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A study of electrospun nanofibers and diatomaceous earth materials for the extraction of alkaloids, flavonoids and aromatic amines in various matrices

A thesis submitted in fulfillment of the requirement for the degree of Master of Science in Chemistry

By

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Dedication

To my mom and my dad with all my love

Acknowledgements

I would like to thank the Almighty God for keeping me and sustaining me during this journey.

Prof. Nelson Torto: Thank you very much Prof. for your mentorship and guidance. You helped me realise my potential and I thank you for helping me to believe in myself.

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ABSTRACT

The thesis explored the use of different sorbent materials in solid phase extraction method development. The methods included the use of the polymeric Agilent Bond Elut Plexa solid phase extraction and electrospun polymer-silica composite sorbents for clean-up and pre-concentration. Sample clean-up for alkaloids (hydrastine and berberine) in goldenseal, *Hydrastis canadensis* and flavonoids (quercetin, kaempferol and isorhamnetin) in *Ginkgo biloba* was achieved using Bond Elut Plexa SPE sorbent. Clean-up of flavonoids in *Ginkgo biloba* was also achieved using electrospun polymer-silica composite (polystyrene-silica, polyacrylonitrile-silica and nylon 6-silica) sorbents. All analysis of flavonoids and alkaloids was carried out using an Agilent 1200 Series HPLC coupled with a diode array detector. Good peak separation was achieved in less than 6 min employing an Agilent ZORBAX Eclipse Plus C18 column (4.6 x 75 mm, 3.5 μm) at 35 ^{o}C. The mobile phases employed were 0.1% phosphoric acid/methanol gradient and 0.5% phosphoric acid/methanol (40:60) for alkaloids and flavonoids respectively. The calibration curves exhibited linearity up to 120 μg mL^{-1} with correlation coefficients of more than 0.9980. The recoveries ranged from 73-109% with relative standard deviation of less than 5% for all analytes.

Agilent Chem Elut supported liquid extraction was employed for the development of a sample preparation method for the determination of 24 banned aromatic amines from azo dyes in textile following the EU standard method EN 14362-1:2003 (E) and the Chinese standard method GB/T 17592-2006. The supported liquid extraction was effective in the extraction of the aromatic amines from textile (cotton, wool and polyester/cotton [80%:20%]). Most of the recoveries obtained were conforming to the minimum requirements set in the EN 14362-1:2003 (E) standard method and the relative standard deviations were less than 15%. Good peak separation was obtained within 70 min run time using the Agilent Zorbax SB-Phenyl column (4.6 mm x 250 mm, 5-micron) or the Agilent DB-35 MS (J & W) (30 m x 0.25 mm, 0.25 μm film thickness.

It was demonstrated that the polymeric Agilent Bond Elut Plexa, electrospun nanofibers and diatomaceous earth were effective in extraction of alkaloids, flavonoids and aromatic amines in different matrices. The developed methods were simple, rapid and reproducible.

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LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
BPA	Bisphenol A
DMF	Dimethylformamide
DZP	Dual zone phases
GC-MS	Gas Chromtography- Mass Spectrometry
HCL	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography-Diode Array Detector
ISRP	Internal surface reverse phases
KOH	Potassium hydroxide
LC-MS	Liquid Chromatography- Mass Spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave assisted extraction
MFP	Mixed functional phase
MIP	Molecularly imprinted Polymers
MSPD	Matrix solid phase dispersion
N	Number of theoretical plates
NaCl	Sodium chloride
NP-SPE	Normal phase SPE

NTP	National Toxicology Programme
PAHs	Polycyclic aromatic hydrocarbons
PAN	Polyacrylonitrile
PBDEs	Polybrominated diphenyl ethers
PDMS	Polydimethylsiloxane
PFE	Pressurised fluid extraction
PHWE	Pressurised Hot Water Extraction
PS-DVB	Polystyrene-divinylbenzene
PTFE	Polytetrafluoroethylene
PT-SPE	SPE pipette tip
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RAM	Restricted access media
RP-SPE	Reverse-phase SPE
RSD	Relative standard deviation
SBSE	Stir-Bar sorptive extraction
SEM	Scanning electron microscope
SEM-EDX	Scanning electron microscopy-Energy dispersive X-ray detection
SFE	Supercritical fluid extraction
SHP	Shielded hydrophobic phases
SLE	Supported liquid-liquid extraction
SPE	Solid Phase Extraction
SPE-GC	Solid phase extraction-Gas chromatography
SPE-LC	Solid phase extraction-liquid chromatography

SPME	Solid Phase Micro-extraction
SPS	Semi-permeable surfaces
TBBPA	Tetrabromobisphenol
TEM	Transmission electron microscope
TEOS	Tetraethylorthorsilicate
THF	Tetrahydrofuran
USEPA	U.S. Environmental Protection Agency
V_B	Breakthrough volume
Vm	Equilibrium volume
V_R	Retention volume
4-NP	4-nonylphenol

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CHAPTER 1: INTRODUCTION

This chapter provides an overview of sample handling procedures and sample preparation techniques. It discusses the theory of classical and modern sample preparation techniques. It also shows the important role the new sample preparation techniques play so as to meet the demands for high throughput analysis.

1.1 SAMPLE HANDLING

Sample handling is any action applied to the sample before the analytical procedure. Sample handling is a fundamental process in chemical analysis as it has an effect on the integrity of results. Poor sample handling could lead to loss of analyte due to instability or also could result in the addition of analyte through contamination and therefore end results would not be a true reflection of the collected sample [1]. Sampling, sample storage and sample preparation are the common steps involved in sample handling. Most of the analysis time is spent on these steps [2] (see Fig. 1.1).

