °C, respectively. 1 µL of each sample was injected in the splitless mode.

The GC parameters were as described previously. The MS was operated in electron impact ionization mode with electron energy of 70 eV. The ion source, quadrupole and transfer line temperatures were held at 230, 150 and 280 °C, respectively. Target compounds were monitored in selected ion monitoring (SIM) mode.

A custom made electrospinning technique set-up was employed to fabricate electrospun nanofibers. It consisted of a power supply, a 10 mL glass syringe with stainless needle from

Poulsen GmbH (Berlin, Germany) mounted on a new Era, NE-1000 programmable syringe pump (New York, USA).

The morphology of the nanofibers was studied with the aid of Vega Tescan (TS5136ML) scanning electron microscope (SEM) (Brno, Czech Republic) operating at an accelerated voltage of 20 kV after gold sputter coating. Lambda 25 Perkin-Elmer spectrophotometer, by Perkin-Elmer (Santa Clara, CA, USA) was used to detect the concentration of chlorophyll, copper(II) phthalocyanine, quercetin and kaempferol at 680, 610, 312 and 350 nm, respectively, using a 1-cm cell.

4.3 Thermal degradation studies of alkaloids in goldenseal during selective PHWE

The extraction and degradation of thermo-labile alkaloids present in goldenseal; hydrastine and berberine, were investigated employing water as the only extraction solvent. The effect of temperature, pressure flow rate and extraction time on extraction yields employing PHWE were monitored with HPLC-DAD. The extraction yields were compared to those achieved through reflux and ultrasonic extraction methods.

4.3.1 Ultrasonication extraction

Goldenseal roots were ground and homogenized, and then extracted employing a modified method from Hartonen et al [276]. 5 g was mixed with 50 mL methanol then sonicated for 4 h at 80 °C. The extracts were then filtered using a hydrophobic PVDF 0.45 µm Millipore Millex – HV membrane filter (Billerica, MA, USA). The methanolic extracts were diluted 1:3 with water, the pH adjusted to 7 with 0.1 M KOH and then analysed by HPLC-DAD. The experiment was performed in triplicates. The procedure was repeated for goldenseal capsules.

4.3.2 Reflux extraction

5 g of the root samples were ground and homogenized and then mixed with 200 mL methanol, refluxed for 6 h with continuous stirring then cooled to room temperature, following a modified method from Hartonen et al [276]. The extracts were then filtered

using a hydrophobic PVDF 0.45 μm Millipore Millex – HV membrane filter (Billerica, MA, USA). The methanolic extracts were diluted 1:3 (v/v) with water, the pH adjusted to 7 with 0.1 M KOH and then analysis was conducted with HPLC-DAD. The experiments were performed in triplicates and the same procedure was repeated for goldenseal capsules.

4.3.3 PHWE

4.3.3.1 Extraction experiment

2 g of goldenseal roots which were previously ground and homogenized were mixed with 2 g of sea sand, to avoid conglomeration and any void space in the extraction cell and then placed in the PHWE oven (Fig 3.6). The extraction cell was filled with ultra pure water and then pre-heated for 5 min in the static mode. To optimize the temperature, pressure, flow rate and time required for extraction, the temperatures were varied from 100 to 160 °C, pressures from 10 to 100 bars, flow rate from 0.5 to 1.5 mL min⁻¹ and extraction time was varied from 5 to 60 min using water as the only solvent in the dynamic mode. The extracts were collected in a glass vial and mixed with methanol (3:1 v/v). The pH was then adjusted to 7 with 0.1 M KOH and analyzed with HPLC-DAD. The experiment was performed in triplicates and the procedure was repeated for goldenseal capsules. The optimization parameters are as summarized in Table 4.1.

Table 4.1. Parameters optimized for PHWE extraction

Parameter	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Temperature (°C)	Varied	140	140	140	140
Pressure (bars)	50	Varied	50	50	50
Flow rate (mL min ⁻¹)	1	1	Varied	1	1
Sample size (g)	2	2	2	Varied	2
Extraction time (min) (n=3)	15	15	15	15	Varied

4.3.3.2 Degradation experiment

Thermal degradation of hydrastine was evaluated in a pure standard and in the goldenseal extracts from section 4.4.3.1. 3 mL of a 100 µg mL⁻¹ (n=3) freshly prepared hydrastine standard was put in an autoclave (Rodwell Scientific Instruments, Basildon,

UK) and then heated to 160 °C for 15 min and a few bars, with a pre-heating time of 5 min, prior to HPLC-DAD analysis. The results were compared to the ones before heating in an autoclave. A similar experiment was conducted with the goldenseal extracts; maintaining the same conditions employed for the pure standard.

4.3.4 Analytical parameters

A stock solution containing 1000 µg mL⁻¹ alkaloids was diluted to obtain standard solutions in the concentration range of 0–120 µg mL⁻¹ so as to establish the linearity range. Analysis was carried out in triplicate by injecting 5 µL of each solution. Peak areas were plotted against the corresponding concentrations to obtain a calibration curve. Intra and inter-day precisions were established by analyzing 20, 60 and 100 µg mL⁻¹ of the alkaloids solution, three times on the same day and on three consecutive days, respectively. Accuracy was determined by analyzing known concentrations of the alkaloids, viz., 20, 60 and 100 µg mL⁻¹ spiked sample in triplicate followed by the determination of the percent recovery. The signal to noise ratio was evaluated at 3:1 and 10:1 for limit of detection (LOD) and limit of quantification (LOQ), respectively.

4.4 Pressurized hot water extraction of alkaloids in goldenseal with in-situ ultrasonication

In this study, ultrasound-assisted extraction and PHWE (US-PHWE) were combined for extraction of hydrastine and berberine from goldenseal. Soxhlet extraction was used as a reference technique, for comparison.

4.4.1 Ultrasound assisted and pressurized hot water extraction

PHWE and UAE were assessed using the experiments already described in sections 4.3.1 and 4.3.3.1 respectively. However, in the UAE experiment, the methanol used for extraction was replaced by ultra pure water. All experiments were performed at 80 °C, the maximum temperature of ultrasonic bath.

4.4.2 Ultrasound assisted pressurized hot water extraction (US-PHWE)

In order to lower the temperatures employed in PHWE while improving the efficiency as well as decreasing chances of degradation of thermo-labile analytes, PHWE was coupled to ultrasound. In this experiment, the oven in the PHWE equipment was replaced by an ultrasonic bath which had a maximum temperature of 80 °C. 2 g of goldenseal roots which were previously ground and homogenized were mixed with 2 g of sea sand; placed in the extraction cell and then put in the ultrasonic bath (80 °C). Ultrasound was applied for 5–20 min.

The ultrasonic frequency was investigated between 20 and 36 KHz. Extractions were carried out at a flow rate of 1 mL min⁻¹ and a pressure of 40 bars using water as the only solvent. The extracts were collected in a glass vial and then mixed with methanol (3:1 v/v). The pH was adjusted to 7 with 0.1 M KOH and then analyzed by HPLC-DAD. The experiment was performed in triplicates.

4.5 Pressurized hot water extraction as an optimal templates removal method from molecularly imprinted polymers

The efficiency of water under subcritical conditions as the sole leaching solvent (without modifiers) in the removal of templates from MIPs was evaluated. PHWE was applied to MIPs with subsequent monitoring of template removal and template bleeding by an ultraviolet spectrophotometer. The templates were washed-off and the extraction efficiency (EE) was compared to that of Soxhlet and ultrasonic extraction methods.

4.5.1 Preparation of chlorophyll, quercetin and phthalocynine MIP particles

For the synthesis of the three model colored MIPs (quercetin, chlorophyll and phthalocynine), a bulk polymerization method [277] was employed with MAA and EGDMA as the functional and cross linking monomers in the ratio of 1:5. The mixtures were refluxed in either THF or ethanol at 65, 75, or 80 °C; for 6 h for chlorophyll, 4 h for quercetin and 9 h for phthalocynine MIPs. The resultant polymer monoliths were ground to powders with particle sizes of less than 45 µm in diameter, and then introduced to the PHWE set up, Soxhlet or ultrasonic extraction for template removal. Thereafter, the particles were left to dry in open air overnight ready to be used for rebinding experiments. Control polymers referred to as non-imprinted polymers (NIPs), without the imprinting templates for chlorophyll, quercetin or copper(II) phthalocynine were prepared following a similar procedure.

4.5.2 Thermo-gravimetric analysis of the MIPs

Thermo-gravimetric analysis (TGA) of the MIPs powders was evaluated for the MIPs stability. This was to ascertain that The MIPs do not degrade at the high temperatures that PHWE usually operates at, for maximum benefits.

4.5.3 Template removal methods

4.5.3.1 Pressurized hot water extraction

To wash off the templates, 800 mg of the MIPs were extracted in a 34 mL PHWE extraction cell with water as the solvent. All extraction procedures were carried out at a flow rate of 2 mL min⁻¹; temperature; 220 °C and pressure; 70 bars for chlorophyll and phthalocynine MIPs. For quercetin, the optimized temperature was slightly higher at 235 °C. Aliquots of the washings from the PHWE set-up were then collected at 10 min intervals until the detected absorbance of the templates in subsequent washings was constant.

4.5.3.2 Soxhlet extraction and ultrasonic extraction

800 mg of the MIPs were extracted using up to 9 times fresh 80 mL MeOH aliquots at 70 °C for up to 16 h. Washings were collected every 2 h to determine the absorbance of the templates.

4.5.4 Determination of the absorbance of the templates in the washings using UV spectrophotometry

The absorbance of the different templates in the washings was determined with a UV spectrophotometry. This was carried out in triplicates for each washing method. Statistical methods were then used to determine the mean values and the %RSD. From the values, plots of absorbance against time of collection for each washing was constructed for each MIP and extraction method (see Figs. 5.7, 5.8, 5.9).

4.5.5 Template bleeding evaluation

To assess if there were any remnants of the templates (template bleeding) in the washed MIPs, 800 mg of the dry, washed MIP powders were extracted by employing the three different extraction methods with water or MeOH modified with acetic acid (9:1 v/v). Acetic acid was chosen as the modifier as it has been used to enhance the elution strength of solvents during desorption studies [278, 279]. Absorbance of templates from the washings were determined with the UV spectrophotometer so as to ascertain that there was no further change in the template bleeding concentrations detected by each method. The experiments were performed in triplicates.

4.5.6 Evaluation of performance of the MIPs after template removal

Equilibrium rebinding experiments were used to evaluate the performance of the MIPs after removing the templates. An optimal quantity of the washed MIP (800 mg) was mixed with 5 mL aliquots of 10% standards (w/v) of each of the templates for an optimal

equilibration time of 25 min. The mixtures were centrifuged for 2 min at 2000 rpm after which the absorbance of each of the templates in the supernatant was determined and the percentage of the template bound to a relevant MIP calculated to give recovery results. These were performed in triplicates with the accompanying percentage relative standard deviations (%RSD) calculated to give an estimation of the precision of the methods.

4.6 Pressurized hot water extraction coupled to molecularly imprinted polymers for simultaneous of extraction and clean-up of pesticides residues in edible and medicinal plants of the Okavango Delta, Botswana

In this study, an in-cell clean-up approach is proposed. The selectivity of PHWE was improved by coupling it with chlorophyll MIP (prepared in section 4.6) in the same extraction cell for the determination of organochlorine pesticides residue levels in various edible and medicinal plants of the Okavango Delta, Botswana. The MIP was designed to target chlorophyll which is an interfering compound in GC-ECD/MS analysis.

4.6.1 Sampling and study area

Plant samples (Table 4.2) were manually collected and wrapped with aluminum foil between January and September 2010 in the experimental area of the Okavango Delta located in the north east of Botswana, see Fig 4.2 for all sampling sites. Upon arrival at the lab the plants were dried at 40 °C for 72 h in an air recirculation oven, model Hewlett Packard 5890 (Blue Island, IL, USA). The stalks were separated from the leaves, ground and homogenized using a mortar and a pestle and then kept away from light in a cardboard until analysis.

Table 4.2 Plant samples collected from the Okavango Delta

Scientific names of plants	Local name (Setswana)	Use
<i>Nymphaea lotus</i>	Tswii	Edible
<i>Cyperus articulatus</i>	Mxowa	Edible/Medicinal
<i>Acalypha filicaulis</i>	Makgonatsotlhe	Medicinal
<i>Cyperus papyrus</i>	Koma	Edible/Medicinal
<i>Phoenix reclinata</i>	Tsaro	Edible

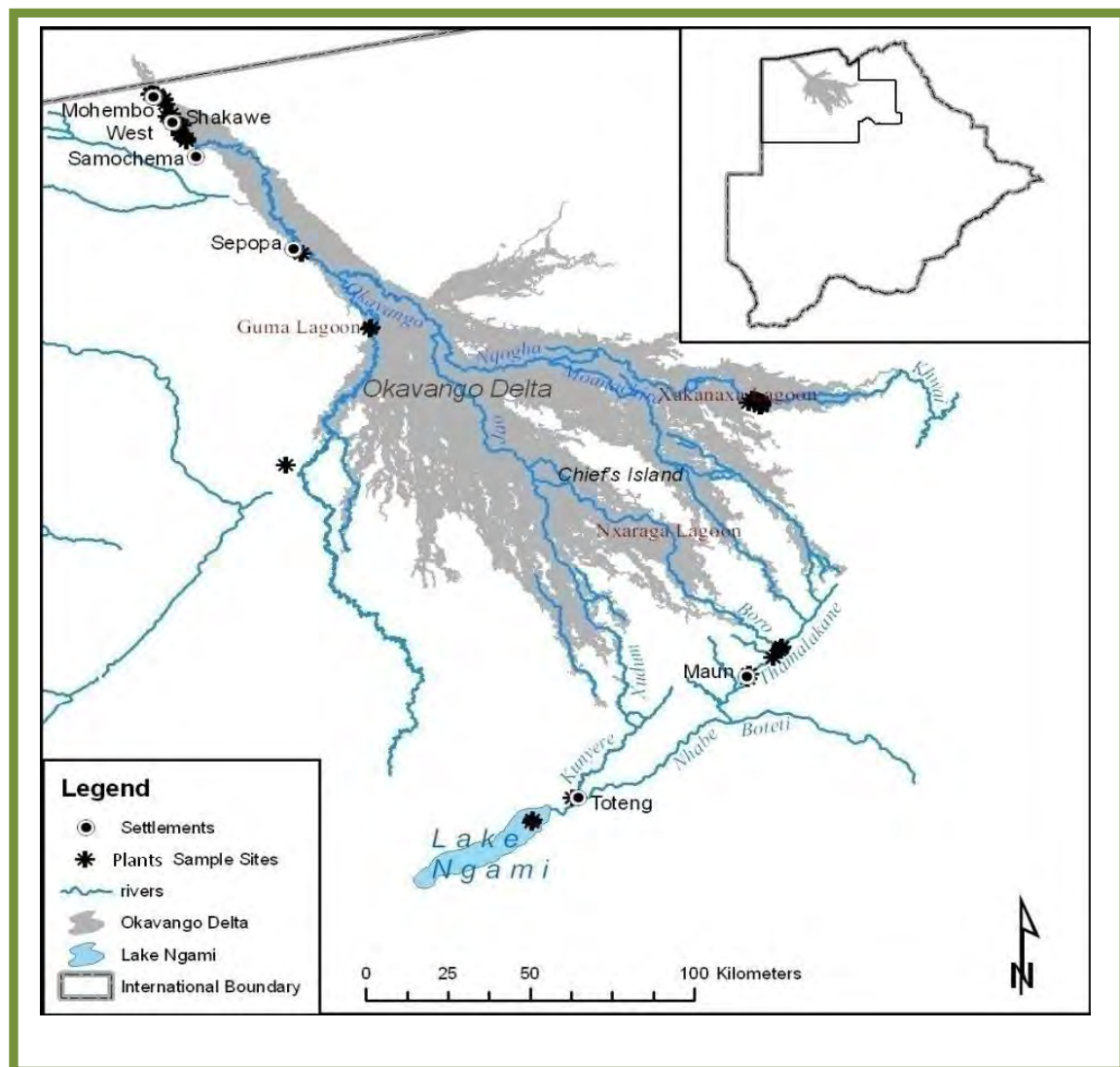


Fig. 4.1. Map of the Okavango Delta showing plants sampling sites [280]

4.6.2 Sample extraction and clean up procedures

4.6.2.1 Pressurized hot water extraction combined with molecularly imprinted polymer clean-up

Extraction and clean-up were performed simultaneously in the same extraction cell. The extraction cell was loaded with 1 g of homogenized plant sample, 1 g of sand, 0.05 g of NaCl and 1 g of a chlorophyll MIP. To optimize the temperature, pressure, flow rate and time required for both extraction and clean-up, the extraction temperatures were varied from 150-260 °C, pressures from 10-100 bars, flow rate from 0.5-1.5 mL min⁻¹ and extraction time was varied from 5-60 min, with a 5 min preheating and equilibration of the oven. The extracts were collected in a glass vial, evaporated at 80 °C under a steady flow of N₂ and then reconstituted in 20 µL of *n*-hexane prior to GC-ECD and GC-MS analysis.

4.6.2.2 QuEChERS AOAC Official Method

The method was modified from European EN Official Method 15662 [37]. 10 g of previously homogenized sample was placed into a 50 mL centrifuge tube (from the SampliQ QuEChERS extraction kit). 15 mL acetonitrile was added to each tube and shaken for 1 min. An Agilent SampliQ QuEChERS extraction salt packet from the kit (p/n 5982-5755) containing 6 g of anhydrous MgSO₄, and 1.5 g of anhydrous NaOAc was added directly to the tubes. Sample tubes were sealed tightly and shaken vigorously for 1 min by hand to ensure that the solvent interacted with the entire sample and crystalline agglomerates were dispersed. Sample tubes were then centrifuged at 4000 rpm for 5 min.

4.6.2.3 Dispersive SPE Clean-up

An 8 mL aliquot was transferred to an Agilent SampliQ QuEChERS dispersive SPE 15 mL tube (p/n 5982-5058), containing 400 mg of PSA and 1200 mg of anhydrous MgSO₄. The tubes were tightly capped and hand shaken vigorously for 1 min. The tubes were centrifuged at 4000 rpm for 5 min. The aliquot from the extract were dried under N₂, and then reconstituted in 20 µL of *n*-hexane prior to analysis by GC-ECD and GC-MS.

A summary of the extraction schemes for the PHWE method and the conventional QuEChERS methods was compared (see Fig. 4.2).