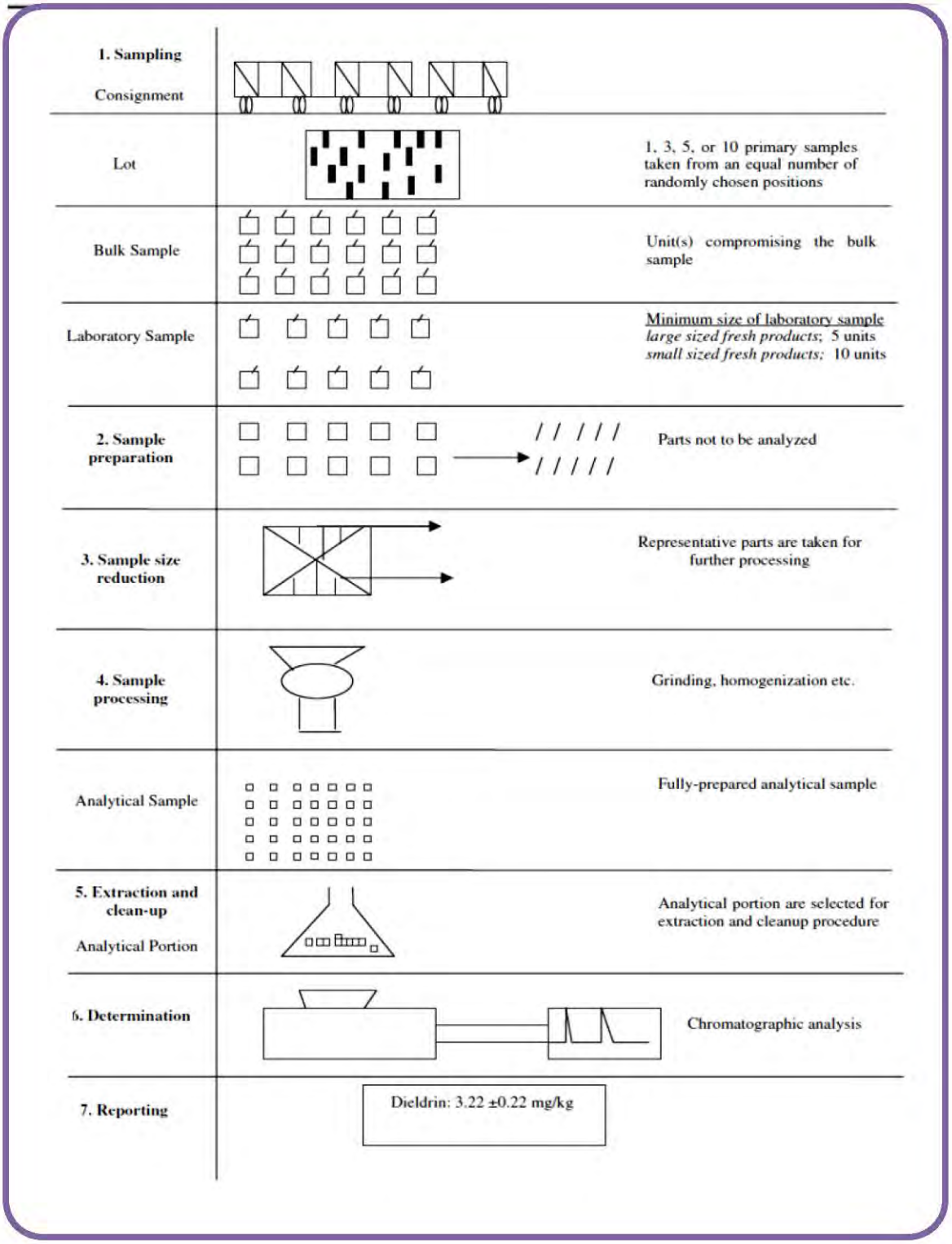


Fig 1.1 Sample handling process [3]

1.2 Sampling, sample collection and storage

Sampling is a process of taking a material that is a representative of the original whole/population. Samples usually originate from heterogeneous or homogeneous material [4]. Incorrect and non representative sample selection is the major source of error in the analysis process. A representative sample is one selected by applying a sampling plan consistent with the definition of the analytical problem addressed [5]. If the collected sample does not represent the population from which it is drawn, then the statistical analysis of the generated data may lead to misinformed conclusions and perhaps costly decisions [4].

In most environmental studies, samples are collected from various locations within a given site. Any subsequent subsamples are then considered as being representative of the site from which they were collected [5]. Incorrect mass reduction could negate the best field sampling designs resulting in highly variable and biased analytical results. An increase in sample size from the bulk material leads to a more representative sample as it approaches the bulk. While this is generally true, there is an upper limit to meet instrumental analysis requirements [4, 5].

Sample analysis cannot always be carried out immediately or within a short time after sampling [6]. Sample storage and preservation is very important because there are often delays between sample collection and analysis. Proper sample preservation ensures that the sample retains its physical and chemical characteristics from the time it is collected to the time it is analysed [7]. To ensure integrity of results care should be taken that proper containers are used, appropriate chemical preservation is carried out and storage requirements are followed [6]. In analysis of biological samples one of the major challenges is the instability of drugs, metabolites and prodrugs [116]. Much attention should be given as the stability of drugs could be affected by storage temperature, enzyme pH, anticoagulants and freeze-thaw cyles. Instability could also occur during the sample preparation process [8].

1.3 Sample preparation

Sample preparation is a process that aims at selective isolation of the analyte of interest from the matrix and if required concentrate the analyte of interest [6]**.** It is necessary to isolate the

desired components from complex matrices because most analytical instruments cannot handle the matrix directly [9, 10]. Sample preparation is the most time consuming and tedious step that yields most of the errors of the total analytical procedure [11]. The process may include multiple steps such as sample drying, homogenization, sieving, extraction, pre-concentration, sample work-up and hydrolysis [7]. Sample type and matrix composition contributes to the degree of difficulty during sample preparation and analyte determination [117]. Samples that are used in environmental and health studies normally occur as liquids or solids [12].

Liquid/aqueous samples can be directly analysed without prior sample preparation if satisfactory results can be obtained. In most cases minimal sample preparation (e.g dilution or filtration) may also be required [118]. Sample preparation for solid samples is more complex than liquid samples. Solid samples must be solubilised to extract the analyte of interest. If the sample is readily soluble, dissolution in a suitable solvent is an easy approach. Most of solid samples such as soils, plant materials and polymers are largely insoluble therefore special extraction techniques need to be employed [13]. A sample preparation technique is chosen based on the sample composition (looking at the possible behaviour of the matrix, chemical knowledge and the trace components to be analysed) [12].

Before sample preparation some preliminary processing like size reduction and drying should be carried out to ensure sample stability and homogeneity. Homogeneous samples resulting from size/particle reduction will in return improve the accuracy and precision of results [7]. Size reduction also improves solubility and extractability. There are various sample reduction techniques as shown in Table 1.1. After sample reduction, a suitable sample preparation technique is chosen. Researchers have been focused on developing new sample preparation techniques that can meet the demands for high throughput analysis.

Table 1.1: Sample reduction techniques [119]

Method	Description
Blending	Mechanical blender chops a semi-soft substance into smaller parts; can also refer to the blending of a non-homogeneous sample into a more consistent form
Chopping	Mechanically cutting a sample into smaller parts
Crushing	Tungsten carbide variable jaw crushers reduce large, hard samples to 1- to 15-mm diameters
Cutting	Cutting mills can reduce soft-to-medium hard materials (< 100 mm diameters) by using rotating and stationary cutting knives; reduced size depends on sieves used in combination with mill
Grinding	Mortar and pestle most popular; mechanical mortar grinders automate and standardize grinding to analytical fineness done manually with a mortar and pestle; both wet and dry grinding are used; fineness of approximate 10-mm diameters can be achieved
Homogenizing	Making a sample more uniform in texture and consistency by breaking down into smaller parts and blending
Macerating	Breaking down a soft material into smaller parts by tearing, chopping, cutting, etc.
Milling	Disks mills pulverize < 20-mm-diameter hard samples by feeding between stationary and rotating disks with adjustable gap settings; generally reduced to 0.1 mm in diameter. Rotor-speed mills combine impact and shearing processes to grind soft-to-medium hard and fibrous materials down to 0.08 mm; ball mills grind material to submicron fineness by developing high grinding energy via centrifugal or planetary actions using agate, tungsten carbide, or PTFE-coated stainless steel balls; a soil mill will gently pulverize dried samples of soils, sludges, clays, and similar material by rotating nylon brushes that throw a sample against a chamber wall
Mincing	Breaking down a meat or vegetable product into smaller parts by tearing, chopping, cutting, dicing, etc.
Pressing	Generally refers to squeezing liquid from a semi-solid material (e.g., plants, fruits, meat) for the purposes of further analysis
Pulverizing	Electromechanically driven rod or vibrating base is used to break particles down mechanically into smaller units; can be performed in wet or dry state; freezer mill can be used with liquid N2 to treat malleable samples
Sieving	Passing a sample through a metal or plastic mesh of a uniform cross-sectional area (square openings from 3 mm to 123 mm) to separate particles into uniform sizes; both wet and dry sieving can be used

1.3.1 Trends in sample preparation

There is increased public awareness that environmental contaminants are a health risk leading to formulation of directives that require routine analysis [14]. The new analysis methods should be accurate, sensitive and rapid. Recent trends in sample preparation include miniaturization, automation, high-throughput performance, on-line coupling with analytical instruments and reduction in solvent volume and time [15]. A lot of traditional sample preparation methods are still in use and the modern methods have been developed so as to achieve:

- use of small initial sample size even for trace analysis
- better specificity and selectivity
- automation or on-line methods to reduce manual operations and sample preparation timeline.
- green chemistry approach with less waste and the use of small volumes or no organic solvents[13].

Sample extraction is usually the first procedure in sample preparation for environmental materials. The main aim of sample extraction is to isolate analyte of interest from the matrix and quantitatively transfer the analyte to another medium, usually an organic solvent [16]. Choice of the extraction process will have an effect on the total analysis time, sample throughput and the analysis cost.

The sample extraction techniques range from the classical soxhlet extraction to modern microwave extraction [16]. The classical methods are the starting point for the development of new, more effective methods which use increased temperature and pressure [17]. New techniques have emerged that will certainly supersede the traditional techniques in the future as new legislation tends to restrict or even ban the use of many common solvents. The modern methods considerably reduce the solvent volumes required and the time needed for the extraction step [18].

There are different extraction processes. A suitable extraction process can be chosen depending on the type of sample (see Fig 1.2). Selectivity can be achieved by manipulating

the extraction temperature or pressure, extraction solvent, pH and use of additives [13]. The increase in temperature has influence in the following:

- increased solubility
- increased diffusion rates and mass transfer
- activation energy of desorption is more readily overcome

The increase in pressure has influence in the following:

- forces liquid into porous material
- Extraction cell fills faster
- Keeps solvent at operating temperature [120]