PHWE-MIP

QuEChERS method

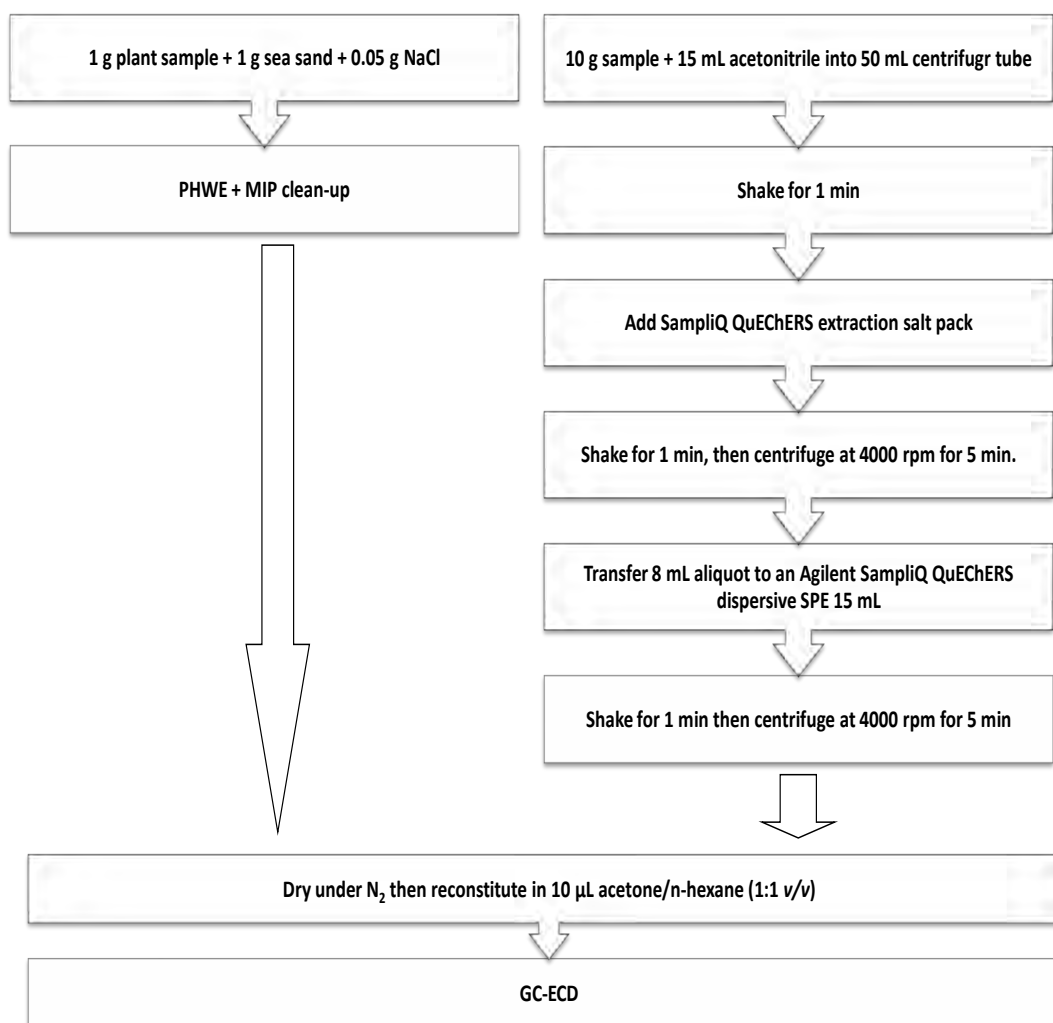


Fig4.2 Comparison of the extraction scheme of QuEChERS method with PHWE-MIP

4.7 Simultaneous extraction, clean-up and desorption of aflatoxins in medicinal plants with pressurized hot water extraction coupled to molecularly imprinted polymers

In this study, the PHWE technique was modified such that two extraction cells were connected in series. The ultimate aim was to perform simultaneous extraction, clean-up and desorption of aflatoxins from medicinal plants, in one step. In this case a class selective MIP was used to target analytes of interest, aflatoxins AFG2, AFG1, AFB2, and AFB1. These were later leached out from the MIP with water at a higher temperature than the one used for extraction. The proposed method was compared with the AOAC Official Method 991.31.

The MIP utilized was prepared as in section 4.6 except that the template this time was aflatoxin AFB1.

4.7.1 Sampling and study area

The medicinal plants investigated in this study were purchased from a local herbal store in Grahamstown (Eastern Cape, South Africa). Table 4.3 lists the medicinal plants under evaluation.

Table 4.3 Medicinal plants from Grahamstown

Scientific names	Local names (Xhosa)	Use
<i>Hydnora Africana</i>	<i>U mavumbuka</i>	Medicinal
<i>Pelargonium reniforme</i>	<i>Intololwana</i>	Medicinal
<i>Cassipourea flanaganii</i>	<i>Ummemezi</i>	Medicinal
<i>Bowiea volubilis</i>	<i>Umagaqana</i>	Medicinal
<i>Kedrostis foetidissima</i>	<i>Utuvishé</i>	Medicinal

4.7.2 PHWE and in-cell clean-up of aflatoxins from plant samples

Two 11 mL cells were serially connected for simultaneous extraction, in-cell clean and pre-concentration (see Fig 4.3). The first cell was loaded with 1 g of the plant sample, 1 g of sand and 0.05 g NaCl. The second cell was attached in series with the first one, and loaded with 1 g of aflatoxin AFB1 MIP, which is class selective to all the aflatoxins investigated. The aflatoxins were then extracted from the first cell. The extraction procedure consisted of filling both extraction cells with ultra pure water and pre-heated; static time 5 min; temperature 180 °C; pressure 60 bars. This was followed by dynamic extraction for 10 min at a flow rate of 0.5 mL min⁻¹. The procedure was such that the extracts from the first cell flow directly into the second cell that contained the MIP to trap the aflatoxins from the extracts.

The first cell and the coupling assembly were removed after extraction was completed, and the cell with the MIP remained in the oven. The MIP-containing cell was inverted and desorbed with pure water at a higher temperature of 190 °C; pressure 60 bars; flow 0.5 mL min⁻¹ for 10 min. The extracts were collected in a clean vial.

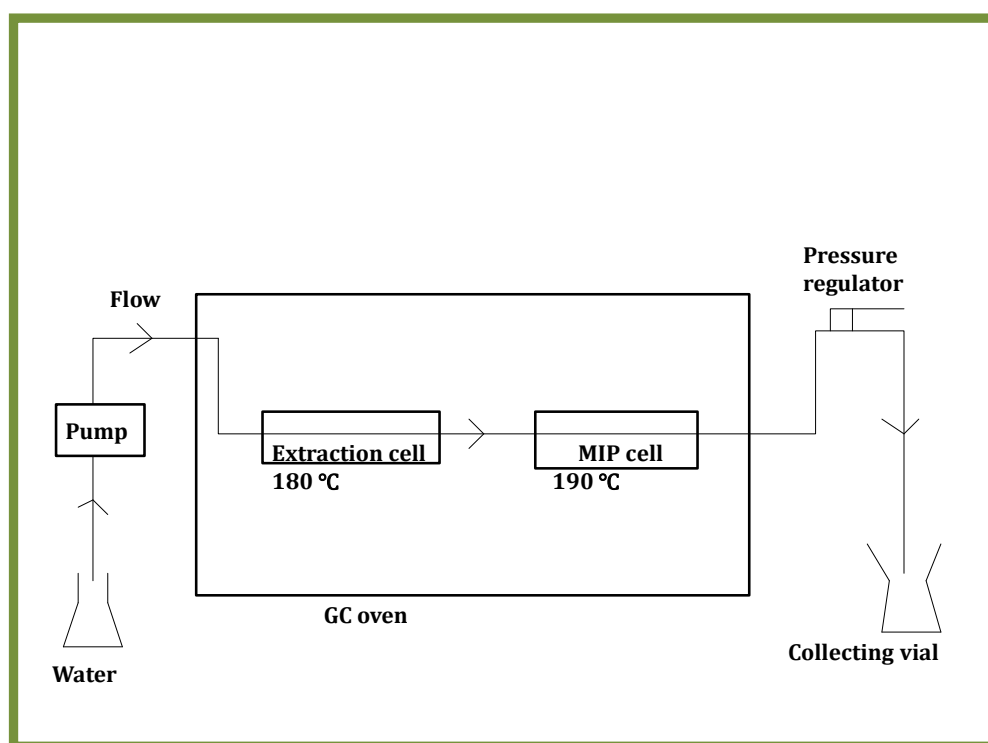


Fig. 4.3 Modified PHWE for serial extractions and clean-up

4.7.3 Sample preparation and immunoaffinity column clean-up of aflatoxins from plants

The method was modified from AOAC Official Method 991.31 [283]. Briefly, 3 g of the ground sample was mixed with 1 g NaCl, dissolved in 15 mL of 70% (v/v) methanol:water in a sealed centrifuge tube. The mixture was sonicated for 30 min followed by centrifugation at 9000 rpm for 10 min. 5 mL of the supernatant was diluted with 15 mL of deionized water and 5 mL phosphate buffer saline (PBS) (pH 7.4). The solution was centrifuged at 9000 rpm for 10 min. The supernatant was filtered through a 0.45 Whatman filter paper. 5 mL of the filtrate was then passed through an immunoaffinity column (Easi-Extract® Aflatoxin) using gravity; washed with 10 mL water and then flushed with air. Final elution was accomplished with 2 mL methanol then diluted with 2 mL water.

4.7.4 Pre-column derivatization

The extracts from 4.8.2 and 4.8.3 were evaporated to dryness with nitrogen. A 5 mL of hexane followed by a 1 mL of TFA were added to each extract. The mixture was vortexed for 1 min, allowed to stand for 5 min and then 3 mL of de-ionized water:methanol (9:1 v/v) were added. The mixtures were further vortexed for 1 min, then the organic layer was allowed to separate from the aqueous layer. The aqueous layer was centrifuged and the supernatant was analyzed with HPLC-FLD.

4.8 Pressurized hot water extraction for desorption of pesticides from electrospun nanofiber sorbents.

In this study, the efficiency of PHWE as a green solvent for desorption of pesticides from electrospun nanofiber sorbent was investigated and monitored using GC-ECD. DDE was used as a model organochlorine pesticide.

4.8.1 Preparation of polystyrene and carbodithioate fuctionalized polystyrene nanofibers

Polystyrene (PS) nanofibers were fabricated by electrospinning according to the following procedure: 2.5 g of PS was dissolved in 10 mL DMF:THF (4:1 v/v) and stirred continuously for 12 h using a magnetic stirrer. The viscous solution formed was then loaded into a 10 mL glass syringe from Poulten GmbH (Berlin, Germany) and mounted on a new Era, NE-1000 programmable syringe pump (New York, USA). The solution was pumped at a rate of 0.10 mL h⁻¹ through a steel needle of 0.58 mm internal diameter. The distance between the needle tip and the collector was kept at 12 cm, while the needle tip and the collector were held at optimized voltages of +15 kV and -5 kV respectively. The fiber diameters were measured using the Scandium 4.0 software [281].

The procedure for preparing the PS nanofibers incorporating carbodithioate was similar to that used for PS except that an optimized mass of 10 mg of the carbodithioate ligand [282]

was added to the PS solution and stirred for another 4 h to obtain a thoroughly mixed solution before electrospinning.

4.8.2 DDE adsorption studies

The adsorption of DDE by PS and carbodithioate incorporated PS nanofibers was investigated. 10 mg of electrospun PS and carbodithioate incorporated PS nanofibers were added to vials containing aliquots of DDE of different concentrations while varying the volume and time. The concentrations, volumes and times under investigation were 0.25-1.0 $\mu\text{g L}^{-1}$, 200-600 μL and 10-30 min respectively. The solutions were stirred, filtered and the fibers were dried under a gentle flow of nitrogen for 2 min, prior to desorption studies. The concentration of DDE remaining in the solution was then determined using GC-ECD. The concentration adsorbed by the fibers was calculated as the difference between the initial and final concentration of solutions. Six replicates of each sample were prepared.

4.8.3 Desorption of DDE from nanofibers by PHWE

For desorption studies, the DDE adsorbed on the fiber was desorbed with PHWE in a dynamic mode using only water as the extraction solvent. To optimize the temperature, pressure, flow rate and time required for desorption, the extraction temperatures were varied from 180-270 $^{\circ}\text{C}$, pressures from 10-100 bars, flow rate from 0.5-1.5 mL min^{-1} and extraction time was varied from 10-60 min, with a 5 min preheating and equilibration of the oven. The eluate was evaporated at 80 $^{\circ}\text{C}$ under a steady flow of nitrogen and then reconstituted in 10-20 μL of *n*-hexane before GC-ECD analysis.

A stock solution containing 500 $\mu\text{g L}^{-1}$ of DDE was diluted to obtain 500 μL solutions in the concentration range of 0.10-2.50 $\mu\text{g L}^{-1}$ to establish linearity. 10 mg of PS nanofibers incorporating carbodithioate were introduced to each concentration ($n=6$), stirred for 20 min then filtered and dried under nitrogen for 2 min. The fibers were then desorbed using PHWE at a flow rate of 0.5 mL min^{-1} , 260 $^{\circ}\text{C}$ and 80 bars in 10 min. The extract was evaporated using nitrogen and subsequently reconstituted in *n*-hexane prior to GC-ECD analysis. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. The intra- and inter-day precisions were established by analyzing 0.10, 1.00 and 0.200 $\mu\text{g L}^{-1}$ DDE standards, on the same day and on three consecutive days after adsorption on the fibers and desorption with PHWE. Analysis was carried out on six replicates of each concentration. The limit of detection (LOD) and limit of quantification (LOQ) were evaluated on the basis of signal to noise ratio of 3:1 and 10:1 respectively.

Chapter 5 Results and Discussion

5.1 Thermal degradation studies of alkaloids in goldenseal during selective PHWE

5.1.1 Optimization of extraction procedures

5.1.1.1 Effects of temperature and pressure in PHWE

The peak areas of hydrastine and berberine were used as evaluation criteria for the optimization of temperature, time and pressure. Of all the parameters evaluated in this experiment, it was observed that generally temperature had the greatest effect while pressure had negligible effect on the extraction efficiency. Data obtained for both hydrastine and berberine ($n = 3$) indicated that the highest extraction yield was obtained within 15 min and it increased with increasing temperature only up to 140 °C. Above 140 °C, extraction yields decreased (see Fig 5.1). This was attributed to a possible degradation since the analytes are thermally labile.

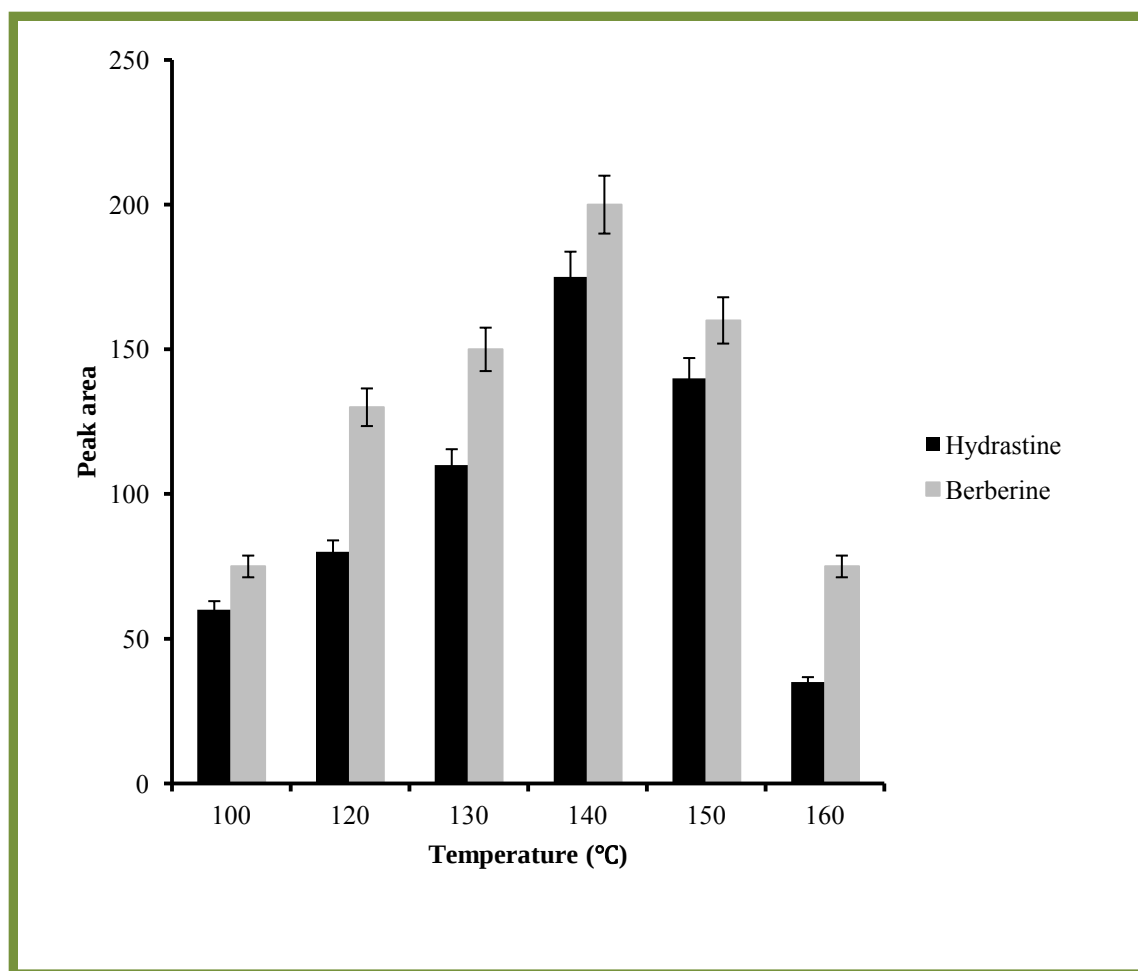


Fig. 5.1. Effects of temperature on the extraction of hydrastine and berberine from goldenseal roots by PHWE at 50 bars, 1 mL min⁻¹ for 15 min.

5.1.2 Comparison of PHWE with conventional methods

Both PHWE and the selected conventional methods were efficient in the extraction of hydrastine and berberine (Table 5.1). At a temperature of 140 °C, pressure of 50 bars and a flow rate of 1 mL min⁻¹ with 15 min extraction time, the total extraction yields of both the alkaloids in the root sample was 43.04 mg g⁻¹ (n=3). These were comparable to those of reflux and ultrasonic extractions (39.07 and 45.67 mg g⁻¹ respectively). At 95%

confidence level (n=3) using the student *t* test, recorded extraction yields for the conventional methods and PHWE are not significantly different. PHWE employed only water which is environmentally friendly, as the extraction medium to achieve comparable yields to those of conventional methods. Moreover, the extraction time was shorter (15 min) compared to over 6 h for the conventional methods.

Table 5.1. Comparison of extraction yields (mg g⁻¹) of alkaloids in goldenseal by PHWE, reflux and sonication

	PHWE	Reflux	Sonication
Alkaloids in goldenseal roots	43.04 (2.84)	39.07 (1.68)	45.67 (2.97)
Alkaloids in goldenseal capsules	34.54 (1.91)	36.65 (2.04)	39.23 (1.78)
RSD (n=3)			

5.1.3 Degradation behavior

The optimized HPLC method was used to further study the degradation behavior of hydrastine under various high temperature conditions using PHWE (see Fig. 5.2 for the chromatograms). Only hydrastine was further investigated due to lack of berberine standard. At the optimal temperature of 140 °C, hydrastine showed only one major peak at 4 min (see Fig. 5.2a). However, at elevated temperatures, 160 and 180 °C, some extra peaks

in the lower time zone were observed (see Figs 5.2b and 5.2c respectively). The extra peaks were assumed to be degradation products rather than new analytes since a pure standard of hydrastine was evaluated for degradation. It was also noted that the number of extra peaks increased as the temperature was increase and that the hydrastine peak completely disappeared. The disappearance of the hydrastine peak was attributed to it changing into its degradation products.