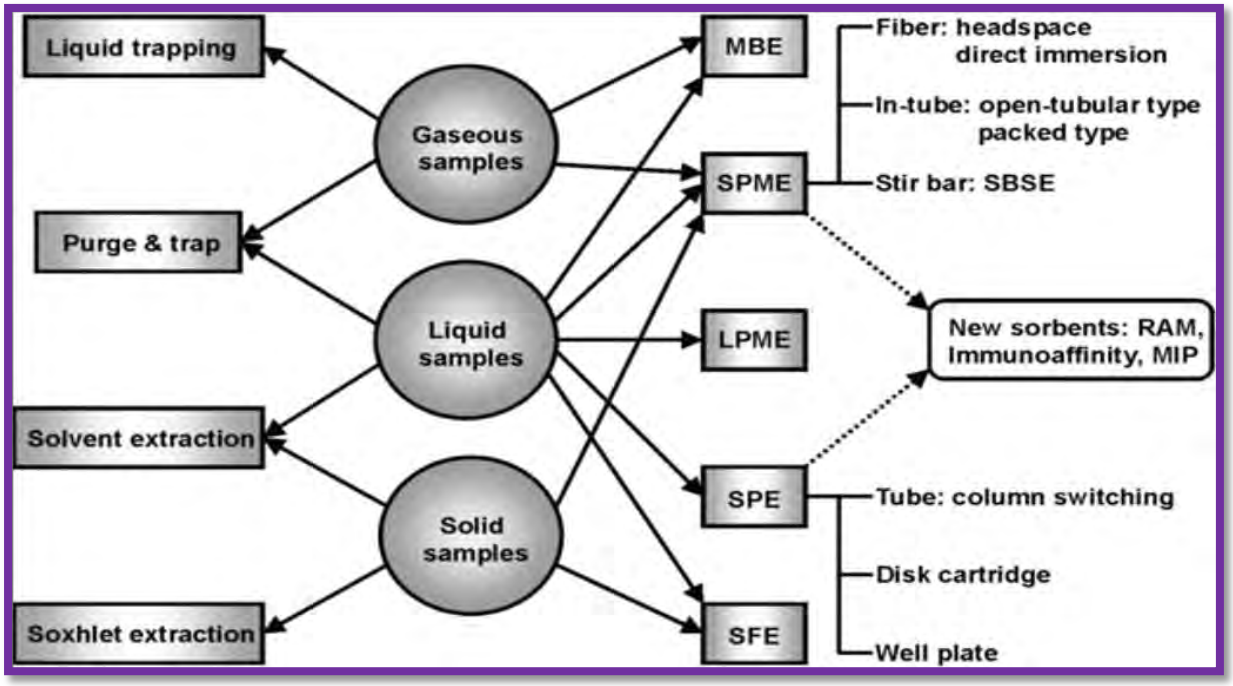


Fig. 1.2:Main extraction techniques for solid, liquid and gaseous samples [15]

1.3.1.1 Modern sample extraction methods for solids

Solvent extraction of solid samples, which is commonly known as "solid–liquid extraction" is one of the oldest techniques for solid sample preparation. It serves, not only to remove and separate compounds of interest from insoluble fractions, but also from other compounds that could interfere with subsequent steps of the analytical process [19]. Most modern liquid–solid extraction techniques use increased temperature or pressure to facilitate the rate of extraction [20]. In recent years a number of modern sample extraction techniques such as automated soxhlet extraction, pressurised fluid extraction, pressurised hot water extraction and solid phase extraction have been widely used.

1.3.1.1.1 Automated Soxhlet Extraction

Soxhlet extraction is a process whereby the solid sample is placed in an extraction chamber and the solvent is heated to reflux and continuously extract analyte from the sample matrix. The disadvantages of conventional soxhlet extraction as compared to other techniques are the long time required for extraction and the large volume of extractant wasted. Large volumes of extractant are not only expensive to dispose-off, but could also be the source of additional environmental challenges. Samples are usually extracted at the solvent boiling point over long periods, which can result in thermal decomposition of thermolabile target species [19]. Despite the challenges, soxhlet extraction has been so far applied for organic compound extraction from solid matrices due to its high extraction efficiency. It has been applied to a wide variety of compounds such as PAHs, 4-nonylphenol (4-NP) and bisphenol A (BPA), polybrominated diphenyl ethers (PBDEs), PCBs and tetrabromobisphenol A(TBBPA) [21].

Conventional soxhlet extraction has been used as a starting point for the development of a variety of modifications aiming at bringing soxhlet closer to that of the more recent techniques for solid sample preparation (see Fig.1.3). Shortening leaching times with the use of auxiliary forms of energy and automating the extraction assembly is employed [19]. Automated soxhlet extraction was approved in 1994 by the Environmental Protection Agency (EPA) as a standard method [21]. In an automated soxhlet system there is an optical level sensor instead of a siphon and its advantage is that it allows more cycles per hour to be

carried out reducing the extraction time from about 24 h to 2 h and thus makes the extraction more efficient and faster than the traditional soxhlet [16]. The extraction is carried out in three stages which are:

- **boiling-** The sample is immersed in a boiling solvent and hence it provides rapid extraction of soluble material.
- **rinsing –** The sample is raised out of the boiling solvent and get rinsed by the condensed solvent that drips through and rinses out the residuals.
- **solvent recovery-** The condensed solvent is collected in a collection vessel and could be reused and the extracted material is concentrated in the extraction cup [19].

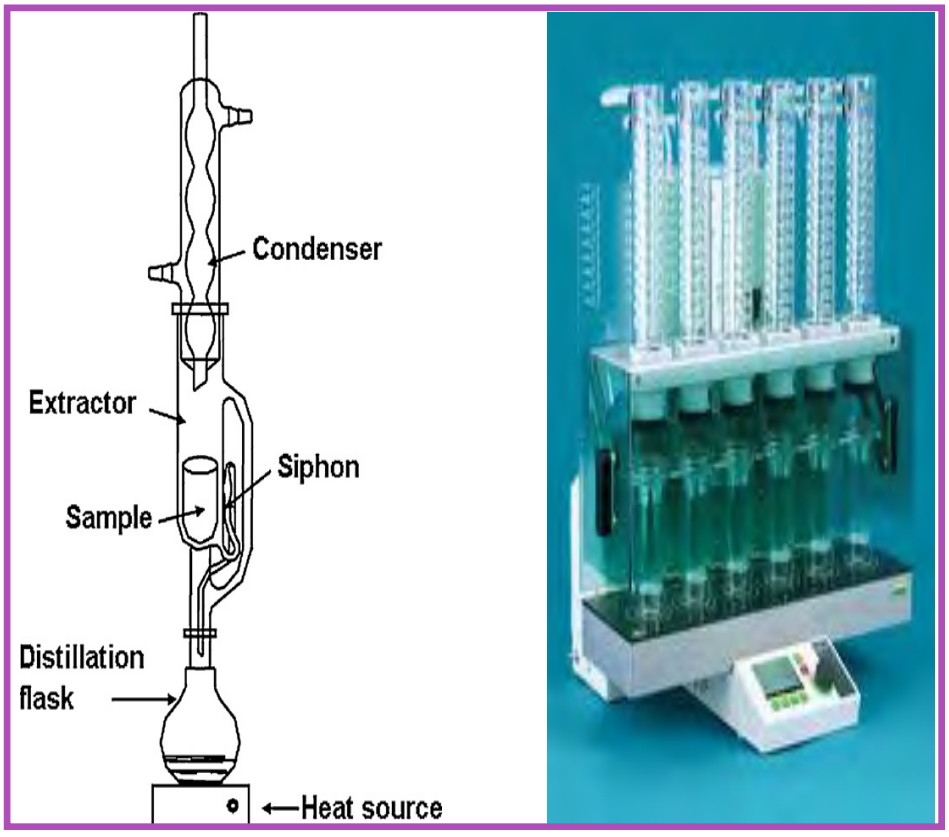


Fig.1.3a: Traditional soxhlet extraction setup[19] **Fig. 1.3b: Modern soxhlet extraction setup from BUCHI [20]**

1.3.1.1.2 Pressurised Fluid Extraction (PFE)

Pressurized fluid extraction (PFE) is an environmental friendly method as it uses environmental friendly solvents [14]. PFE is one of the latest technologies to be approved for extraction of the solid samples [16] and works similar to soxhlet extraction except that in PFE, the solvent is near its critical region. It uses conventional liquid solvents at high temperatures and high pressures to extract compounds from solid materials in less than 30 minutes using small solvent volume [22]. Supercritical carbon dioxide or carbon dioxide-organic modifier is the fluid that extracts the sample in PFE and it has both properties of liquids and gases [16]. Higher temperature increases the ability of the solvent to solubilize the analyte, decreases the viscosity of liquid solvents therefore resulting in better penetration of the solvent into the matrix. The use of higher pressure facilitates the extraction of the analytes from samples by improving the solvent to access the analyte trapped in the matrix pores [23].

Adjusting the fluid properties by regulating pressure, temperature and the content of modifiers enables SFE to perform selective extractions. SFE is essentially an analyte- and matrix-independent technique that provides cleaner extracts than the time-consuming classical procedures. The main use of SFE is for the extraction of persistent organic pollutants from environmental samples [22] (see Fig 1.4).

Advantages of PFE are:

- method development time is minimized
- it can easily be automated
- less solvent is used compared to traditional extraction methods
- enhanced solubilisation properties due to solvents heated above their boiling point

The disadvantages are:

- everything is extracted due to rigorous extraction conditions therefore it is non-selective
- concentration step is necessary as extracted samples have greater volume of solvent

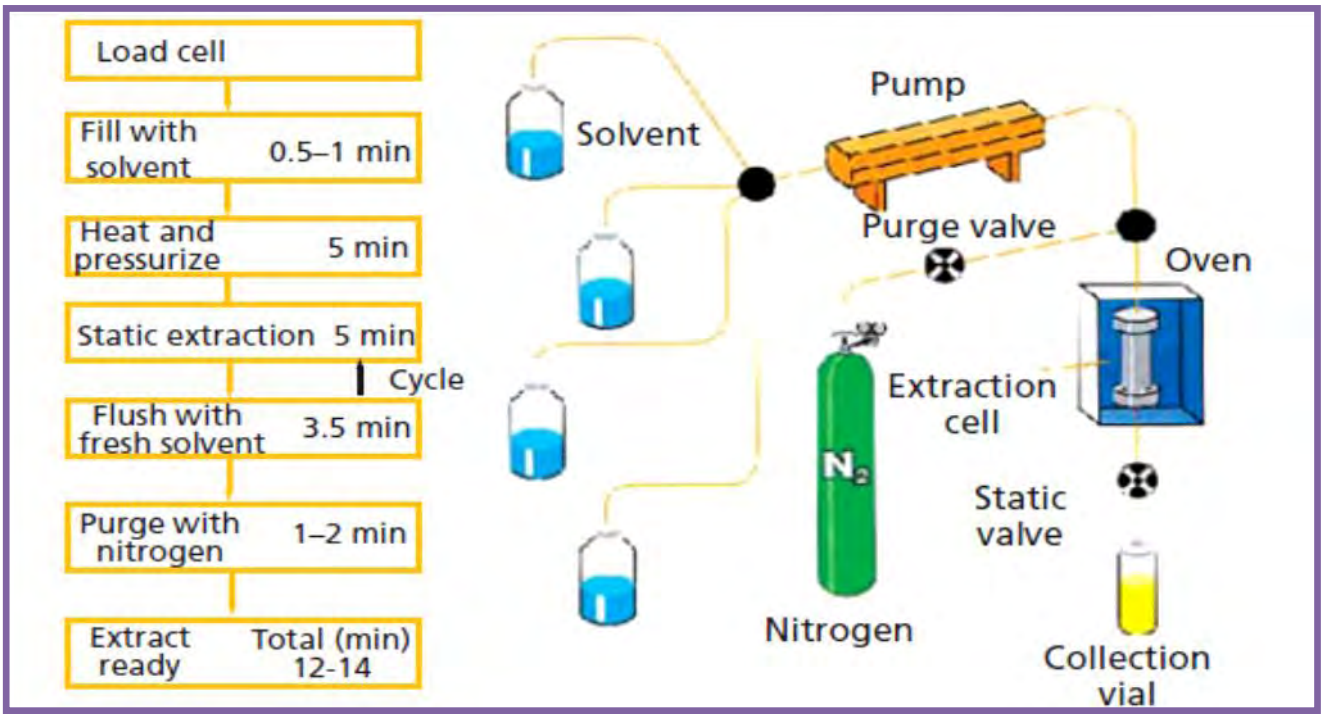


Fig 1.4: Pressurised fluid extractor (Dionex) [20]

1.3.1.1.3 Pressurised Hot Water Extraction (PHWE)

Pressurised hot water extraction is a PLE type that employs water as extraction solvent. PHWE is fast, selective and can be easily automated [24]. The temperatures used are between 100 and 374 ^{o}C and at a pressure that is sufficient to keep water in a liquid state and the polarity is lowered [25]. The lowered polarity of the water is similar to that of ethanol or acetone therefore could be used for the extraction of less polar compounds [26].

The main factor that affects extraction efficiency is temperature, high diffusion, low viscosity, and low surface tension [21]. PHWE is mainly applied in the extraction of food, environmental and natural products. Care should be taken when using this technique as degradation, hydrolysis or oxidation of the target compounds could occur at the elevated temperatures.