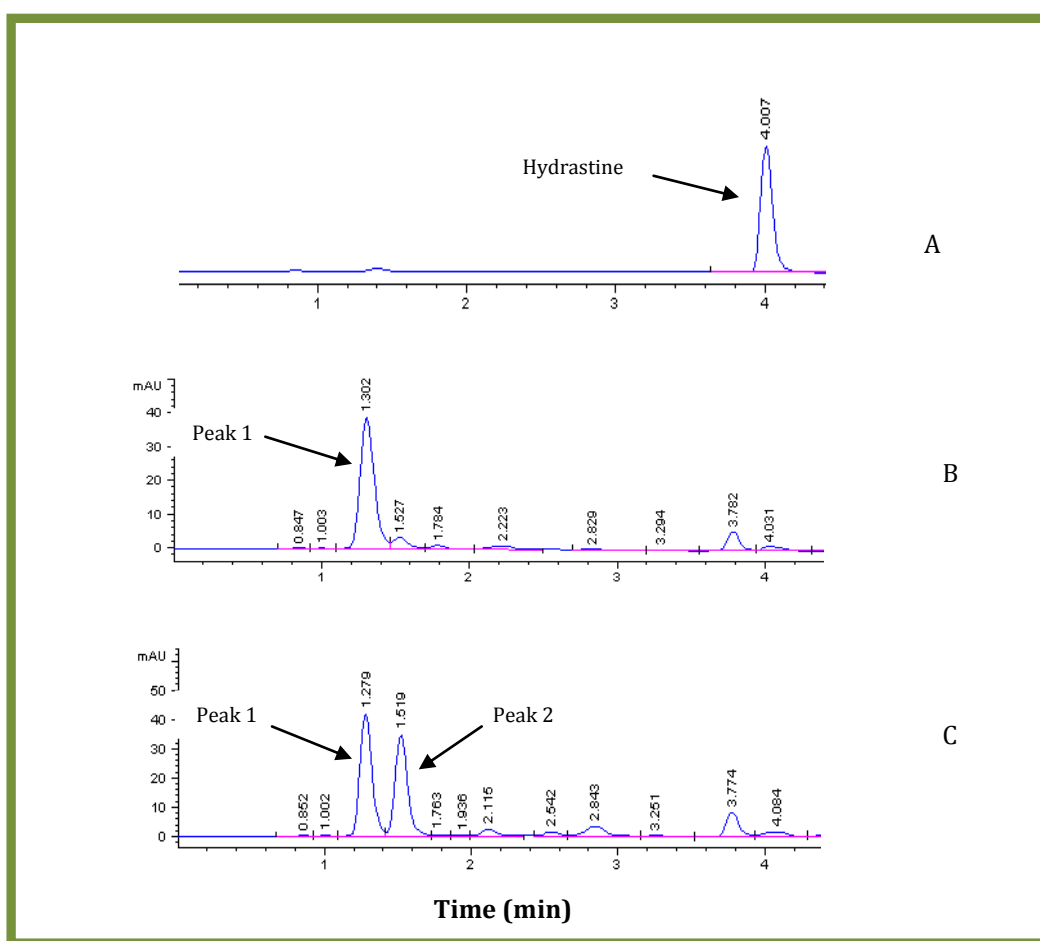


Fig. 5.2. Chromatograms showing degradation products of hydrastine standard ($100 \mu\text{g mL}^{-1}$) at (a) 140°C , (b) 160°C and (c) 180°C 50 bars, 15 min.

5.1.4 Extraction/degradation curves

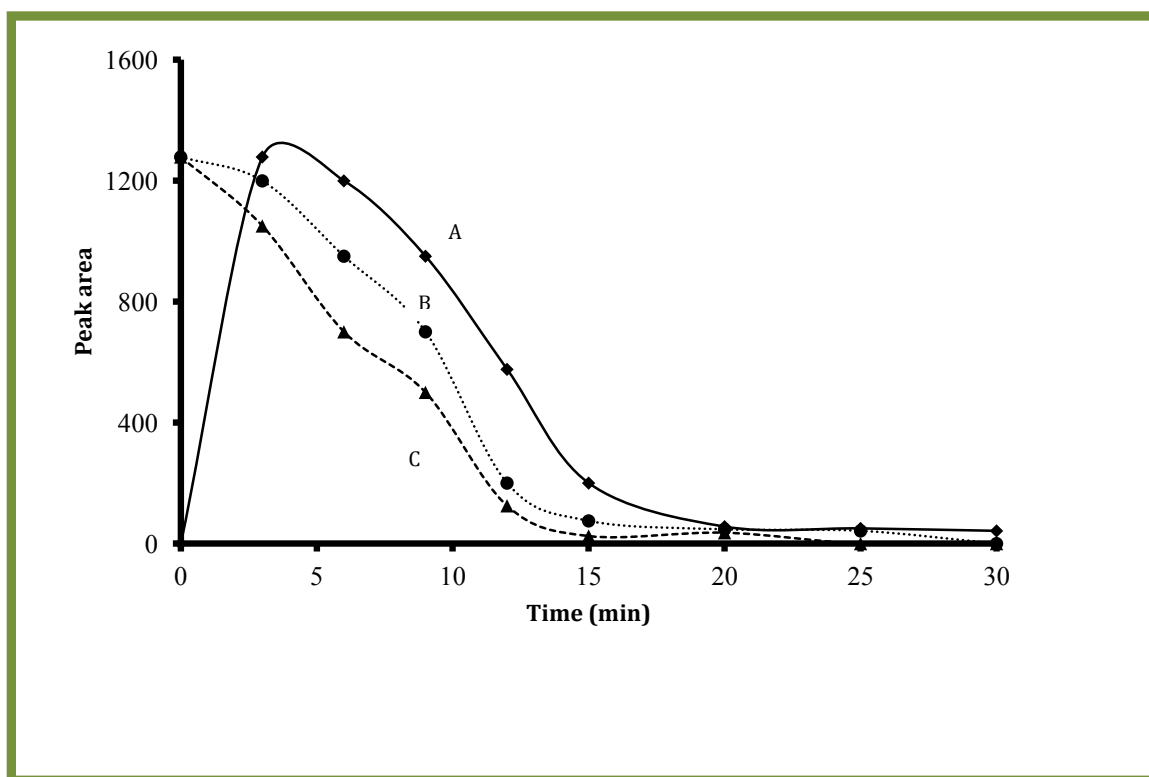


Fig. 5.3 Extraction curve at 140 °C (A), and degradation curves for sample (B) and a standard (C) of hydrastine at 160 °C at 50 bars, 1 mL min⁻¹ for 15 min.

Figure 5.3a represents the extraction profile of hydrastine while 5.3b and 5.3c represent the degradation of hydrastine for goldenseal root sample and standard respectively. It was observed that degradation started immediately at the beginning of the experiment even during the 10 min pre-heating stage. Within the first 10 min of the experiment, 25% of the original sample had already degraded while 41% of the standard had degraded. It was also observed that, the rate of degradation for the standard was faster than that of the sample.

The difference in the degradation pattern was attributed to the matrix presence which seemed to possess a 'protective effect' on the sample. The root matrix possibly consisted of some other alkaloids or other antioxidants that could be responsible for slowing down the degradation of hydrastine observed in Fig. 5.3b. Petersson *et al* [287] observed a similar effect when they studied role of the matrix in the degradation experiments of anthocyanins in red cabbage.

5.1.5 Kinetics of hydrastine thermal degradation

Figure 5.4 shows the relationship between the concentration of the hydrastine standard and time over a temperature range of 100-160 °C. As expected, it was observed that the concentration decreased with time and that alkaloids were more rapidly degraded at higher temperatures. A plot of the logarithm of concentration against time resulted in pseudo first-order reaction kinetics (results not shown).

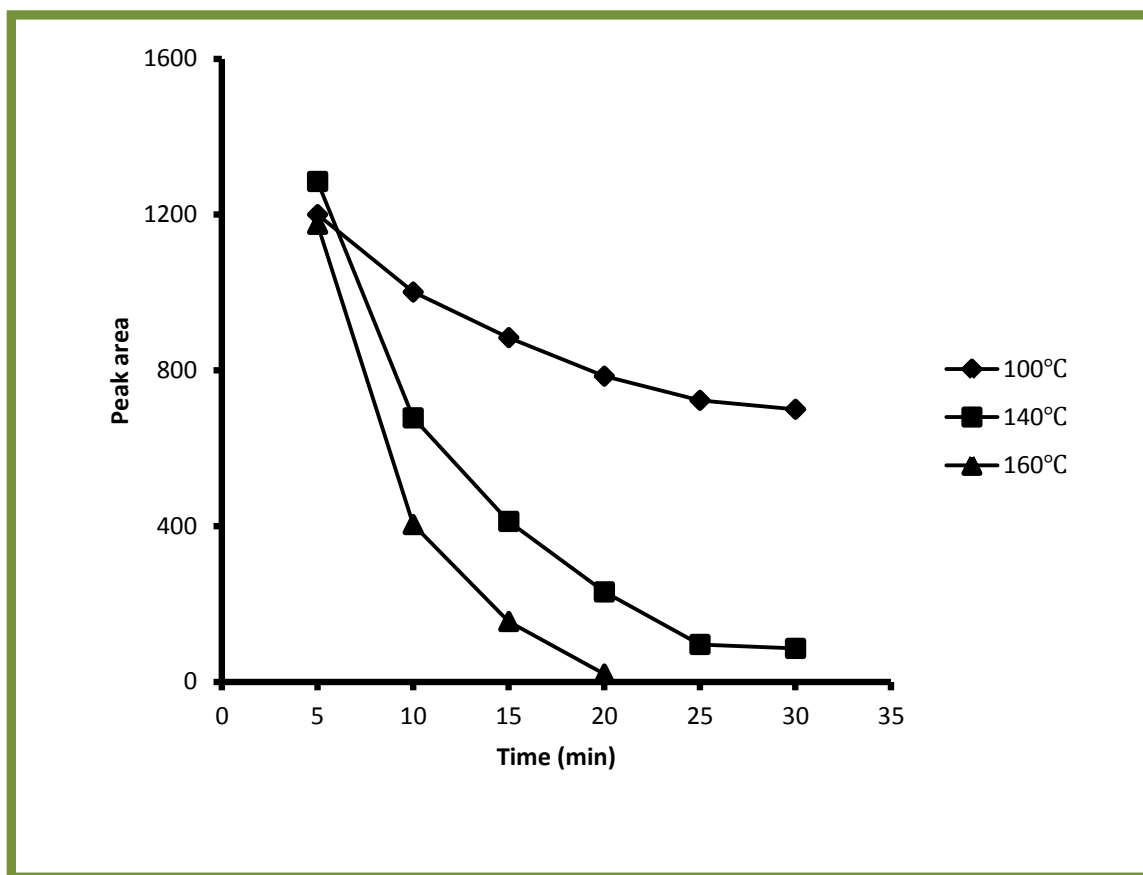


Fig. 5.4 Relationship between the concentration of hydrastine standard and time

5.1.6 Peak identification

The degradation products were tentatively identified using mass spectrometry (MS). The MS data was compared to molar masses of the fragmentation pattern based on literature. Only one match corresponding to hydrastinine was found at m/z value of 208 (see peak 1 in Fig 5.2) [283]. However, the rest of the degradation products were not positively identified.

5.1.7 Analytical parameters

The method was found to be linear in the concentration range of 0–120 $\mu\text{g mL}^{-1}$ ($r^2 = 0.9992$) for both the alkaloids. The data for triplicate analysis showed that the % R.S.D. for each investigated concentration was $<0.25\%$. The % R.S.D. for intra- and inter-day precision at three different concentrations, viz., 20, 60, and 100 $\mu\text{g mL}^{-1}$ was $<0.40\%$. Furthermore, good recoveries were obtained (over 95%) when samples were spiked with known concentration of the drug at 20, 60, and 100 $\mu\text{g mL}^{-1}$. The limits of detection (LOD) and quantification (LOQ) were found to be 0.50 and 1.65 $\mu\text{g mL}^{-1}$ respectively for hydrastine and 0.47 and 1.55 $\mu\text{g mL}^{-1}$ respectively for berberine.

5.2 Pressurized hot water extraction of alkaloids in goldenseal with in-situ ultrasonication

5.2.1 Effect of ultrasound in extraction

The results showed that the extraction yield increased with temperature in all extraction methods while pressure had negligible effects on the extraction yields. However, PHWE method obtained lower yields compared to US-PHWE at 80 °C and a flow of 0.5 mL min⁻¹. Temperatures higher than 80 °C were not investigated as it was the maximum temperature possible with the ultrasonic bath; therefore it was a limiting parameter.

The general accepted explanation of ultrasound enhancement is that the effects of ultrasonic waves on plant material breaks down the cells and releases their contents into the extraction medium [285]. Since a dried plant material was used in this case, extraction took place in two stages; 1. The plant material was soaked in the solvent to facilitate the swelling and hydration; 2. Mass transfer of analytes from material to solvent by diffusion and osmosis processes then followed. Ultrasound treatment accelerated the second stage; resulting in advanced hydration, reduced diffusion resistance, improved mass transfer and therefore better extraction yields. The ultrasonicated tissues absorbed an extra volume of the solvent compared to the ones which were not sonicated.

As it is demonstrated in Fig. 5.5, the ultrasound improved the extraction yields considerably. In 12 min, the extraction yields achieved by employing US-PHWE were 36.3

mg g⁻¹. This was almost double the extraction yields achieved when PHWE was employed without ultrasonication (19.5 mg g⁻¹).

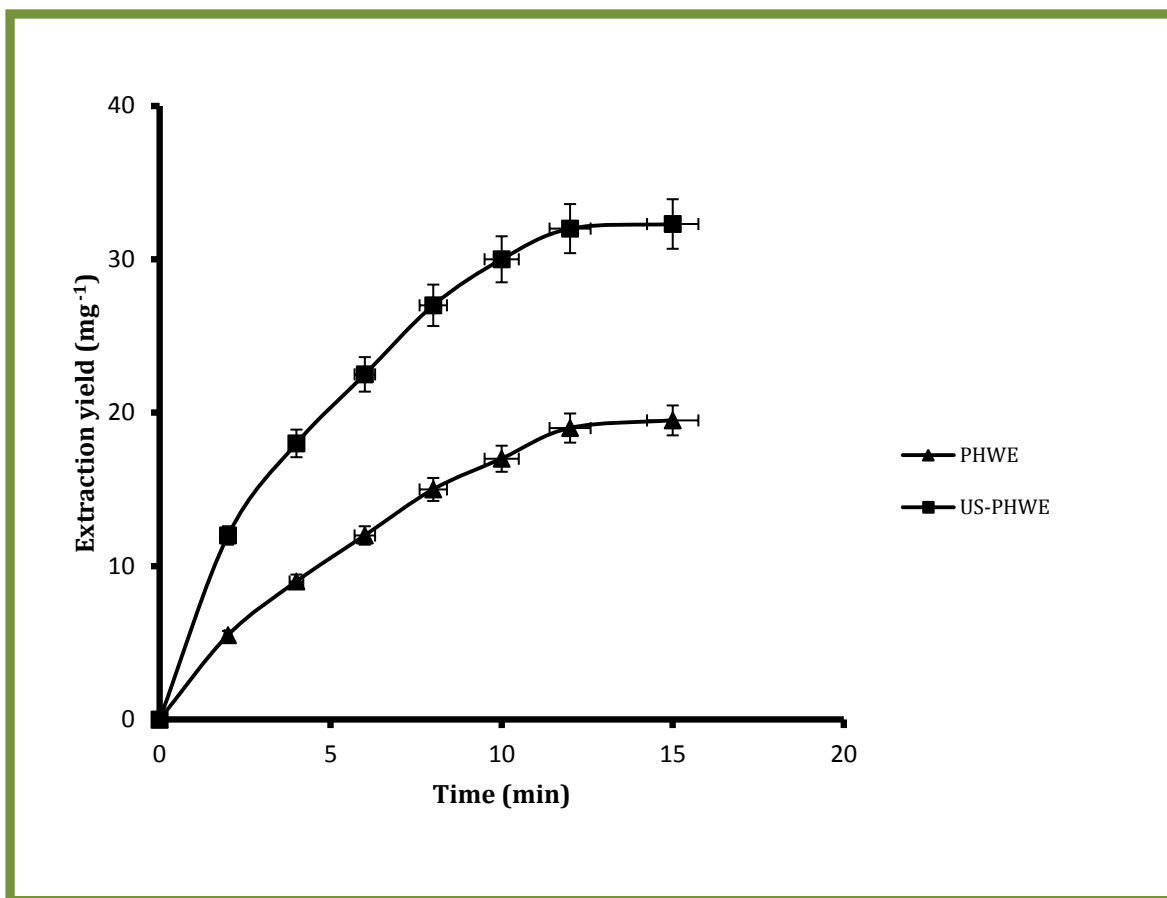


Fig. 5.5 Effect of time of ultrasound applied on the PHWE of berberine from goldenseal

5.2.2 Effect of ultrasonic frequency

The enhancement effect of 25 KHz was better than that of 35 KHz. The lower the ultrasound frequency, the longer the ultrasonic cycle. The cavitation bubbles had sufficient time to grow and collapse thereby contributing to the intensification of the ultrasonic

cavitation. Low frequency therefore had a significant influence on the swelling hydration of dried plants hence better extraction efficiency. The acoustic absorptivity of medium increased rapidly with the increase of frequency, which reduced the ultrasonic enhancement effect hence lower extraction yields.

5.2.3 Comparison with conventional methods

Although US-PHWE achieved better extraction yields than PHWE without ultrasonication in 12 min extraction time, they were statistically lower when compared to Soxhlet extraction in real samples (95% confidence level). When the extraction time was increased to 30 min, the extraction yields were comparable. Addition of modifiers could also improve the extraction yields of US-PHWE. However, when a critical comparison is established between the conventional Soxhlet extraction method and the proposed extraction methods, it should be noted that extraction time was decreased drastically from 6 h to 30 min. Moreover, the solvent utilized in the extraction procedure was only water which is environmentally friendly.

5.3 Pressurized hot water extraction as an optimal templates removal method from molecularly imprinted polymers

5.3.1 TGA results for the molecularly imprinted polymers

The TGA plots (see Fig. 5.6) showed that all the MIPs were stable at temperatures where the optimum extraction was realized (220 and 235 °C). The temperatures at which the MIPs were stable are as marked by the initial horizontal sections of the plots, after which there are sharp drops indicating some degradation of the MIPs. During the washing of the MIPs the temperature of the hot water was maintained at optimal temperatures of 220 and 235 °C to ensure that the integrity of the MIPs was not compromised in the process.

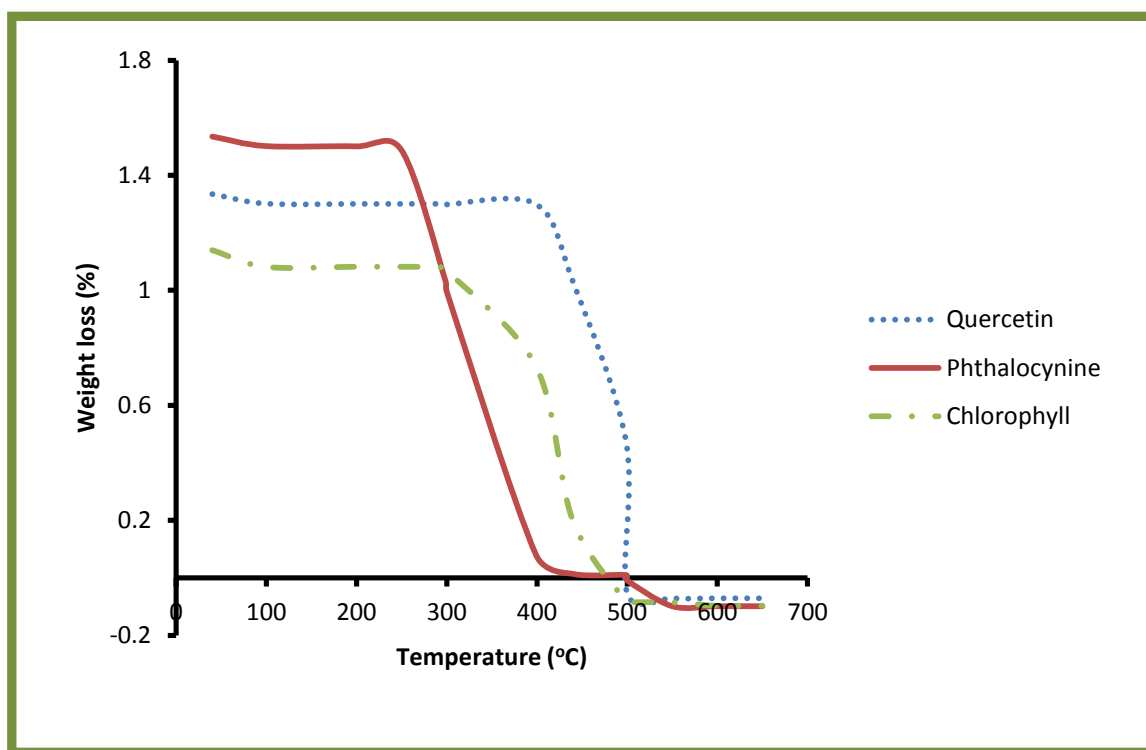


Fig. 5.6. A TGA plot for the MIPs

5.3.2 Template removal method

Template removal is a critical step in the preparation of most MIPs. If there are remaining template molecules in the MIPs, less cavities will be available for rebinding. This will result in decrease in the efficiency as well as errors in analytical application. Following the procedures described in section 4.6.2, the templates were thoroughly washed off their MIPs so as to free recognition sites for selective binding during the rebinding experiments. The concentration (absorbance) of the templates as determined by the UV spectrophotometer decreased with time in all cases until it remained constant with continued washing. This marked the point at which the templates were completely removed by a particular method of extraction (see Figs. 5.7, 5.8, 5.9). Optimal removal of

template was also marked by the great loss of the distinct, bright colours of the MIPs to slightly white or white after washing.