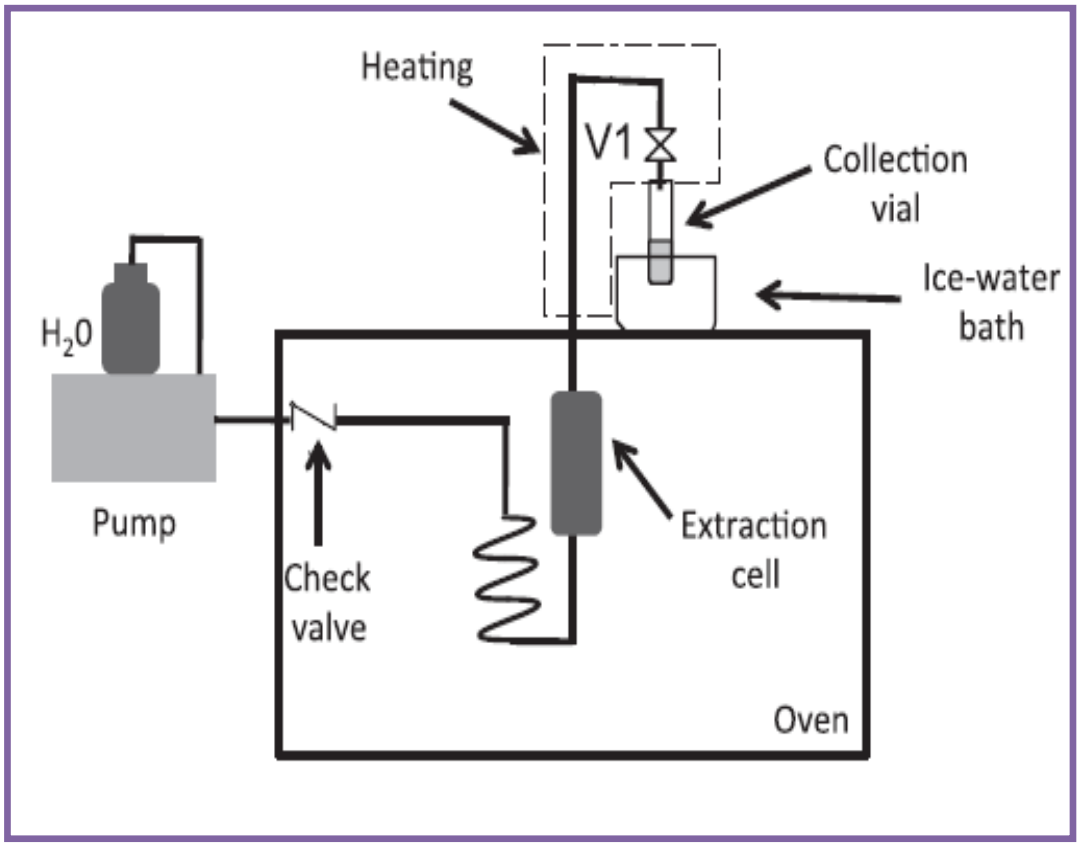


Fig. 1.5: Schematic of pressurised hot water extraction [24]

1.3.1.1.4 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction has been widely used due to its unique solvating properties. Supercritical carbon dioxide is employed as the extraction solvent with its advantages being low toxicity, low reactivity, low critical temperature and pressure [27]. A gas or a liquid is compressed under pressure and heated past its critical point and at that critical state the fluid takes many properties of both gas and liquid. The viscosity is lower than that of liquids, and the diffusion coefficients are higher resulting in more efficient extraction. Highly selective extractions are also achieved by varying pressure and temperature [18].

An SFE system consists of a high pressure pump, extraction cell and a modifier. The pump delivers the fluid to the extraction cell which contains the sample and kept at the appropriate pressure and temperature. The modifier is added to enhance solvation properties [18]. The process involves five sequential steps of wetting the matrix with the supercritical fluid, partitioning of the analyte from matrix into the supercritical fluid, diffusion of analyte from matrix, elution from extraction cell and collection of analyte [121].

The advantages of SFE over traditional extraction techniques is the use of low volumes of organic solvents [28]. SFE can extract non polar to moderately polar compounds. SFE has added selectivity and wide range of applications in environmental, food and polymer analysis. SFE provides short extraction time, mild pressure and temperature which are conducive for the preservation of the integrity of functional compounds of environmental samples, food and natural products [14]. The disadvantages are limited polarity of supercritical carbon dioxide, there is strong matrix effect and also care should be taken when controlling variables to obtain reproducibility.