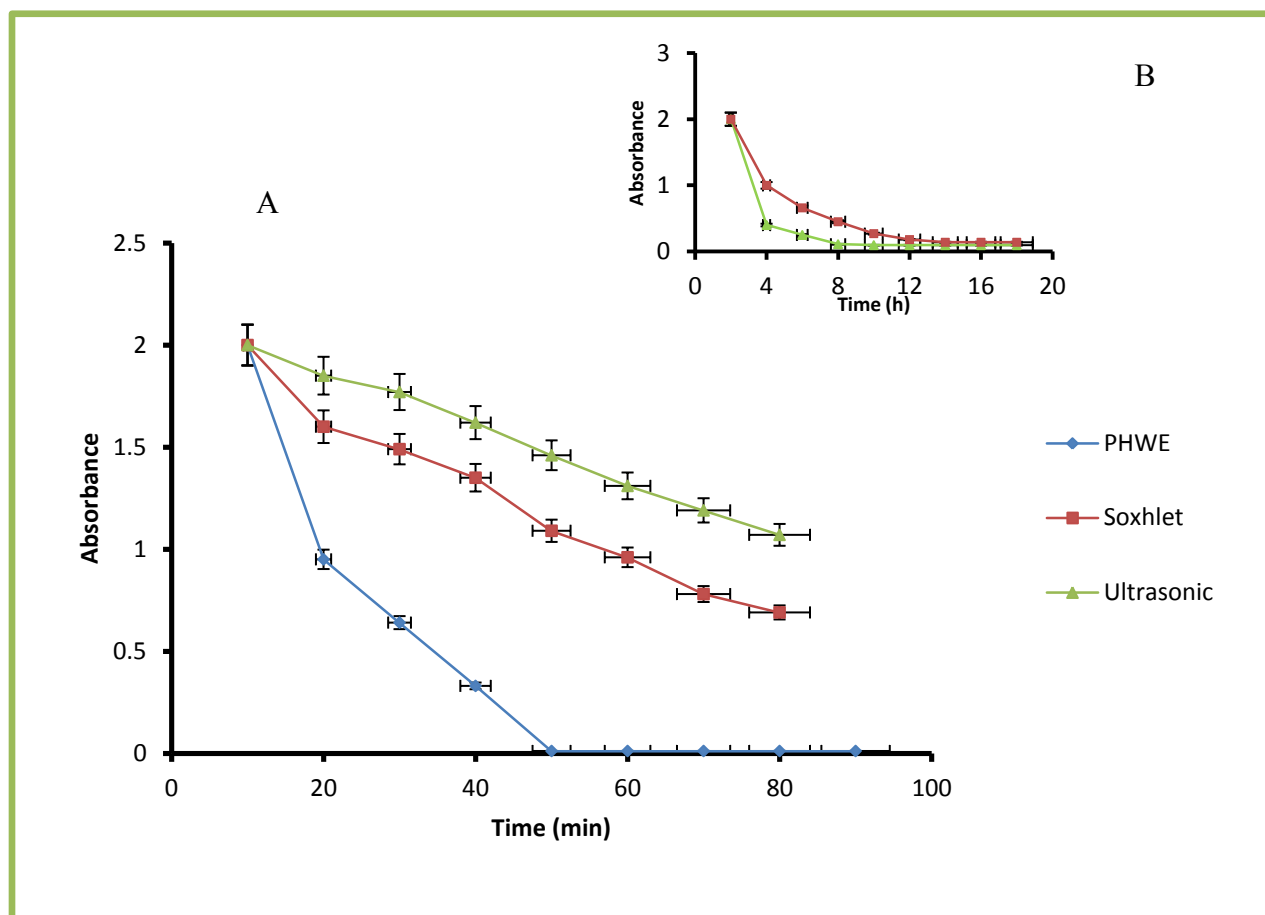


Fig. 5.7. Absorbance of chlorophyll in each washing at (A) 10 min intervals for the three extraction methods and (B) at 2 h intervals for the two that took longer to complete the extraction.

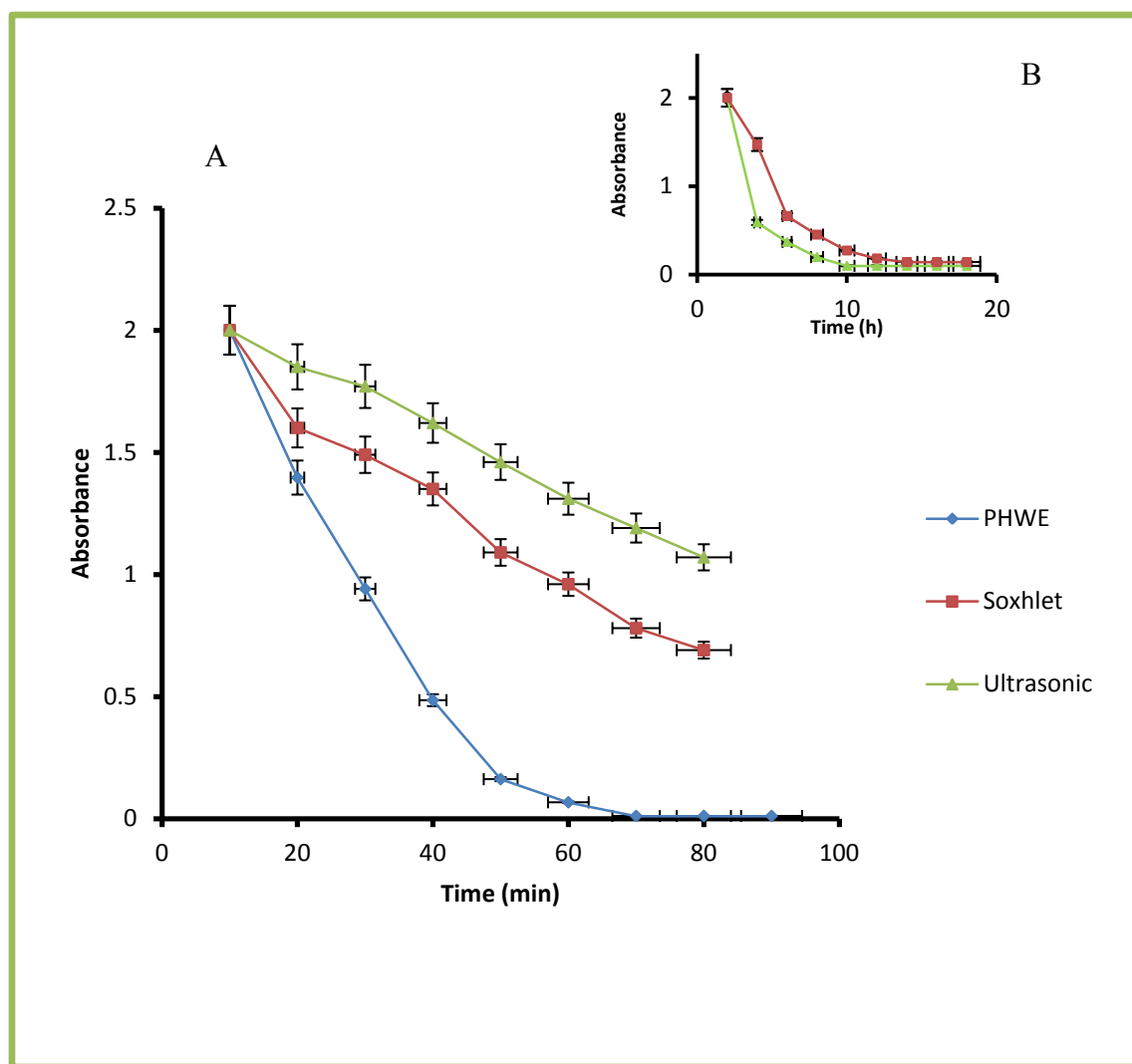


Fig. 5.8 Absorbance of quercetin in each washing at (A) 10 min intervals for the three extraction methods and (B) at 2 h intervals for the two that took longer to complete the extraction.

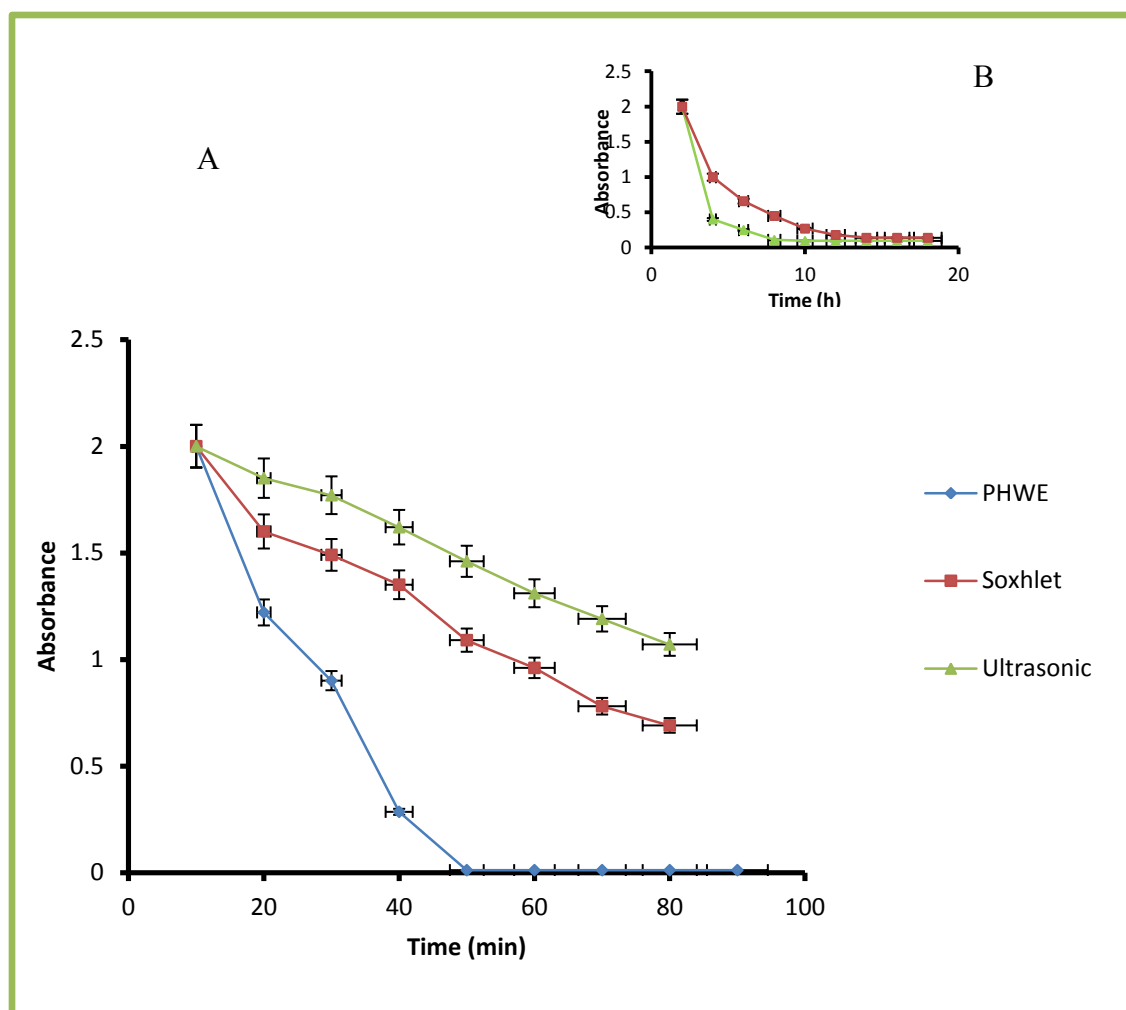


Fig. 5.9 Absorbance of phthalocynnine in each washing at (A) 10 min intervals for the three extraction methods and (B) at 2 h intervals for the two that took longer to complete the extraction.

According to the plots, complete process of washing-off the templates took under 70 min for all the MIPs when using PHWE (see Figs 5.7, 5.8, 5.9). This was advantageous as the extraction time was relatively very short compared to the Soxhlet and ultrasonic extraction methods which took several hours (see Figs 5.7B, 5.8B, 5.9B). Furthermore PHWE used an environmentally friendly solvent (water) to achieve the same or better results.

Conventional methods of removing templates like Soxhlet and ultrasonic extraction employ organic solvents to achieve optimal extraction which have detrimental effects to the environment.

5.3.3 Template bleeding

Figure 5.10 showed that template removal by PHWE was better than the other two methods employed as marked by the much lower template bleeding concentrations (0.01%) or non detectable in some cases. The relatively higher bleeding concentrations (over 1.00%) by Soxhlet and ultrasonic methods were a clear indication that the methods were not exhaustive in washing-off templates from MIPs.

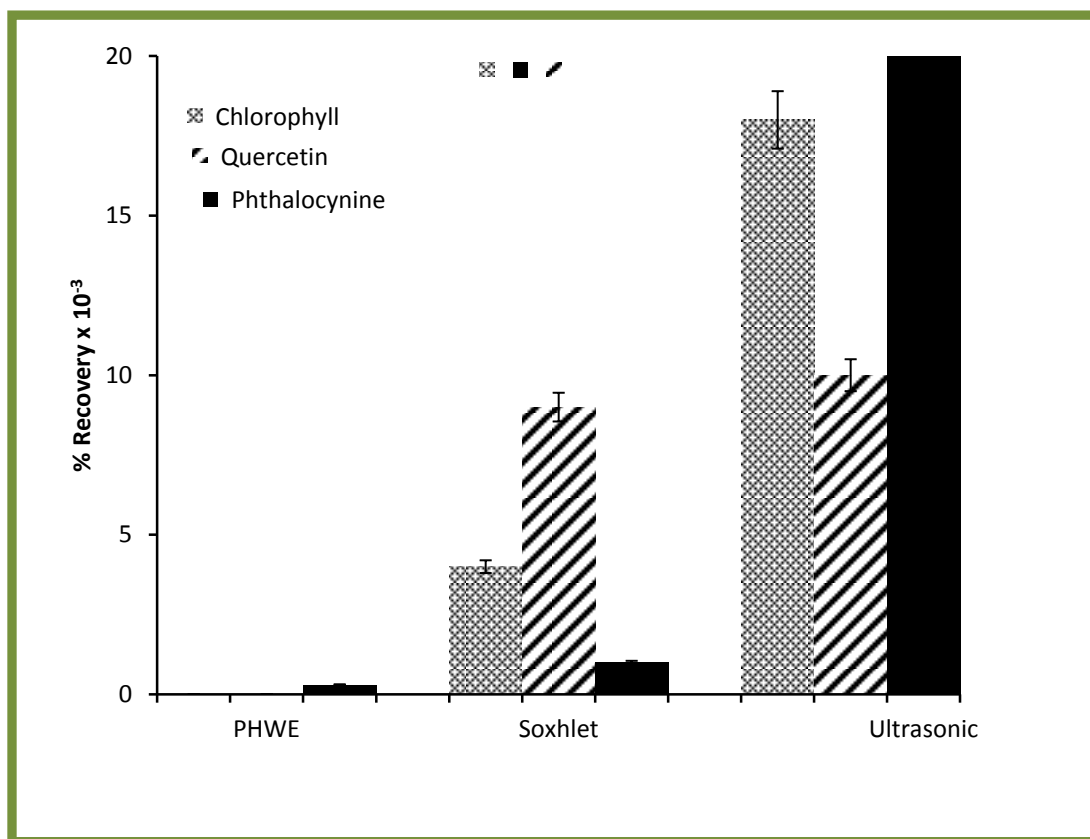


Fig. 5.10 Template removal by different extraction methods

5.3.4 Performance after template removal

There is a possibility of destroying/distorting the binding sites after extractions with different methods. The recoveries of the templates re-adsorbed by the MIPs post-extraction were used to evaluate if the binding sites were still intact. On average the MIPs adsorbed over 99.6% of templates when PHWE was employed to wash the MIPs and less than 94.5% for the other two methods (Table 5.2). The high percentage recoveries are a demonstration that the recognition sites of the MIPs were not destroyed/distorted and still had excellent selectivities even after employing the methods of extraction.

Table 5.2 Recoveries of MIPs after extraction with different methods (n=3)

MIP	Recovery (%)			
	Analyte	PHWE	Soxhlet	Ultrasonic
Chlorophyll	Chlorophyll	99.99 (0.7)	95.19 (2.64)	94.92 (2.51)
Quercetin	Quercetin	99.96 (1.76)	95.96 (1.37)	95.83 (1.44)
Phthalocyanine	Phthalocynine	99.98 (2.46)	95.41 (0.89)	94.64 (1.52)

5.4 Pressurized hot water extraction coupled to molecularly imprinted polymers for simultaneous of extraction and clean-up of pesticides residues in edible and medicinal plants of the Okavango Delta, Botswana

5.4.1 Pesticides detected employing PHWE-MIP

The PHWE-MIP method achieved simultaneous extraction and clean-up of pesticides from medicinal plants in the same extraction cell. PHWE employed an optimal temperature of 260 °C, pressure of 80 bars and flow rate of 1 mL min⁻¹ in 10 min for the extraction. On the other hand, the MIP performed a successful in-cell clean-up by selectively removing the interfering chlorophyll prior to GC-ECD/MS analysis. A total of nine pesticides were detected when the proposed method was employed in all the medicinal plants investigated. A representative chromatogram of the pesticides detected from *N. lotus* is depicted in Fig. 5.11. All other chromatograms from the rest of the plants showed a similar pattern.

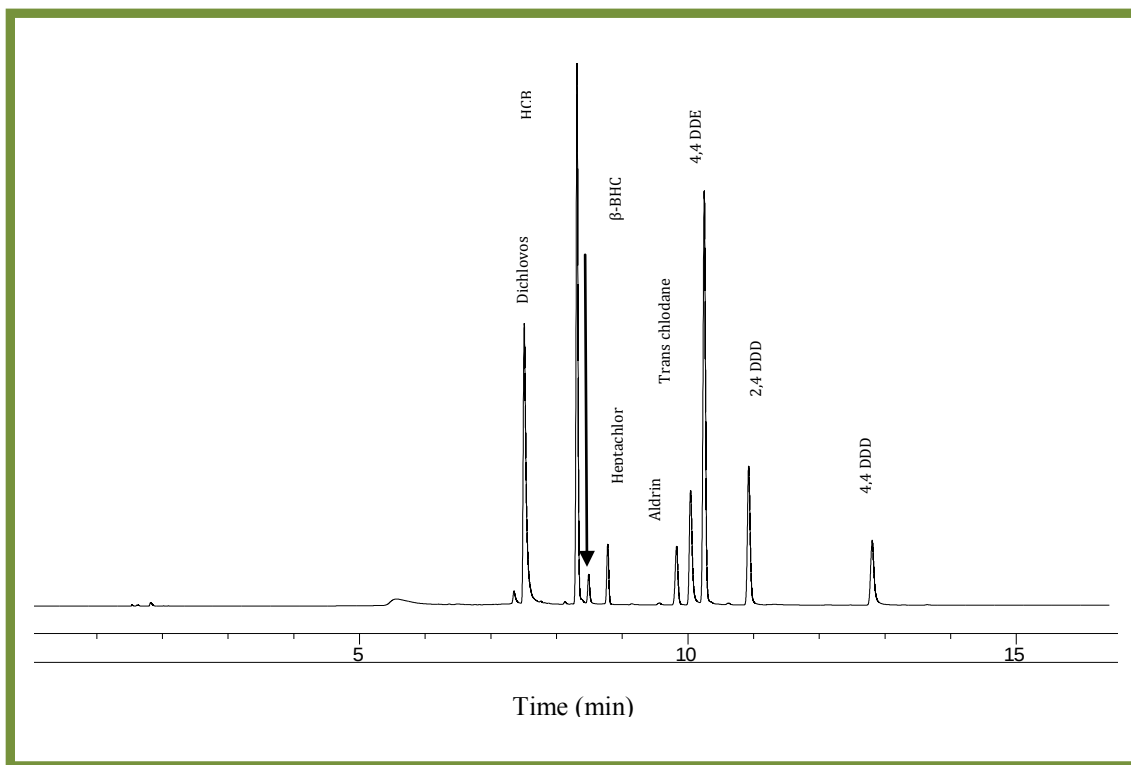


Fig. 5.11 A chromatogram of pesticides detected from *N. lotus* when PHWE was combined with a chlorophyll MIP.

5.4.2 Pesticides detected employing QuEChERS method

The extraction and clean-up of pesticides from medicinal plants was also achieved employing QuEChERS method. However, the detection of pesticides with planar structures (HCB, 4,4 DDD, 2,4 DDE and dichlorvos) was significantly reduced relative to the PHWE-MIP method. Only seven pesticides were detected (see Fig 5.12) from the same samples as the ones evaluated with the PHWE-MIP method. Currently, GCB is employed to remove the interfering chlorophyll during pesticides residue analysis in the dSPE step of the QuEChERS

method. While GCB is effective in removing chlorophyll from plant samples, it also removes planar pesticides

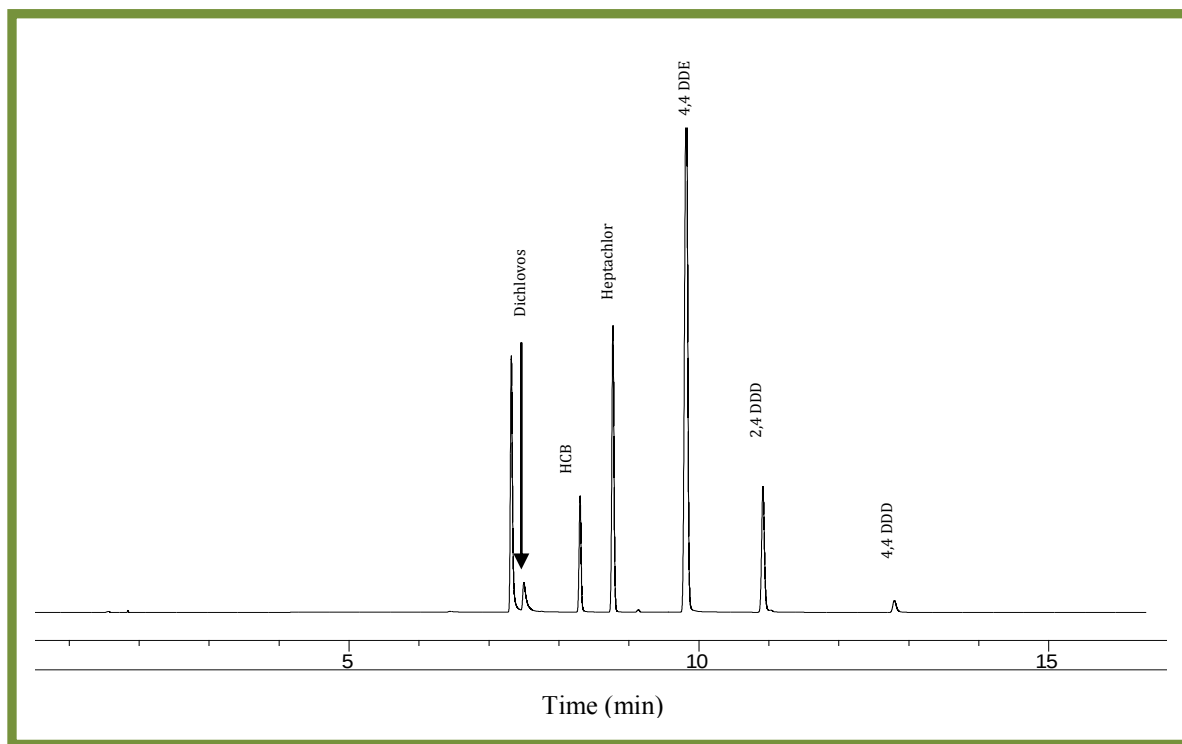


Fig. 5.12 A chromatogram of pesticides detected from *N. Lotus* when QuEChERS method was employed.

5.4.3 Comparison of the proposed PHWE-MIP method with the QuEChERS method

The linearity was determined by the analysis of all plant samples spiked at six levels of concentration, between 0.5 and 2.5 $\mu\text{g kg}^{-1}$, to obtain the calibration curves. The assays were acquired by GC-ECD. The linearity was evaluated by determining the correlation

coefficients, which were all greater than 0.999 for all the plants. The LOD and LOQ of the method were calculated at 3SD and 10SD respectively and they ranged from 0.06-0.3 $\mu\text{g kg}^{-1}$. Student's *t*-test was used to statistically compare the recovery and repeatability data of the two methods. At 95% confidence level, the analysis revealed no significant difference between the mean values of both methods. The overall range of recoveries for the various pesticides at 3 different spiking levels (0.1, 0.5 and 1.5 $\mu\text{g kg}^{-1}$) for both methods is as summarized in Table 5.3. However, it should be noted that the recoveries and repeatability of HCB; 4,4 DDD; 2,4 DDD; 4,4 DDE and dichlorvos were significantly reduced in the QuEChERS method compared to the proposed method by a factor of more than half.

This could be due to the fact that GCB has a layered planar structure. The structures of the pesticides are also planar and smaller, making them to be trapped easily between GCB layers as their geometry complement each other. Besides reduction in recoveries of pesticides of planar structures (see Table 5.3), there was more sample handling steps involved when the QuEChERS method was employed compared to the PHWE-MIP (see Fig. 4.2). There is usually loss of analytes and or contamination when there are too many sample handling step. Moreover, acetonitrile which is very toxic to the environment was employed in the QuEChERS method during the extraction step.

The PHWE-MIP method on the other hand was very selective to the targeted chlorophyll and it utilized only water for extraction, which is environmentally friendly. Furthermore, with the in-cell clean-up approach, there is a possibility for automaton and higher sample

throughput and hence better quality of the results. The method was also simple and hence is proposed for the monitoring of pesticides residue analysis.

Table 5.3 Recoveries (%) of pesticides employing both methods for all the plant samples

	<i>Nymphaea lotus</i>		<i>Cyprus articulates</i>		<i>Acalpha vilicaulis</i>		<i>Cyprus papyrus</i>		<i>Phonix reclinata</i>	
	QuEChERS	PHWE- MIP	QuEChERS	PHWE- MIP	QuEChERS	PHWE- MIP	QuEChERS	PHWE- MIP	QuEChERS	PHWE- MIP
4,4'-DDD	58.3	84.7	45.8	78.6	25.8	94.8	45.5	88.6	68.3	85.4
4,4'-DDE	42.9	87.1	36.4	93.1	45.2	89.3	34.6	87.0	58	90.2
Heptachlor	89.2	90.1	91.4	87.2	78.3	78.3	89.9	87.3	58.5	65.6
TransChlodane	78.4	76.4	89.3	89.3	81.3	78.5	87.4	89.3	85.5	78.3
Dichlorvos	25.7	89.6	67.3	89.4	56.3	92.3	67.2	95.3	50.3	86.3
α-BHC	84.9	90.2	89.5	91.4	96.3	91.4	94.6	90.2	94.4	88.4
HCB	17.3	94.6	12.4	89.4	14.7	94.6	0	78.3	28.0	87.4
2,4 DDD	43.7	85.3	33.8	78.2	27.3	89.4	25.0	76.9	26.5	85.4
Aldrin	83.5	89.8	83.2	78.4	78.4	87.4	78.9	79.1	70.3	69.99

RSD <10

5.4.4 Application of PHWE-MIP method to real samples

The proposed method was employed in the analysis of real samples. The levels of the pesticides accumulation in roots and leaves of different plants are presented in Table 5.4. The results showed a wide variation in the quantities of the pesticides in different tissues of the various plant species. Generally, it was observed that the roots accumulated higher concentration levels of the pesticides. This could be due to the fact that the roots were more exposed to the water and sediments which are reported to contain high levels of organochlorine pesticides [285]. Among the plant species, *N. lotus* recorded the highest concentration of all the pesticides detected in the root portion. *N. lotus* was more exposed to the water than all other plants since it is aquatic, therefore there could be direct contact with the contaminated water and sediments.

Table 5.4 Concentration of pesticides ($\mu\text{g kg}^{-1}$) detected in different plant parts

Pesticide	<i>Numphea lotus</i>		<i>Cyperus articulatus</i>		<i>Acalypha filicaulis</i>		<i>Cyperus papyrus</i>		<i>Phoenix reclinata</i>	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
4,4 DDD	1.41	± 2.47	0.35	01.11	0.53	0.53	0.5	0.56	0.68	0.25
	0.02	±0.01	±0.03	±0.01	±0.01	±0.71	±0.01	±0.01	±0.05	±0.01
4,2 DDD	0.32	0.33	0.16	0.9	0.64	1.06	0.36	0.65	0.58	0
	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	
4,4 DDD	0.3	0.53	0.26	0.76	0.25	0.6	0.25	0.22	0.58	0.65
	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01
HCB	0.28	0.71	1.69	0.7	0.38	0.5	0.692	1.0	0.85	0.25
	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01
B-BHC	1.33	1.39	0.21	0.65	0.61	0.46	0.21	0.6	0.5	0.36
	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01
Trans	0	0	0.33	0	0.94	0.31	0.46	0.5	0.94	0.58
chlordane			±0.01			±0.01	±0.01	±0.01	±0.01	±0.01
Aldrin	0.35	0.74	0.26	0.8	0.06	0.92	0.36	0.7	0	0.14
	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01	±0.03		±0.02
Dichlovos	0.54	0.32	0	0.56	0.54	0.25	0.25	0.	0.25	0.54
	±0.01	±0.01		±0.01	±0.01	±0.01	±0.01			±0.01
Heptachlor	0.80	1.31	0.23	0.61	0.35	0.23	0	1.9	0.2±0.01	0.59
	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01		±0.04		±0.01

The plant samples were divided into three regions of sampling sites; the Panhandle, the lower and the upper part of the Delta, indicated on the map (see Fig. 5.13). It was observed that plants from the Panhandle part of the delta contained the lowest concentration of the pesticides ($0.1 - 0.6 \mu\text{g kg}^{-1}$), while the plants from the lower part of the delta contained the highest concentrations ($0.8 - 1.1 \mu\text{g kg}^{-1}$). The trend may be due to the direction of flow of

the water as well as the low topographic gradient of the Delta which has led to the low flow rates [285]. Low flow rates allow partitioning of the water insoluble components such as pesticides onto suspended matter that subsequently settles to the bottom of the river becoming part of the sediment. Thus pesticides are more likely to be adsorbed onto organic-rich sediment relative to the sandy sediment, characteristic of the Panhandle as reported by Daka et al. [286].

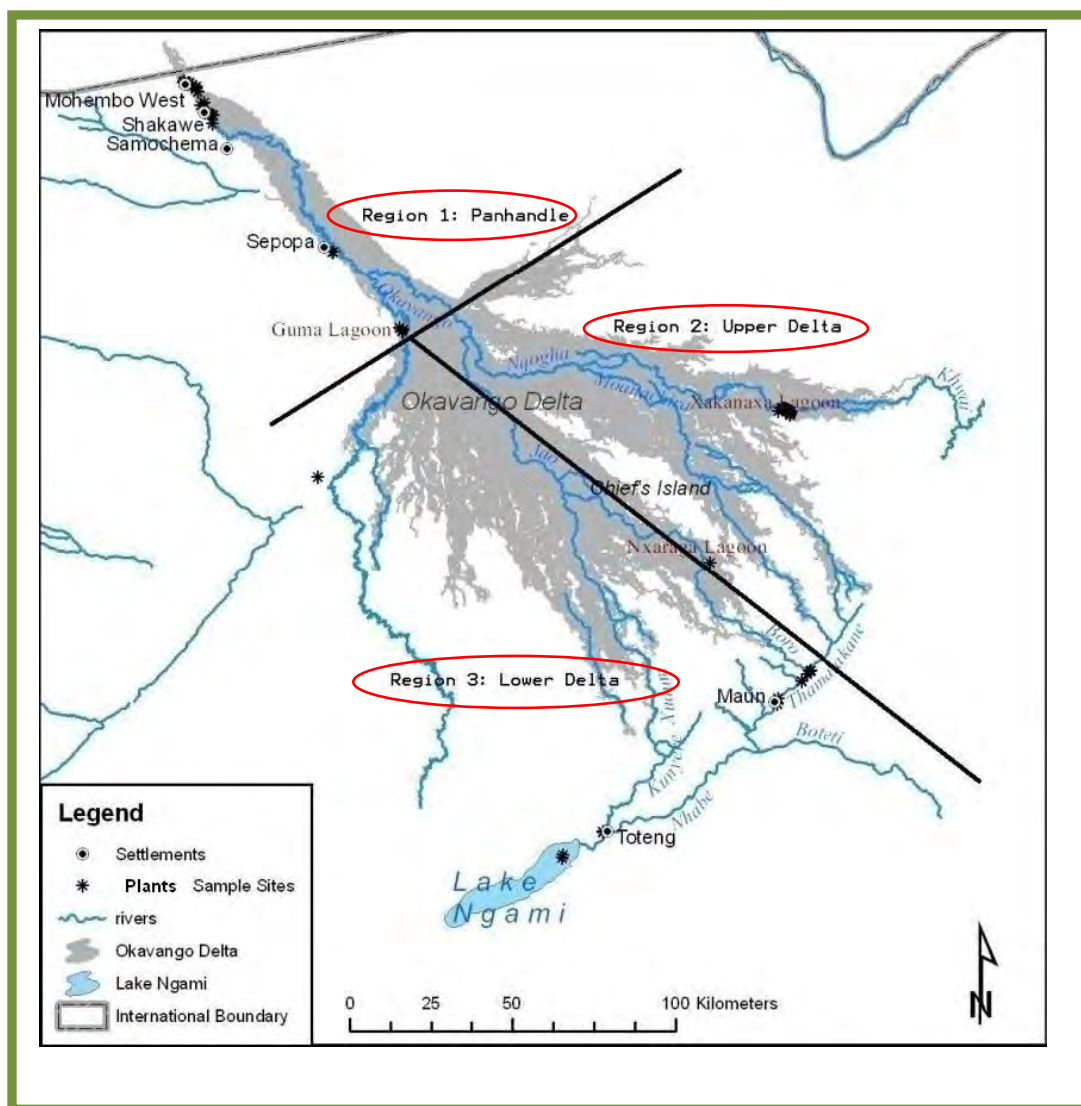


Fig. 5.13 Map of the Okavango Delta showing 3 regions of the plants sampling sites [280]

In aqueous and marine environments, OCPs tend to have strong affinities for suspended particulates and accumulate in sediment. The determination of OCP levels in sediments can therefore indicate the level of contamination and bioaccumulation in aquatic organisms and plants. DDT was employed for aerial spraying by health authorities until late 1990

[287]. The presence of DDT metabolites in plant samples at the peripheries of the delta could be due to the fact that the areas act as final catchments for the water. Lake Ngami, for example, is at the receiving end of the delta (see Fig. 5.13), and is not fed by any other water source. The sediments in peripheral areas are rich in organic matter, capable of accumulating considerable quantities of the pesticides by adsorption thereby transferring them to the plants that feed on the water. Alternatively, there could be a subsistent input of pesticides employed on vegetable farming as well as industrial application by the riparian community.

OCPs such as HCB and aldrin are employed in agriculture due to their effectiveness against various pests. OCPs are capable of travelling long distances over a considerable period of time. The possibility of them being employed somewhere within the Okavango River Basin could not be overruled. It should be noted however, that the concentration recovered from all the plants was still lower than MRLs of $250 \mu\text{g kg}^{-1}$ (see Fig. 5.14), of organochlorine pesticides in solid matrices; EPA method 3545 [288]. However, it is important to understand the factors influencing transport bound contaminants.

2,4 DDD, 4,4 DDD, 4,4 DDE, HCB and aldrin were positively confirmed with a MS.

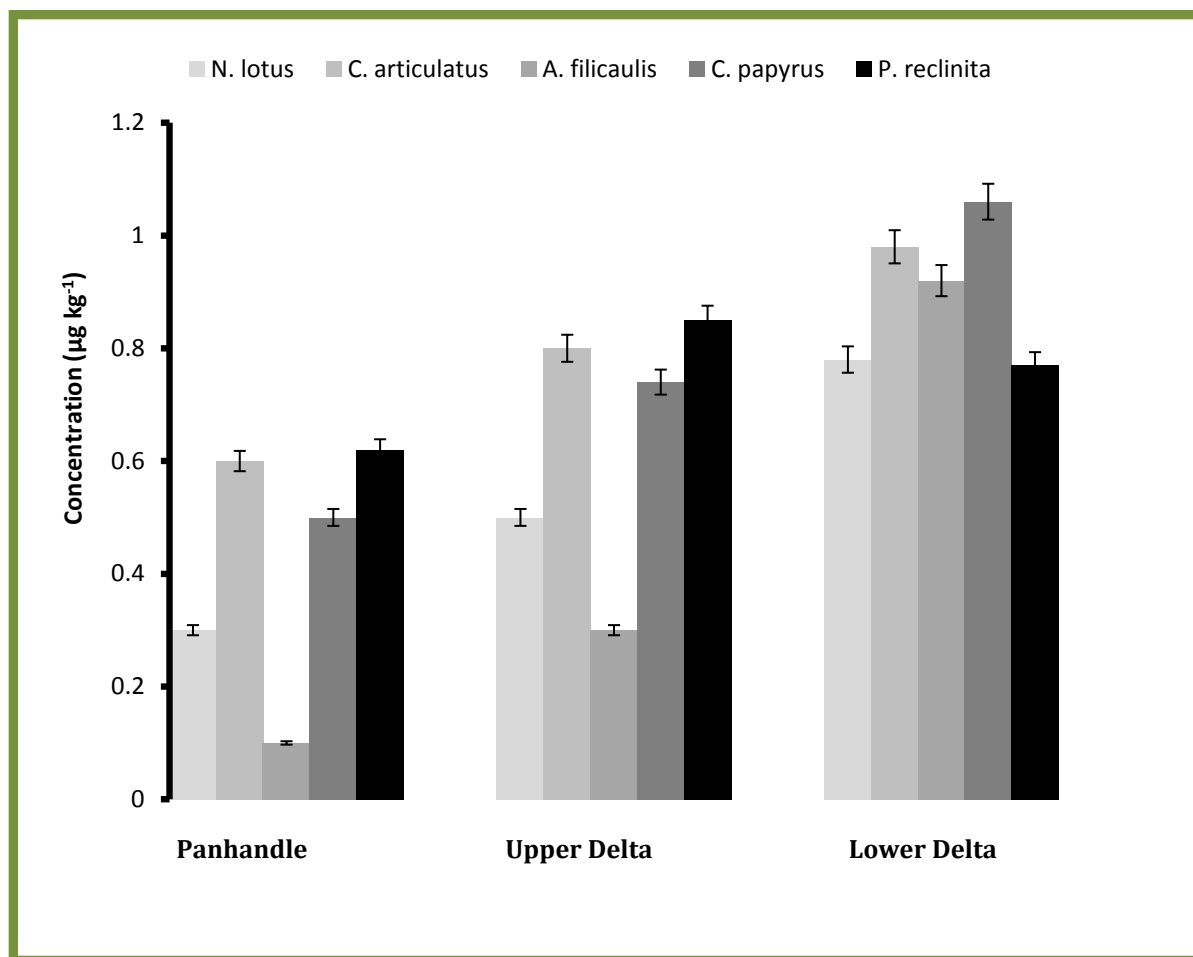


Fig. 5.14 Concentration of 4,4DDE in different plants from all sampling regions. All other pesticides show a similar trend.