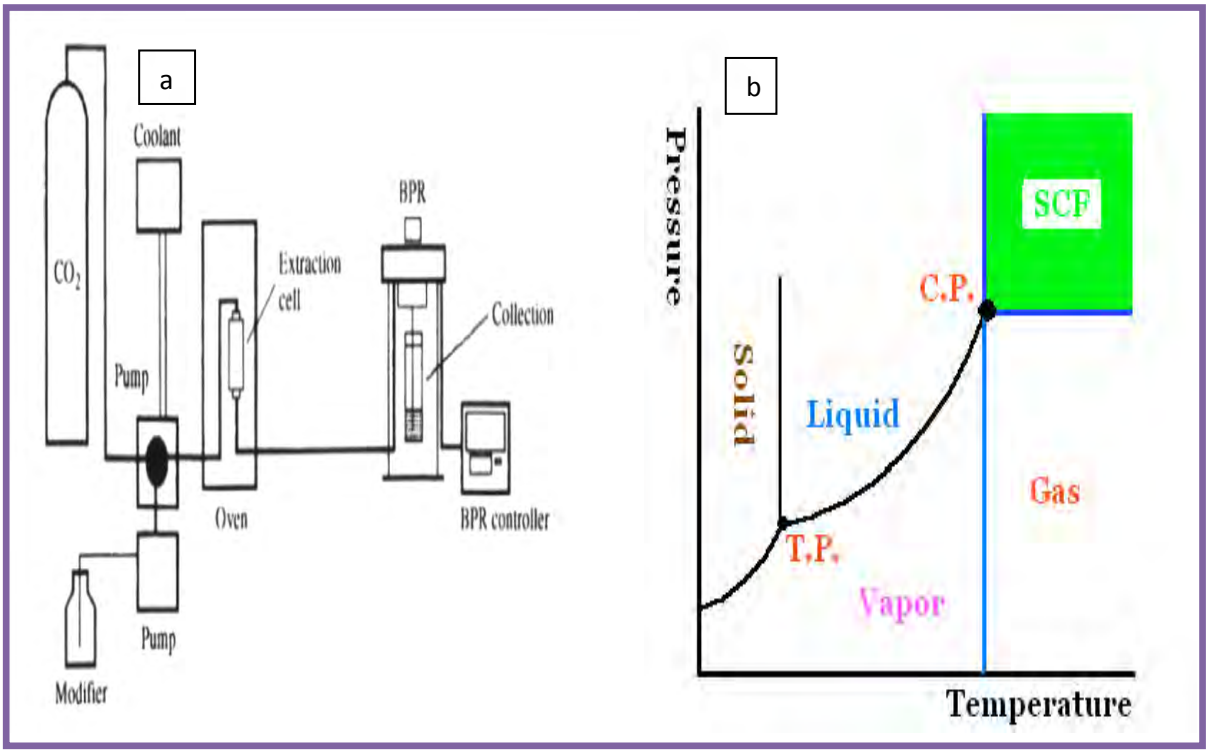


Fig. 1.6a Schematic of a basic SFE system [25] and 1.6 b SFE profile

1.3.1.1.5 Microwave Assisted Extraction (MAE)

Microwave assisted extraction uses energy of microwave radiation to selectively extract target compounds from various matrices. It is an innovative solvent extraction technology whereby solvents are heated quickly and efficiently [29]. There is a limitation of solvent choice since the solvents that could be used are the ones that absorb microwave radiation (solvent with permanent dipole leading) [21]. The application of microwave energy to the samples may be achieved by two technologies of closed vessels (under controlled pressure and temperature), or open vessels (at atmospheric pressure) [18]. An extraction could be performed at higher temperatures using a closed system which results in drastic reduction of extraction time [29]. The other advantage of MAE is that unlike classical conductive heating methods, the whole sample is heated simultaneously [30] (see Fig 1.7).

MAE is mainly used for the extraction of organic compounds from solid matrices associated with environmental samples, pharmaceuticals, polymer/plastic industries and the food industry. U.S. Environmental Protection Agency (USEPA) approved Method 3546 for microwave extraction of organic compounds from soils, sludges and sediments [21, 29].

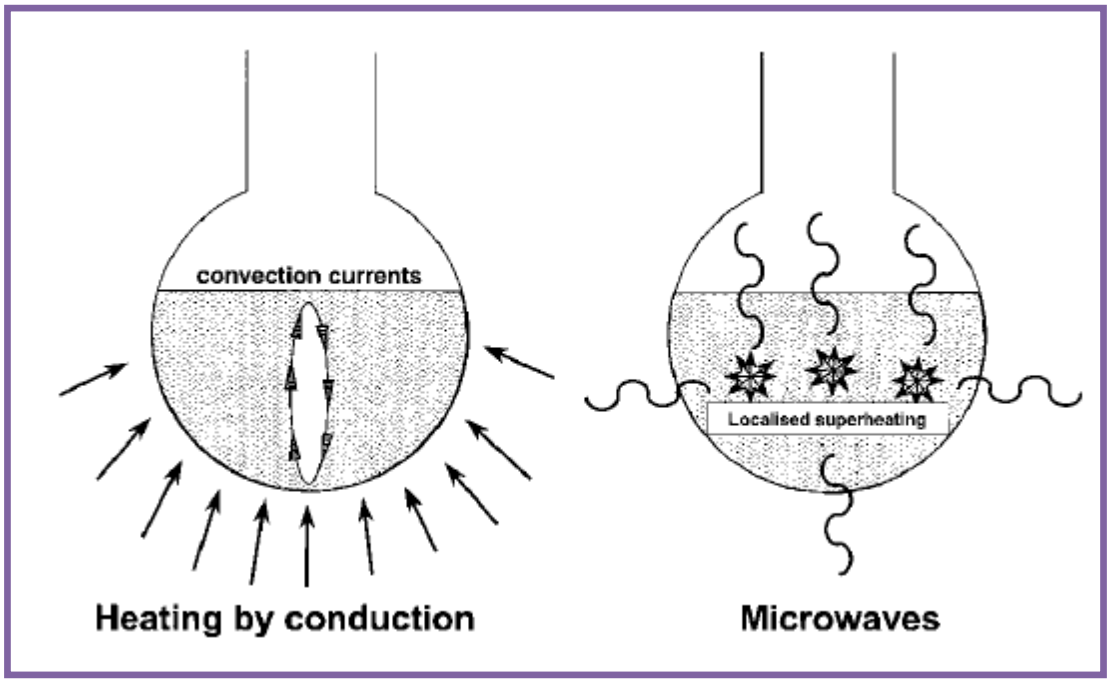


Fig. 1.7: Conduction in the classical method of extraction and microwave irradiation in MAE [30]

1.3.1.1.6 Matrix Solid Phase Dispersion (MSDP)

Matrix solid phase dispersion has proven to be applicable to the analysis of drugs, pollutants, naturally occurring constituents and other compounds from complex animal and plants matrices [31, 32]. MSPD extraction method is suitable for biological samples as compared to other extraction methods because it does not depend on non polar solvents that cannot easily penetrate the largely aqueous matrices of biological samples [13]. The process involves homogenization or blending of a small amount of sample with an abrasive solid support material. The most commonly used being silica based sorbents (C18- and C8-) [31] in a pestle and mortar. The force applied during grinding disrupts the gross architecture and the smaller sample components pieces are able to be dispersed by hydrophobic or hydrophilic interactions over the surface of the solid sorbent support [33]. The blend is then packed in a pre-fitted column or on top of solid phase extraction sorbent and analyte of interest can be eluted with appropriate solvent [33] (see Fig. 1.8).

The advantage of the technique is that there is minimal usage of sample and solvent, the process is easy to follow, it can be used in field analysis, there are reduced costs and its

ability to simultaneously perform extraction and clean-up in a single step [34]. It also uses mild extraction conditions (room temperature and pressure) [21].