5.5 Simultaneous extraction, clean-up and desorption of aflatoxins in medicinal plants with pressurized hot water extraction coupled to molecularly imprinted polymers

5.5.1 PHWE procedure

Aflatoxins AFG2, AFG1, AFB2 and AFB1 were serially extracted and cleaned up successfully by employing a flexible approach of PHWE-MIP followed by analysis with-HPLC-DAD (see a typical chromatogram is in Fig 5.15). Optimum extraction was achieved in the first extraction cell employing water at a temperature of 180 °C, pressure of 60 bars and a flow rate of 0.5 mL min⁻¹ for 10 min. The selectivity of PHWE was reduced at high temperature, resulting in co-extraction of other components of the plant material. Selectivity was therefore enhanced by performing in-cell clean-up in the second extraction cell using a class selective MIP for the aflatoxins AFG2, AFG1, AFB2 and AFB1. The solvent strength was increased by increasing the temperature of water for elution of the aflatoxins from the MIP in the second extraction cell. The conditions employed for elution of the aflatoxins were; temperature of 190 °C; pressure of 60 bars and a flow rate of 0.5 mL min⁻¹ for 10 min.

The procedure offers clear advantages as compared to the normal PHWE. It involves loading two extraction cells with sample and a selective MIP sorbent respectively; then extracting the sample; trapping the desired analytes on the sorbent; removing the sample cell, and then eluting the trapped material through from the sorbent. The MIP sorbent is very selective to aflatoxins, the bulk of matrix passes through during the extraction.

Moreover, only water was employed as a solvent for both extraction and elution. However, the strength of the 'solvent' was improved by just changing the temperature.

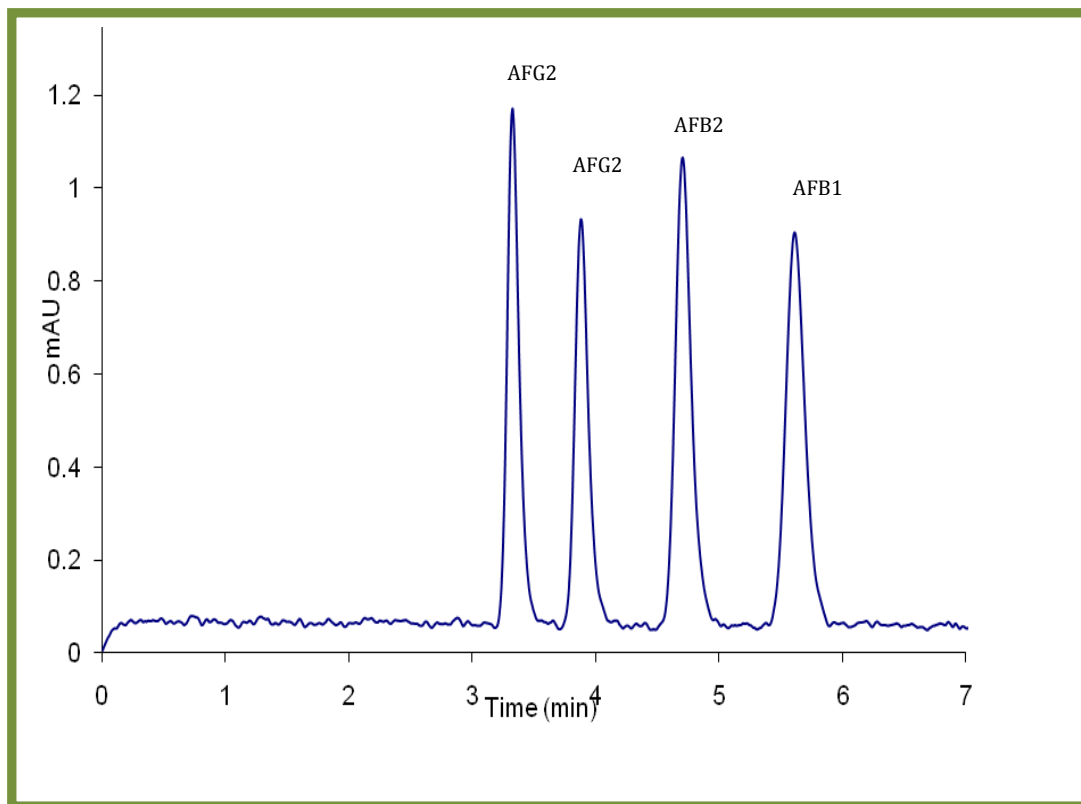


Fig. 5.15. A typical chromatogram of aflatoxins B1, B2, G1, and G2.

5.5.2 Comparison of the AOAC Official Method with the PHWE method

The PHWE in-cell clean-up method presented was applicable for quantitative determination of aflatoxins AFG2, AFG1, AFB2 and AFB1 in medicinal plants samples at a wide concentration range. Calibration curves were constructed by plotting the peak area

versus the concentration (0.1–1.0 µg L⁻¹) of aflatoxins dissolved in 70% methanol. The calibration curves of the four aflatoxins exhibited linearity with r² greater than 0.999. The RSD of five replicate analyses of medicinal plants spiked with aflatoxins was less than 5%, indicating high reproducibility of the method. Detection and quantitation limits of the aflatoxins were 0.06 µg L⁻¹ and 0.3 µg L⁻¹, respectively. The concentration of aflatoxins in the test samples (*C*) was calculated by the following equation:

$$C = A \times V \times D \times \frac{K}{W} \quad 5.1$$

where *A* is the concentration of aflatoxins determined from the calibration curve (µg L⁻¹); *V* is final volume of eluate (2 mL); *D* is a dilution factor (if necessary); *K* is a volume factor given by (15/20)×(15/5); *W* is the weight of sample used for the analysis (3 g). The recovery was given by the ratio of aflatoxins calculated using above equation to the concentration spiked on the medicinal plants. As observed in Table 5.5, the analysis of several samples by the developed method achieved comparable recoveries with the AOAC method (at 95% confidence level, n=5).

Table 5.5 Recoveries of aflatoxins by PHWE-MIP and AOAC methods

Medicinal plant/Method	Recoveries of aflatoxins (%)			
	G2	G1	B2	B1
<i>Pelargonium reniforme</i>				
PHWE	87.7 (3.7)	79.4(2.3)	78.5 (4.1)	68.3 (4.0)
AOAC	89.6 (1.0)	76.9 (5.0)	81.1 (3.4)	71.1 (2.3)
<i>Cassipourea flanaganii</i>				
PHWE	76.0 (4.4)	70.2 (4.6)	82.7 (3.2)	78.9 (2.6)
AOAC	73.1 (3.22)	71.8 (2.1)	86.3 (4.0)	82.8 (2.2)
<i>Bowiea volubilis</i>				
PHWE	77.0 (4.3)	72.2 (4.8)	68.7 (4.5)	80.4 (0.6)
AOAC	72.3 (0.1)	70.8 (5.0)	67.7 (4.3)	78.1 (2.6)
<i>Kedrostis foetidissima</i>				
PHWE	79.5 (0.6)	76.4 (4.0)	84.8 (1.7)	64.8 (2.9)
AOAC	81.4 (1.7)	76.9 (2.9)	79.1 (0.8)	60.4 (4.7)
<i>Hydnora africana</i>				
PHWE	89.2 (2.2)	82.1 (2.2)	75.8 (3.4)	74.8 (3.8)
AOAC	80.2 (1.9)	79.5 (4.8)	74.3 (3.1)	56.7 (4.3)

Analysis of plant samples by the developed method showed that the PHWE-MIP extraction yields were comparable or higher than the AOAC method (Table 5.6). The proposed method offered simultaneous extraction and clean-up which results in reduction in analysis costs; solvent consumption; manual labour and improved quality of analysis.

Table 5.6 concentrations of aflatoxins by PHWE-MIP and AOAC methods

Medicinal plant/Method	Concentration of aflatoxins ($\mu\text{g kg}^{-1}$)			
	G2	G1	B2	B1
<i>Pelargonium reniforme</i>				
PHWE	7.7 ± 0.7	9.4 ± 0.3	8.5 ± 1.1	8.3 ± 0.4
AOAC	9.6 ± 0.1	6.9 ± 0.5	11.1 ± 0.4	7.1 ± 0.2
<i>Cassipourea flanaganii</i>				
PHWE	6.0 ± 0.4	7.2 ± 0.6	2.7 ± 0.2	8.9 ± 0.6
AOAC	7.1 ± 0.2	7.8 ± 0.1	6.3 ± 0.4	12.8 ± 0.9
<i>Bowiea volubilis</i>				
PHWE	7.0 ± 0.3	7.2 ± 0.8	8.7 ± 0.5	8.4 ± 0.6
AOAC	6.3 ± 0.1	10.8 ± 0.5	6.7 ± 0.3	7.1 ± 0.6
<i>Kedrostis foetidissima</i>				
PHWE	9.5 ± 0.6	16.4 ± 0.4	8.8 ± 0.7	4.8 ± 0.9
AOAC	11.4 ± 0.7	6.9 ± 0.9	9.1 ± 0.8	6.4 ± 0.7
<i>Hydnora africana</i>				
PHWE	9.2 ± 0.2	2.1 ± 0.2	5.8 ± 0.4	4.8 ± 3.8
AOAC	8.2 ± 0.9	9.5 ± 0.8	4.3 ± 0.3	5.7 ± 4.3

5.6 Pressurized hot water extraction for desorption of pesticides from electrospun nanofiber sorbents.

5.6.1 Electrospinning

. Studies in our laboratory showed that a mixture of DMF:THF (4:1 v/v) was suitable for dissolving PS into solutions. The high conductivity of DMF and volatility of THF favoured the formation of smooth nanofibers during electrospinning [289, 290]. Hence, by varying the solution concentration, flow rate, applied voltage and distance between the needle tip and the collector, it was determined that 25% (w/v) PS gave smooth and bead-free nanofibers when electrospun at a feed rate of 0.10 mL h⁻¹ (see Fig. 5.16). The fiber diameters ranged from 130-500 nm. Such nanofibers with smooth morphologies and small diameters are characterized by high specific surface area preferred for a sorbent.

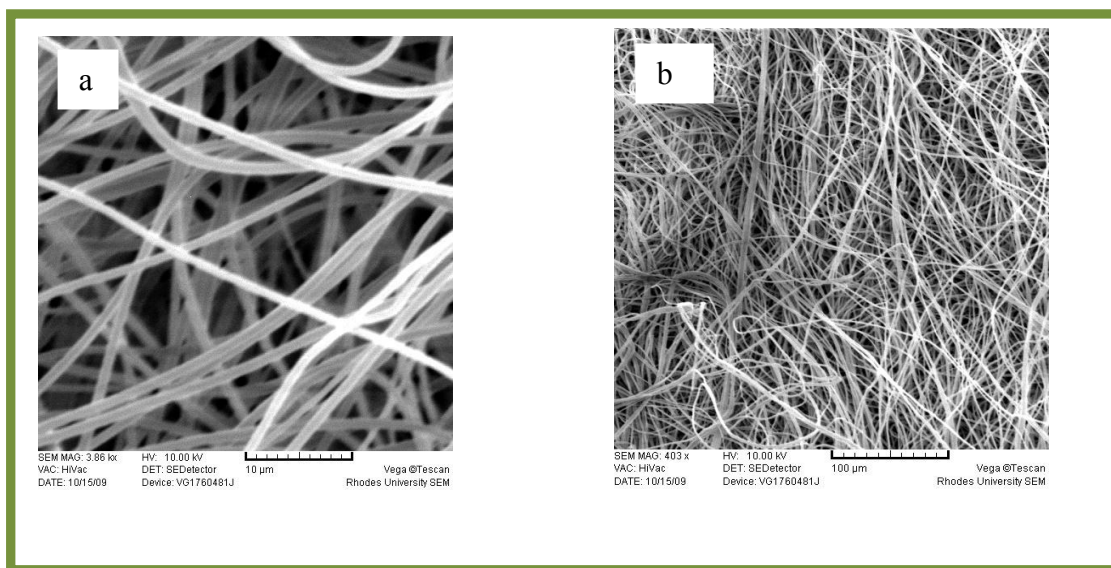


Fig. 5.16 SEM images of (a) Polystyrene (b) Carbodithioate-functionalized polystyrene nanofibers. (25% w/v)

5.6.2 Choice of nanofiber sorbents

The performance of nanofibers as sorbents in SPE largely depends on their surface morphology and the composition of polymer. The two nanofibers, one based on PS and the other, a PS incorporating carbodithioate were evaluated to determine their effectiveness as sorbent material for DDE. As illustrated in Table 5.7, the PS nanofiber incorporating carbodithioate gave better sorption recoveries than the native PS nanofiber. This is possibly due to the carboxyl groups and carbothiol group of the carbodithioate as they improved nanofibers wettability resulting in a strong adsorption affinity for the target analyte [291]. Although, there are large numbers of potential binding sites in

unfunctionalized PS nanofibers, the relatively higher hydrophobicity of the nanofibrous backbone may have obstructed them from interacting efficiently with the target compound.

Table 5.7 Adsorption (%) of DDE (0.25-1.00 $\mu\text{g L}^{-1}$) on 10 mg electrospun PS and carbodithioate functionalized PS nanofibers.

Concentration of analyte ($\mu\text{g L}^{-1}$)	Sample volume (μL)	% Sorption recovery on PS nanofibers (RSD)	% Sorption recovery on PS functionalized nanofibers (RSD)
0.25	500	38.7 (3.6)	82.5 (3.2)
0.50	200	14. (4.1)	30.6 (1.7)
	300	27.9 (3.6)	32.5 (2.3)
	400	37.8 (4.8)	34.8 (3.5)
	500	43.7 (3.7)	94.6 (1.5)
	600	41.3 (5.3)	84.1 (3.8)
0.75	500	40.2 (3.7)	80.5 (2.7)
1.00	500	38.7 (4.5)	63.1 (4.3)

5.6.3 Effect of contact time on sorption process

The contact time between the sorbent and the analyte is an important factor that can affect the rate of sorption. The results indicated that the analyte was initially adsorbed rapidly, until the sorbent got saturated. The equilibration times for maximum uptake of DDE on PS and carbodithioate functionalized PS nanofibers did not exceed 20 min. Incorporation of

carbodithiote in the PS doubled the surface interaction of the analyte with the sorbent material as reflected by the higher adsorption rate at optimal conditions.

5.6.4 Effects of sample volume on sorption process.

Sample volume is an important parameter that may affect the loading rate of analyte on a sorbent during sample preparation. If the volume of sample exceeds the breakthrough volume, it is possible that some of the target compound may not be adsorbed and get flushed away by the sample solution. Table 5.7 shows that optimum enrichment was achieved at 500 μL sample volumes. When the volume was increased to 600 μL , the recoveries declined significantly. The volume of sample used in this procedure was relatively much lower than the one used in conventional sorbents such as octadecylsilica SPE [292].

5.6.5 Optimization of temperature and time for the PHWE technique.

As illustrated in Table 5.8, optimum desorption was achieved at 260 $^{\circ}\text{C}$, 80 bar in 10 min employing only water at 0.5 mL min^{-1} . This is because at a higher temperature and elevated pressure, water exhibits fast diffusion and low viscosity, capable of extracting medium and non-polar compounds. A steady increase in desorption recoveries was noticed when the temperature was raised gradually from 180 to 260 $^{\circ}\text{C}$. However, a complete desorption was not achieved at 260 $^{\circ}\text{C}$, possibly as a result of precipitation of some of the analyte, as the water cooled [292] and due to DDE being hydrophobic in nature. A further increase in temperature to 270 $^{\circ}\text{C}$ resulted in lowering in desorption recoveries. This was probably due to partial degradation of some of the analyte.

Table 5.8: Desorption (%) of DDE sorbed onto the carbodithioate nanofiber from the spiked stock solution ($0.5 \mu\text{g L}^{-1}$) by PHWE technique at 80 bars and varying temperatures.

Pressurized hot water temperature ($^{\circ}\text{C}$)	Flow rate (mL min^{-1})	Duration of hot water flow (min)	Desorption recovery of DDE (%) RSD
180	0.5	20	20.5 (3.2)
	0.5	30	34.2 (3.1)
	0.5	40	34.2 (3.0)
	0.5	50	34.2 (3.4)
200	0.5	30	57.7 (3.8)
230	0.5	30	74.7 (2.5)
250	0.5	30	79.4 (4.1)
260	0.5	10	93.8 (3.8)
270	0.5	30	78.9 (3.5)

RSD: (n=6).

The GC-ECD based method employing nanofiber sorbents was found to be linear in the concentration range of $0.10\text{-}2.50 \mu\text{g L}^{-1}$. The correlation coefficient (r^2), slope and intercept values were 0.998, 0.00143 and 0.0150 respectively, with relative standard deviation (RSD), of less than 5% ($n=6$). The %R.S.D. for intra- and inter-day precision at three different concentrations; 0.10 , 1.00 , and $0.20 \mu\text{g L}^{-1}$ were less than 4.3%. The LOD and LOQ were found to be 0.000234 and $0.00765 \mu\text{g L}^{-1}$ respectively.

Chapter 6 Conclusion

This thesis has demonstrated the robustness and versatility of PHWE technique. The selectivity of extraction by PHWE can be improved by combining it with selective sorbents such as MIPs. Simultaneous extractions and in-cell clean-up can be performed to obtain purified extracts that are ready for instrumental analysis; all in line with current trends in analytical chemistry to automate and streamline the analytical procedures so as to reduce the analysis costs, solvent consumption, manual labour and increase the quality of analysis. PHWE has many advantages that make it a good alternative to traditional methods such as Soxhlet and solid-liquid extractions. Using water at elevated temperature and pressure during the extraction does not only improve the extraction yield, but also decreases time and solvent consumption. However, thermo-labile analytes may be degraded if the temperatures used are too high. Therefore a careful optimization of the extraction parameters must be performed. Alternatively, it can also be combined with other extraction techniques such as ultrasonication.

References

- [1] G. Romanik, E. Gilgenast, A. Przyjazny, M. Kamiński, *Biochem. Biophys. Methods* 70 (2007) 253.
- [2] P. C. Abhilash, N. Singh, *Bull Environ Contam Toxicol* 81 (2008) 604.
- [3] P.K. Gupta, M. Hubbard, B. Gurley, H. P. Hendrickson, *J Pharm. Biomed. Anal.* 49 (2009) 1021.
- [4] N. Fidalgo-Used, E. Blanco-González, A. Sanz-Medel, *Anal. Chim. Acta* 590 (2007) 1.
- [5] B. Kinsella, J. O'Mahony, E. Malone, M. Moloney, H. Cantwell, A. Furey, M. Danaher, *J. Chromatogr. A* 1216 (2009) 7977.
- [6] L. Ramos, J.J. Ramos, U.A. Th. Brinkman, *Anal Bioanal Chem* 381 (2005) 119.
- [7] M. Waldebäck, *Pressurized Fluid Extraction. A Sustainable Technique with Added Values*, Acta Universitatis Upsaliensis, Uppsala, 2005.
- [8] C. Nerín, *Anal Bioanal Chem* 388 (2007) 1001.
- [9] <http://www.aoac.org/Dietary-Supplement-web-site/Sampling.pdf>.
- [10] X. Fu, Y. Liao, H. Liu, *Anal Bioanal Chem* 381 (2005) 75.
- [11] W. Horwitz, L.R. Kamps, K.W. Boyer, *J. Assoc. Off. Anal. Chem.* 63 (1980) 1344.
- [12] M. Stoeppler, *Sampling and sample preparation*, Springer, Berlin 1997.
- [13] J. J. Pawliszyn, *Anal. Chem.* 75 (2003) 2543.
- [14] J.V Sweedler *Anal Bioanal Chem* 373 (2002) 321.
- [15] T. Hyotylainen, *Anal Bioanal Chem* 394 (2009) 743.
- [16] R.E Majors, *Liq Chromatogr Gas Chromatogr* 16 (2003) 71.
- [17] R.M Smith, *J Chromatogr. A* 1000 (2003) 3.

- [18] V .R. Meyer, LC GC Eur. 15 (2002) 398.
- [19] R.E Majors, R.E., LC GC Int. 5 (1991) 12.
- [20] V.F Samanidou, I.N.; Papadoyannis, Sample Preparation Prior to HPLC, Marcel Dekker, J. Cazes (Ed), Florida USA, pp 1-17, (2003).
- [21] C. L. Arthur, J. Pawliszyn, Anal Chem 62 (1990) 2145 – 2148.
- [22] I. Rodriguez, E. Rubi, R. Gonzalez, J.B. Quintana, R. Cela, Anal. Chim. Acta 537 (2005) 259 - 266.
- [23] A. Handley, Ed.; Extraction Methods in Organic Analysis; Sheffield Academic Press: Sheffield, UK, 1999.
- [24] S. Stern, Membrane Separation Technology; Elsevier: Amsterdam, 1995.
- [25] K. Pratt, J. Pawliszyn, Anal. Chem. 64 (1992) 2101.
- [26] M. Yang, M. Adams, J. Pawliszyn, Anal. Chem. 68 (1996) 2782.
- [27] H. Kataoka, H.L. Lord and J. Pawliszyn, J. Chromatogr. A 880 (2000) 35.
- [28] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P. Revilla-Ruiz, J. Hernandez-Mendez, J. Chromatogr. A 1089 (2005) 1.
- [29] R.M. Smith, J. Chromatogr. A 975 (2002) 31.
- [30] Y. J. Xue, J. Pursley, M. E. Arnold, J Pharm Biomed Anal 34 (2004) 369.
- [31] E. Thurman, M. Mills, Solid-Phase Extraction; John Wiley: New York, 1998.
- [32] Y. Ito, H. Oka, Y. Ikai, H. Matsumoto, J. Chromtogr. A 898 (2000) 95.
- [33] M.C. Hennion, J. Chromatogr. A 856 (1999) 3.
- [34] C. Dietz, J. Sanz, E. Sanz, R. Muñoz-Olivas, C. Cámara, J. Chromatogr. A 1153 (2007) 114.
- [35] C F Poole, S A Schuette, Contemporary Practxe of Chromatography, Elsevler, Amsterdam (1984) 429.

- [36] K. Ridgeway, S.P.D Lalljie, R.M. Smith, J Chromatogr. A 1153 (2007) 36.
- [37] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J. Schenck, J AOAC Int 86 (2003) 412.
- [38] S. J. Lehotay, K. Mastovska, A.R. Lightfield, J. AOAC Int., 88 (2005) 615.
- [39] N. Fontanals, R.M. Marce, F. Borrull, Trends Anal. Chem. 24 (2005) 394.
- [40] T. A. Gehring, L. G. Rushing, M. I. Churchwell, J. Agric. Food Chem. 44 (1996) 3164.
- [41] C.P. Desilets, M.A. Rounds, F.E. Regnier, J. Chromatogr. 544 (1991) 25.
- [42] C. Shafer, D. Lubda, J. Chromatogr. A 909 (2001) 73.
- [43] S. Souverain, S. Rudaz, J.-L. Veuthey, J. Chromatogr. B 801 (2004) 141.
- [44] E. Blahova, L. Bovanova, E. Brandsteterova, J. Liq. Chromatogr. 24 (2001) 3027.
- [45] K. Heinig, F. Bucheli, J. Chromatogr. B 769 (2002) 9.
- [46] C. He, Y. Long, K. Li, F. Liu, J. Biochem. Biophys. Methods 70 (2007) 133
- [47] N.D. Bertoncini, V. Pichon, M.C. Hennion, LC-GC Eur. 14 (3) (2001) 162.
- [48] K. S. Boss, A. Rudolphi, LC-GC Int. 15 (1997) 602.
- [49] N. Delaunay, V. Pichon, MC. Hennion, J. Chromatogr. B 745 (2000) 15.
- [50] A. Moser, M.A. Nelson, D.S. Hage In: J.M. Van Emon, Bioanal. Tech. CRC Press, New York (2006) 337.
- [51] N. Delaunay-Bertoncini, M.-C. Hennion, J. Pharm. Biomed. Anal. 34 (2004) 717.
- [52] M.C. Hennion and V. Pichon, J. Chromatogr. A 1000 (2003) 29
- [53] E. Watanabe, Y. Yoshimura, Y. Yuasa, H. Nakazawa, Anal. Chim. Acta 433 (2001) 199.
- [54] V. Pichon, A.I. Krasnova, M.C. Hennion, Chromatographia 60 (2004) S221-S226.
- [55] J. Gilbert, E. Anklam, Trends Anal. Chem. 21 (2002) 468.
- [56] C.K. Holtzapple, S.A. Buckley, L.H. Stanker, J. Agric. Food Chem. 47 (1999) 2963.

- [57] A.A.M. Stolker, P.L.W.J. Schwillens, L.A. Van Ginkel, U.A.Th. Brinkman, J. Chromatogr. A 893 (2000) 55.
- [58] H.X. Chen, Q.P. Deng, L.W. Zhang, X.X. Zhang, Talanta 78 (2009) 464.
- [59] E. Turiel, A. Martin-Esteban, Anal. Chim. Acta 668 (2010) 87.
- [60] S.A Piletsky, A.P.F Turner, Electroanalysis, 14 (2002) 317.
- [61] T. Pap, G. Horvai, J. Chromatogr. B 804 (2004) 167
- [62] K. Haupt, K. Mosbach, Chem. Rev. 100(2000) 2495.
- [63] <http://www.infu.tu-dortmund.de/english/Projects/MIP>.
- [64] E. Caro, R.M. Marcé, F. Borrull, P.A.G. Cormack, D.C. Sherrington Trends Anal. Chem. 25 (2006) 143.
- [65] L.I. Andersson, Bioseparation 10 (2002), 353.
- [66] C. Baggiani, L. Anfossi C. Giovannoli, Anal. Chim. Acta 591 (2007) 29.
- [67] V. Pichon, F. Chapuis-Hugon, Anal. Chim. Acta 622 (2008) 48.
- [68] C.M. Lok, R. Son, Int. Food Res. J. 16 (2009) 127.
- [69] S. Chigome, G. Darko, N. Torto, Analyst 136 (2011) 2879.
- [70] S. Chigome, N. Torto, Anal. Chim. Acta (2011) article in press.
- [71] S.V. Fridrikh, J.H. Yu, M.P. Brenner G.C. Rutledge Phys Rev Lett 90 (2003) 144501.
- [72] J.J Stankusa, J. Guan, K. Fujimoto, W.R Wagner, Biomaterials 27(2006) 735.
- [73] W. E. Teo, S. Ramakrishna, Nanotechnology 17 (2006) R89.
- [74] Z.M. Huang, Y.Z. Zhang, M. Kotaki, S. Ramakrishna, Compos. Sci. Technol. 63 (2003) 2223.
- [75] J.D. Schiffman, C.L. Schauer, Biomacromolecules 8 (2007) 594.
- [76] J.D. Schiffman, C.L. Schauer, Biomacromolecules 8 (2007) 2665.

- [77] J.D. Schiffman, Masters of Engineering; Cornell University, 2004.
- [78] L. Li, L.M. Bellan, H.G. Craighead, M.W. Frey, *Polymer* 47 (2006) 6208.
- [79] Y. Ji, B. Li, S. Ge, J.C. Sokolov, M.H. Rafailovich, *Langmuir* 22 (2006) 1321.
- [80] R. Gopal, S. Kaur, C.Y. Feng, C. Chan, S. Ramakrishna, S. Tabe, T. Matsuura, *J. Membr. Sci.* 289 (2007) 210.
- [81] L. Wannatong, A. Sirivat, P. Supaphol, *Polym. Int.* 53 (2004) 1851.
- [82] K.W. Kim, K.H. Lee, M.S. Khil, Y.S. Ho, H.Y. Kim, *Fibers and Polymers* 5 (2004) 122.
- [83] S.Y. Chew, J. Wen, E.K.F. Yim, K.W. Leong, *Biomacromolecules* 6 (2005) 2017.
- [84] E.R. Kenawy, J.M. Layman, J.R. Watkins, G.L. Bowlin, J.A. Matthews, D.G. Simpson, G.E. Wnek, *Biomaterials* 24 (2003) 907.
- [85] Y.M. Shin, M.M. Hohman, M.P. Brenner, G.C. Rutledge, *Polymer* 42 (2001) 09955.
- [86] G. Taylor, "Disintegration of water drops in an electric field", *Proc. R. Soc. London, Ser. A* 1964, 280, 383–397.
- [87] D.H. Reneker, A.L. Yarin, H. Fong, S. Koombhongse, *J. Appl. Phys.* 87 (2000) 4531.
- [88] T. Subbiah, G.S. Bhat, R.W. Tock, S. Parameswaran, S.S. Ramkumar, *J. Appl. Polym. Sci.* 96 (2005) 557.
- [89] Y.Q. Cai, G.B. Jiang, J.F. Liu, Q.X. Zhou, *Anal. Chem.* 75 (2003) 2517.
- [90] Y.Q. Cai, G.B. Jiang, J.F. Liu, Q.X. Zhou, *Anal. Chim. Acta* 494 (2003) 149.
- [91] Y.Q. Cai, Y.E. Cai, S.F. Mou, Y.Q. Lu, *J. Chromatogr. A* 1081 (2005) 245.
- [92] K. Jinno, M. Ogawa, I. Ueta, Y. Saito, *Trends Anal. Chem.* 26 (2007) 27.
- [93] Z.M. Huang, Y.Z. Zhang, M. Kotaki, S. Ramakrishna, *Compos. Sci. Technol.* 63 (2003) 2223.
- [94] I. Arambarri, M. Lasa, R. Garcia, E. Millán, *J. Chromatogr. A* 1033 (2004) 193.

- [95] J.F. Peng, J.F. Liu, H.L. Hu, G.B. Jiang, *J Chromatogr A* 1139 (2007) 165.
- [96] M. Algarra, V. Jiménez, P. Fornier de Violet, M. Lamotte, *Anal Bioanal Chem* 382 (2005) 1103.
- [97] <http://www.chromatography-online.org/topics/derivatization.html>.
- [98] A. Poletti, A. Groppi, C. Vignali, M. Montagna, *J. Chromatogr. B* 713 (1998) 265.
- [99] H.H. Maurer, J.W. Arlt, *J. Chromatogr. B* 714 (1998) 181.
- [100] T. Toyooka, *Modern Derivatization Methods for Separation Science*, Wiley, New York, 2002.
- [101] T. Andersson, Parameters affecting the extraction of polycyclic aromatic hydrocarbons with pressurized hot water. Doctoral thesis, University of Helsinki, 2007.
- [102] A. Mustafa, C. Turner, *Anal. Chim. Acta* 703 (2011) 8.
- [103] V. Camel, *J. Chromatogr. A* 975 (2002) 3.
- [104] C.C. Teo, S.N. Tan, J.W.H. Yong, C.S. Hew, E.S. Ong, *J. Chromatogr. A* 1217 (2010) 2484.
- [105] C. Turner, M. Waldebäck, Separation extraction and concentration processes in the food, beverage and nutraceutical industries, in: S. Rizvi (Ed.), in: *Series in Food Science, Technology and Nutrition*, vol. 202, Woodhead Publishing, Cambridge, 2010.
- [106] C. Turner, J.W. King, L. Mathiasson, *J. Chromatogr. A* 936 (2001) 215.
- [107] K. Srinivas, J.W. King, J.K. Monrad, L.R. Howard, C.M. Hansen, *J. Food Sci.* 74 (2009) E342.
- [108] E. Bjorklund, C. Turner, L. Karlsson, L. Mathiasson, B. Sivik, J. Skogsmo, *J. Supercrit. Fluids*, (1996) 56.
- [109] V. Camel, *Spectrochimica Acta B* 58 (2003) 1177 – 1233.
- [110] V. Lopez-Avila, *Crit. Rev. Anal. Chem.* 29 (1999) 195.

- [111] M.D. Luque de Castro and L.E. Garcia-Ayuso, *Anal. Chim. Acta*, 369 (1998) 1.
- [112] <http://www.epa.gov>.
- [113] L.J. Wang, C.L. Weller, *Trends Food Sci. Technol.* 17 (2006) 300.
- [114] <http://www.homechemistry.org/view/soxhletextractor>.
- [115] R. Zarnowski, Y. Suzuki, *Journal of Food Composition and Analysis* 17 (2004) 649.
- [116] P.K. Mamidipally and S.X. Liu, *European Journal of Lipid Science and Technology* 106 (2004) 122.
- [117] H. Li, L. Pordesimo, J. Weiss, *Food Research International* 37 (2004) 731.
- [118] J.L. Luque-Garcia, M.D. Luque de Castro, *Trends Anal. Chem.* 22 (2003) 41.
- [119] M.D. Luque de Castro and L.E. Garcia-Ayus, *Anal. Chim. Acta* 369 (1998) 1.
- [120] M. Suhaj, *J. Food Comp. Anal.* 19 (2006) 531.
- [121] M.D. Luque de Castro, F. Priego-Capote, *J. Chromatogr. A* 1217 (2010) 2383.
- [122] T. J Mason, L. Paniwnyk, J. P. Lorimer, *Ultrasonics Sonochemistry* 3 (1996) 253.
- [123] F. Priego-Capote, M.D. Luque de Castro, *Trends Anal. Chem.* 23 (2004) 644.
- [124] V. Lopez-Avila, *Crit. Rev. Anal. Chem.* 29 (1999) 195.
- [125] N. Hanari, Y. Horii, T. Okazawa, J. Falandysz, I. Bochentin, A. Orlikowska, T. Puzyn, B. Wyrzykowska, N. Yamashita, *J. Environ. Monit.* 6 (2004) 305.
- [126] J. Pan, Y.-L. Yang, Q. Xu, D.-Z. Chen, D.-L. Xi, *Chemosphere* 66 (2007) 1971.
- [127] P. Isosaari, A. Hallikainen, H. Kiviranta, P.J. Vuorinen, R. Parmanne, J. Koistinen, T. Vartiainen, *Environ. Pollut.* 141 (2006) 213.
- [128] P. Rodr'iguez-Sanmart'ın, A. Moreda-Pi'neiro, A. Bermejo-Barrera, P. Bermejo-Barrera, *Talanta* 66 (2005) 683.
- [129] N. Ratola, S. Lacorte, A. Alves, D. Barcel'ó, *J. Chromatogr. A* 1114 (2006) 198.

- [130] M. Jin, Y. Zhu, J. Chromatogr. A 1118 (2006) 111.
- [131] K.E.C. Smith, G.L. Northcott, K.C. Jones, J. Chromatogr. A 1116 (2006) 20.
- [132] C. Domeño, M. Blasco, C. Sánchez, C. Nerín, Anal. Chim. Acta 569 (2006) 103.
- [133] V. Camel, Trends Anal. Chem. 19 (2000) 229.
- [134] C.S. Eskilsson, E. Bjorklund, J. Chromatogr. A 902 (2000) 227.
- [135] L. Cai, J. Xing, L. Dong, C. Wu, J. Chromatogr. A 1015 (2003) 11.
- [136] M. Barriada-Pereira, E. Concha-Grana, M.J. Gonzalez-Castro, S. Muniategui-Lorenzo, P. L'opez-Mahía, D. Prada-Rodríguez, E. Fernández-Fernández, J. Chromatogr. A 1008 (2003) 115.
- [137] M. Barriada-Pereira, M.J. González-Castro, S. Muniategui-Lorenzo, P. L'opez-Mahía, D. Prada-Rodríguez, E. Fernández-Fernández, J. Chromatogr. A 1061 (2004) 133.
- [138] M. Barriada-Pereira, M.J. González-Castro, S. Muniategui-Lorenzo, P. L'opez-Mahía, D. Prada-Rodríguez, E. Fernández-Fernández, Chemosphere 58 (2005) 1571.
- [139] N. Carro, I. Garcia, M. Llompart, Analusis 28 (2000) 720.
- [140] G. Wittmann, T. Huybrechts, H. Van Langenhove, J. Dewulf, H. Nollet, J. Chromatogr. A 993 (2003) 71.
- [141] S. Bayen, H.K. Lee, J.P. Obbard, J. Chromatogr. A 1035 (2004) 291.
- [142] S. Bayen, O. Wurl, S. Karuppiyah, N. Sivasothi, H.K. Lee, J.P. Obbard, Chemosphere 61 (2005) 303.
- [143] T. Pena, L. Pensado, C. Casais, C. Mejuto, R. Phan-Tan-Luu, R. Cela, J. Chromatogr. A 1121 (2) (2006) 163.
- [144] B.E. Richter, B.A. Jones, J.L. Ezzell, N.L. Porter, N. Avdalovic, C. Pohl, Anal. Chem. 68 (1996) 1033.

- [145] L.J. Fitzpatrick, O. Zuloaga, N. Etxebarria, J.R. Dean, *Rev. Anal. Chem.* 19 (2000) 75.
- [146] H. Bautz, J. Polzer, L. Stieglitz, *J. Chromatogr. A* 815 (1998) 231.
- [147] D. Steytler, A. Grandison, M. Lewis (Eds.), *Separation Processes in the Food and Biotechnology Industries: Principles and Applications*, Woodhead Publishing Ltd., Abington, England, 1996.
- [148] E. Ramsey, Q. Sun, Z. Zhang, C. Zhang, W. Gou, *J. Environ. Sci.* 21 (2009) 720.
- [149] G. Brunner, *J. Food Eng.* 67 (2005) 21.
- [150] E. Schütz, *Chem. Eng. Technol.* 30 (2007) 685.
- [151] http://www.teamonslaught.fsnet.co.uk/co2_info.htm.
- [152] J.A. Mendiola, M. Herrero, A. Cifuentes, E. Ibáñez, *J. Chromatogr. A* 1152 (2007) 234.
- [153] E. Björklund, C. Sparr-Eskilsson, W. Paul, T. Alan, P. Colin, in: P. Worsfold, A. Townshend, C. Poole (Eds.), *Encyclopedia of Analytical Science*, Elsevier, Oxford, England, 2005, p. 597.
- [154] L.S. Daintree, A. Kordikowski, P. York, *Adv. Drug Deliv. Rev.* 60 (2008) 351.
- [155] R. Bauza, A. Ríos, M. Valcárcel, *Anal. Chim. Acta* 391 (1999) 253.
- [156] S. Mitra, *Sample Preparation Techniques in Analytical Chemistry*, John Wiley & Sons, Inc., Hoboken, USA, 2003.
- [157] W. Huang, Z. Li, H. Niu, D. Li, J. Zhang, *J. Food Eng.* 89 (2008) 298.
- [158] http://www.appliedseparations.com/supercritical/SFE_Education/Prime.asp.
- [159] M. Zougagh, M. Valcárcel, A. Ríos, *Trends Anal. Chem.* 23 (2004) 399.
- [160] A. J. King, J. W. Readman, J. L. Zhou, *Enviro. Geochem.* 25 (2003) 69.
- [161] Q. Lang, C. M. Wai, *Talanta* 53 (2001) 771.

- [162] S. Espinosa, M.S. Diaz, E.A. Brignole, J. Supercrit. Fluids 45 (2008) 213.
- [163] J.C. Chuang, K. Hart, J.S. Chang, L.E. Boman, J.M. Van Emon, A.W. Reed, Anal. Chim. Acta 444 (2001) 87.
- [164] M. Herrero, J.A. Mendiola, A. Cifuentes, E. Ibáñez, J. Chromatogr. A 1217 (2010) 2495.
- [165] B.E. Richter, B.A. Jones, J.L. Ezzell, N.L. Porter, N. Avdalovic, Anal. Chem. 68 (1996) 1033.
- [166] E.S. Ong, J.S.H. Cheong, D. Goh, J. Chromatogr. A 1112 (2006) 92.
- [167] B. Kaufmann, P. Christen, Phytochemical Analysis 13 (2002) 105.
- [168] S.B. Hawthorne, Y. Yang, D.J. Miller, , Anal Chem 66 (1994) 2912.
- [169] http://serc.carleton.edu/research_education/equilibria/phaserule.html.
- [170] J.W. King J. Assoc. Off. Anal. Chem. 81 (1998) 9.
- [171] D.J. Miller, S.B. Hawthorne, A.M. Gizir, A.A. Clifford, J. Chem. Eng. Data 43 (1998) 1043.
- [172] D.J. Miller, S.B. Hawthorne, Anal. Chem. 70 (1998) 1618.
- [173] M.S.S. Curren, J.W. King, Anal. Chem. 73 (2001) 740.
- [174] A.A. Clifford, M.D. Burford, S.B. Hawthorne, J.J. Langenfeld, D.J. Miller, J. Chem. Soc. Faraday Trans. 91 (1995) 1333.
- [175] H.J. Vandenburg, A.A. Clifford, K.D. Bartle, S.A. Zhu, J. Carroll, I.D. Newton, L.M. Garden, Anal. Chem. 70 (1998) 1943.
- [176] Y. Yang, S.B. Hawthorne, D.J. Miller, Environ. Sci. Technol. 31 (1997) 430.
- [177] C.A. Meyer, Steam Tables: Thermodynamic and Transport Properties of Steam, American Society of Mechanical Engineers, New York, 1993.
- [178] E.S. Ong, S.M. Len, Anal. Chem. Acta 482 (2003) 81.
- [179] E.S. Ong, S.M. Len, J. Sep. Sci. 26 (2003) 1533.

- [180] E.S. Ong, S.M. Len, J. Chromatogr. Sci. 42 (2004) 211.
- [181] E.S. Ong, S.O. Woo, Y.L. Yong, J. Chromatogr. A 22 (2000) 57.
- [182] E. Kremer, M. Rompa, B. Zygmunt, Chromatographia 60 (2004) S169.
- [183] Y. Cheng, S.M. Li, Int. J. Environ. Anal. Chem. 84 (2004) 367.
- [184] A.J.M. Lagadec, D.J. Miller, A.V. Lilke, S.B. Hawthorne, Environ. Sci. Technol. 34 (2000) 1542.
- [185] T. Anderson, T. Pihtsalmi, K. Hartonen, T. Hyötyläinen, M.L. Riekkola, Anal. Bioanal. Chem. 376 (2003) 1081.
- [186] L. Wennrich, P. Popp, J. Koller, J. Breuste, J. AOAC Int. 84 (2001) 1194.
- [187] S. Rovio, K. Hartonen, Y. Holm, R. Hiltunen, M.-L. Riekkola, Flavour Fragr. J. 14 (1999) 399.
- [188] S.B. Hawthorne, Y. Yang, D.J. Miller, Anal. Chem. 66 (1994) 2912.
- [189] K. Kuosmanen, T. Hyötyläinen, K. Hartonen, J. Å. Jönsson, M.-L. Riekkola, Anal. Bioanal. Chem. 375 (2003) 389.
- [190] K. Kuosmanen, T. Hyötyläinen, K. Hartonen, M.-L. Riekkola, Analyst 128 (2003) 434.
- [191] X. Lou, D.J. Miller, S.B. Hawthorne, Anal. Chem. 72 (2000) 481.
- [192] K. Lüthje, T. Hyötyläinen, M.-L. Riekkola, J. Chromatogr. A 1025 (2004) 41.
- [193] S.B. Hawthorne, C.B. Grabanski, K.J. Hageman, D.J. Miller, J. Chromatogr. A 814 (1998) 151.
- [194] L. Wennrich, P. Popp, J. Breuste, Chromatographia Suppl. 53 (2001) S-380.
- [195] Y. Yang, B. Li, Anal. Chem. 71 (1999) 1491.

- [196] K. Kuosmanen, T. Hyötyläinen, K. Hartonen, M.-L. Riekkola, J. Chromatogr. A 943 (2001) 113.
- [197] S. Kipp, H. Peyrer, W. Kleiböhmer, Talanta 46 (1998) 385.
- [198] M. Herrero, D. Arráez-Román, A. Segura, E. Kenndler, B. Gius, M.A. Raggi, E. Ibáñez, A. Cifuentes, J. Chromatogr. A 1084 (2005) 54.
- [199] J. Kronholm, T. Kuosmanen, K. Hartonen, M.-L. Riekkola, Waste Manage. 23 (2003) 253.
- [200] Y. Yang, M. Belghazi, A. Lagadec, D.J. Miller, S.B. Hawthorne, J. Chromatogr. A 810 (1998) 149.
- [201] P. Karašek, J. Planeta, M. Roth, J. Chem. Eng. Data 51 (2006) 616.
- [202] A.R. Katritzky, S.M. Allin, M. Siskin, Acc. Chem. Res. 29 (1996) 399.
- [203] I. Windal, S. Hawthorne, E. de Pauw, Organohalogen Compd. 40 (1999) 591.
- [204] L.N. Konda, G. Füleky, G. Morovján, J. Agric. Food Chem. 50 (2002) 2338.
- [205] O. Chienthavorn, P. Su-in, Anal. Bioanal. Chem. 385 (2006) 83.
- [206] C. Crescenzi, G. D'Ascenzo, A. Di Corcia, M. Nazzari, S. Marchese, R. Samperi, Anal. Chem. 71 (1999) 2157.
- [207] K. Hartonen, "Supercritical Fluid Extraction and Pressurized Hot Water Extraction – Novel Environmentally Friendly Analytical Techniques, Biological and Environmental Applications", Doctoral thesis, University of Helsinki, Helsinki, 1999, p.26-32.
- [208] M.M. Jiménez-Carmona, J.L. Ubea, M.D. Luquede Castro, J. Chromatogr. A 855 (1999) 625.
- [209] R.S. Ayala, M.D. Luque de Castro, Food Chem. 75 (2001) 109.

- [210] K.J. Hageman, L. Mazeas, C.B. Grabanski, D.J. Miller, S.B. Hawthorne, *Anal. Chem.* 68 (1996) 3892.
- [211] M.S. Krieger, J.L. Wynn, R.N. Yoder, *J. Chromatogr. A* 897 (2000) 405.
- [212] S. Morales-Muñoz, J.L. Luque-García, M.D. Luque de Castro, *Spectrochim. Acta Part B* 58 (2003) 159.
- [212]. M.D. Luque de Castro, personal communication, Brugge, 2/2002.
- [213] M. Mukhopadhyay, P. Panja, *Sep. Purif. Technol.* 63 (2008) 539.
- [214] K. Kiathevest, M. Goto, M. Sasaki, P. Pavasant, A. Shotipruk, *Sep. Purif. Technol.* 66 (2009) 111.
- [215] D. Barceló, in: J. Pawliszyn (Ed), *Sampling and Sample Preparation for Field and Laboratory* , Elsevier, Amsterdam, 2002, p. 255
- [216] L. Chang, Y. Cheng, C. Chang, *Food Chem.* 84 (2004) 279.
- [217] A. Shotipruk, J. Kiatsongserm, P. Pavasant, M. Goto, M. Sasaki, *Biotechnol. Prog.* 20 (2004) 1872.
- [218] C.C. Teo, S.N. Tan, J.W.H. Yong, C.S. Hew, E.S. Ong, *J. Sep. Sci.* 32 (2009) 613.
- [219] A.H. Zaibunnisa, S. Norashikin, S. Mamot, H. Osman, *LWT Food Sci. Technol.* 42 (2009) 233.
- [220] W. Andlauer, P. Furst, *Food Research International* 35 (2002) 171.
- [221] L. Dauchet, P. Amouyel, S. Hercberg, J. Dallongeville, *Journal of Nutrition* 136 (2006) 2588.
- [222] C. Zhang, S.C. Ho, Y. Chen, J. Fu, S. Cheng, F. Lin, *International Journal of Cancer* 125 (2009) 181.

- [223] L.I. Mignone, E. Giovannucci, P.A. Newcomb, L. Titus-Ernstoff, A. Trentham- Dietz, J.M. Hampton, W.C. Willett, K.M. Egan, International Journal of Cancer 124 (2009) 2929.
- [224] C.C. Teo, S.N. Tan, J.W.H. Yong, C.S. Hew, E.S. Ong, J. Chromatogr. A 1182 (2008) 34.
- [225] M.H. Eikani, F.G. Mohammad, M. Mirza, S. Rowshanzamir, J. Food Eng. 30 (2007) 255.
- [226] M.Z. Ozel, H. Kaymaz, Anal. Bioanal. Chem. 379 (2004) 1127.
- [227] M.Z. Ozel, F. Gogus, J.F. Hamilton, A.C. Lewis, Anal. Bioanal. Chem. 382 (2005) 115.
- [228] M. Ollanketo, A. Peltoketo, K. Hartonen, R. Hiltunen, M.L Riekkola, Eur. Food Res. Technol., 215 (2002) 158.
- [229] T. Hyotylainen, LCGC Asia Pacific 12 (2009) 6.
- [230] E. Ibanez, A. Kubatova, F.J. Senorans, S. Caverro, G. Reglero, S.B. Hawthorne, J. Agric. Food Chem, 51 (2003) 375.
- [231] Z.Y Ju, L.R. Howard, J. Food Sci, 70 (2005) S270.
- [232] C. Turner, P. Turner, G. Jacobson, K. Almgren, M. Waldeback, P. Sjöberg, E. Nordberg-Karlsson, K.E. Markides, Green Chem. 8 (2006) 949.
- [233] M. Garcia-Marino, J.C. Rivas-Gonzalo, E. Ibanez, C. Garcia-Moreno, Anal. Chim. Acta, 63 (2006) 44.
- [234] J.W. King, R.D. Grabiell, J.D. Wightman, Proceedings of the 6th International Symposium on Supercritical Fluids ISSF – 28-30 April, Versailles (France).(2003) 409.
- [235] Z. Pineiro, M. Palma, C.G. Barroso, J. Chromatogr. A 1026 (2004) 19.2004.
- [236] M.H. Eikani, F.G. Mohammad, M. Mirza, S. Rowshanzamir, J. Food Eng. 30 (2007) 255.
- [237] C.H.L. Hoa, J.E. Cacace, G. Mazzaa, LWT Food Sci. Technol. 40 (2007) 1637.
- [238] S. Hata, J. Wiboonsirikul, A. Maeda, Y. Kimura, S. Adachi, Biochem. Eng. J. 40 (2008) 44.
- [239] M.G. Marino, J.C.R. Gonzalo, E. Ibanez, C.G. Moreno, Anal. Chim. Acta 563 (2006) 44.

- [240] G.F. Barbero, M. Palma, C. Barroso, J. Agric. Food Chem. 54 (2006) 3231.
- [241] T. Mroczek, J. Mazurek, Anal. Chim. Acta 633 (2009) 188.
- [242] C.H.L. Ho, J.E. Cacace, G. Mazza, Lwt-Food Science and Technology 40 (2007) 1637.
- [243] S.M. Ghoreishi, R.G. Shahrestani, J. Food Eng. 93 (2009) 474.
- [244] A.I. Smeds, P.C. Eklund, R.E. Sjöholm, S.M. Willfor, S. Nishibe, T. Deyama, B.R. Holmbom, J. Agric. Food Chem. 55 (2007) 1337.
- [245] D. Bansleben, I. Schellenberg, A.C. Wolff, J. Sci. Food Agric. 88 (2008) 1949.
- [246] J. Mokgadi, L.C. Mmualefe N. Torto N. "Agilent sampliQ solid phase extraction sorbent in the clean-up of alkaloids in goldenseal by HPLC-DAD". In: Environmental Application Note. Interchim, AT (Ed) Agilent Technologies. United States of America. 2009. 1-6. ISBN: 5990-4188.
- [247] IARC monographs on the evaluation of carcinogenic risks to humans, vol. 82, some traditional medicines, some mycotoxins, naphthalene and styrene. International Agency for Research on Cancer, Lyon, 1993, p. 171.
- [248] A.L. Chiuchiolo, R.M. Dickhut, M.A. Cochran, H.W. Ducklow, Environ. Sci. Technol. 38 (2004) 3551.
- [249] IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56, some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, International Agency for Research on Cancer, Lyon, 1993, p. 245.
- [250] H. Nakata, Y. Hirakawa, M. Kawazoe, T. Nakabo, K. Arizono, S. Abe, T. Kitano, H. Shimada, I. Watanabe, W. Li, X. Ding, Environ. Pollut. 133 (2005) 415.
- [251] www.epa.gov/pesticides, 1.10.2003.
- [252] K.A. Scudamore, S. Patel, Food Addit. Contam. 17 (2000) 407.

- [253] European Commission, Commission Regulation (98/53/EC), Off. J. Eur. Communities: Legis. 1998, L201/93.
- [254] Z. Y. Ju and L. R. Howard, J. Agric. Food Chem. 51 (2003) 5207.
- [255] A. Juan-Garcia, G. Font, C. Juan, Y. Pico, Food Chem. 120 (2005) 1242.
- [256] K. Lüthje, T. Hyötyläinen, M. Rautiainen-Rämä, M.-L. Riekkola Analyst 130 (2005) 52.
- [257] K. Kuosmanen, T. Hyotylainen, K. Hartonen, M.L. Riekkola, Analyst 128 (2003) 434.
- [258] T.A. Andersson, K.M. Hartonen, M.L. Riekkola, J. Chem. Eng. Data 50 (2005) 1177.
- [259] E.P. Lopez, M.D.L. Castro, Anal. Chim. Acta 511 (2004) 24.
- [260] F.J. Rivas, J. Hazard. Mater. B138 (2006) 234.
- [261] S. Gan, E.V. Lau, H.K. Ng, J. Hazard. Mater. 172 (2009) 532.
- [262] J. Kronholma, P.R. Ruiz, S.P. Porras, K. Hartonen, R.C. Martinez, M.L. Riekkola, J. Chromatogr. A 1022 (2004) 9.
- [263] K. Kuosmanen, T. Hyotylainen, K. Hartonen, M.L. Riekkola, J. Chromatogr. A 943 (2002) 113.
- [264] B. van Bavel, K. Hartonen, C. Rappe, M.L. Riekkola, Analyst 124 (1999) 1351.
- [265] J. Tollback, M.B. Bigata, C. Crescenzi, J. Strom, Anal. Chem. 80 (2008) 3159.
- [266] F. Bruno, R. Curini, A. Di Corcia, I. Fochi, M. Nazzari, R. Samperi, Environ. Sci. Technol. 36 (2002) 4156.
- [267] X. Amashukeli, C.C. Pelletier, J.P. Kirby, F.J. Grunthaner, J. Geophys. Res. 112 (2007) 234.
- [268] M. Garcia-Lopez, I. Rodrigue, R. Cela, J. Chromatogr. A 1216 (2009) 6986.

- [269] E. Ibáñez, A. Cifuentes, I. Rodríguez-Meizoso, J.A. Mendiola, G. Reglero, F.J. Señoráns, C. Turner, 2009. Device and procedure for the on-line extraction and drying of complex extracts. Patent: P200900164.
- [270] R.E. Sievers, E.T.S. Huang, J.A. Villa, J.K. Kawamoto, M.M. Evans, P.R. Brauer, *Pure Appl. Chem.* 73 (2001) 1299.
- [271] S. Lindhal, A. Ekman, S. Khan, C. Wennerberg, P. Borjesson, P.J.R. Sjöberg, E.N. Karlsson, C. Turner, *Green Chem.* 12 (2010) 159.
- [272] H. Ping-ping, Y. Ri-fu, Q. Tai-qiu, Z. Wei, L. Chun-mei, *Sep. Sci. Technol.* 45 (2010) 1433.
- [273] I.D. Wilson, U.A.T. Brinkman, *J. Chromatog.* 1000 (2003) 325.
- [274] <http://apps.webofknowledge.com>.
- [275] B.S. Batlokwa, J. Mokgadi, T. Nyokong, N. Torto, *Chromatographia* 73 (2010) 589.
- [276] K. Hartonen, J. Parshintsev, K. Sandberg, E. Bergelin, L. Nisula, M-L. Riekkola, *Talanta* 74 (2007) 32.
- [277] Ong ES, Cheong JSH, Goh D, *J Chromatogr A* 1112 (2006) 92.
- [278] Ellwanger A, Berggren C, Bayoudh S, Crecenzi C, Karlsson L, Owens PK, Ensing K, Cormack P, Sherrington D, Sellergren B *Analyst* 126 (2001) 784.
- [279] Peng-Ju W, Jun Y, Qing-De S, Yun G, Xiao-Lan Z, Ji-Bao C *Chin J Anal Chem* 35(2007) 484.
- [280] B. Mbongwe, M. Legrand, J.M. Blais, L.E. Kimpe, J.J. Ridal, D.R.S. Lean, *Environ. Toxicol. Chem.* 22 (2003) 7.
- [281] Ziabari M, Mottaghitalab A, Haggi A *Braz J Chem Eng* 26 (2009) 53.
- [282] Anderson EB, Long TE *Polymer* 51 (2010) 2447.

[283] E.V. Petersson, J. Liu, P.J.R. Sjöberg, R. Danielsson, C. Turner, *Anal. Chim. Acta.* 663 (2010) 27.

[284] A. Rubel, R. Bierl, *Fresenius J. Anal. Chem.* 364 (1999) 648.

- [285] L.C. Mmualefe N. Torto, P. Huntsman-Mapila, B. Mbongwe, *Microchemical Journal* 91 (2009) 239.
- [286] P.S. Daka, V.C. Obuseng, N. Torto, P. Huntsman-Mapila, *Water SA* 32 (2006) 483.
- [287] M.L.H. Mabaso, B. Sharp, C. Lengeler, *Trop. Med. Int. Health* 9 (2004) 846.
- [288] <http://www.dionex.com/en-us/webdocs/68591-EPA-3545-ASE.pdf>.
- [289] D. Qi , X. Kang, L. Chen, Y. Zhang, H. Wei, Z. Gu *Anal Bioanal Chem* 390 (2008) 926.
- [290] K. Lee, H. Kim, Y. Ra, D. Lee, *Polymer* 44 (2003)1287.
- [291] E.B. Anderson, T.E Long, *Polymer* 51 (2010) 2447.
- [292] B. Bavel, K. Hartonen, C. Rappe, M-L Riekkola, *Analyst* 124 (1999) 1351.