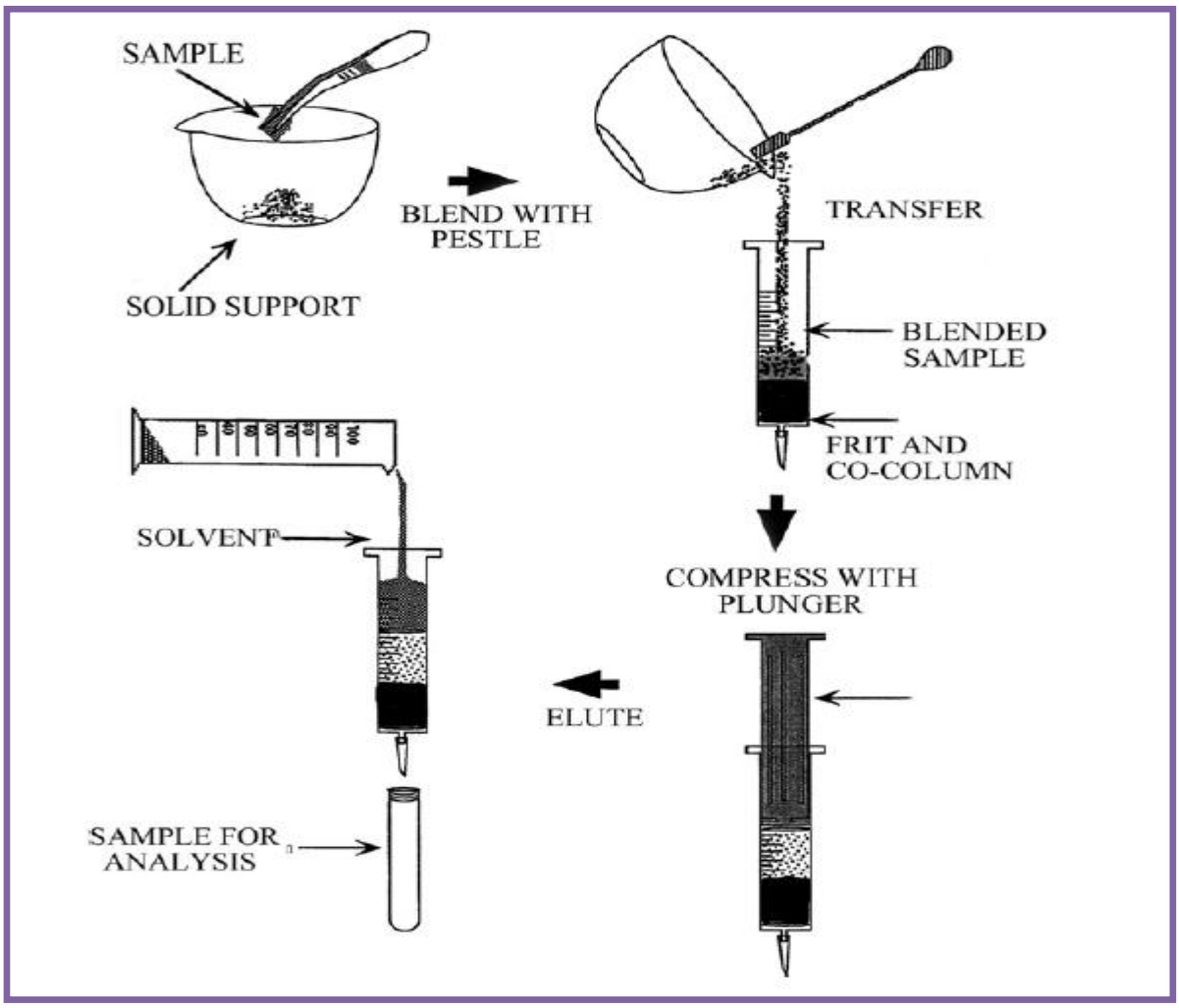


Fig. 1.8: Steps in typical MSPD extraction [32]

1.3.2 Modern sample extraction methods for analytes in solution

Classical methods of extraction of analytes in solution include liquid-liquid extraction, trapping analyte into a column, evaporation of sample to dryness and selective solvation of the analyte [13]. The disadvantages of classical methods are:

- time consuming and for liquid-liquid extraction there is formation of emulsion
- further steps of clean-up may be required [14].

- costly because the methods are labor intensive and large volumes of solvents are required
- large volumes of organic solvents have negative impact on the environment through pollution [16].

Membrane extraction, solid phase extraction (SPE), solid phase micro-extraction (SPME) and stir bar extraction are the modern extraction techniques used for the extraction of analytes in solution. They use less solvent, are not time consuming and could be automated therefore are a better alternative to classical methods. The methods can also be used for further clean-up of extracts obtained from solid samples [13].

1.3.2.1 Membrane Extraction

Membrane extraction involves the use of a membrane that acts as a barrier between two phases. The driving force can either be a concentration gradient, electric potential or pressure difference. The analyte is transported from one phase (donor phase) to the other (acceptor phase) [35] (see Fig. 1.9).

The major applications of membrane extraction are in water desalination, food industry and biomedical engineering [36].

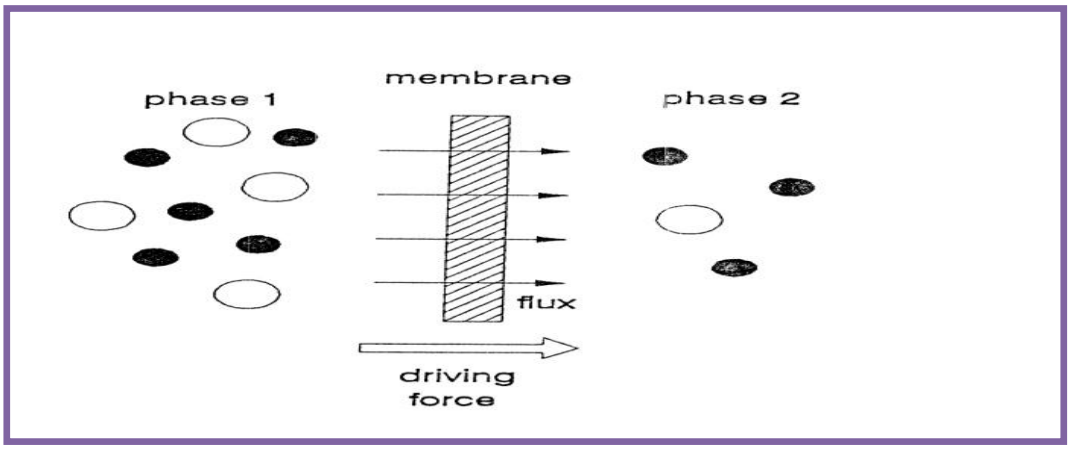


Fig. 1.9: Schematic of membrane extraction [35]

Membrane extraction processes are categorised according to structure and separation mechanism as summarised in Table 1.2.

Table 1.2: Summary of membrane extraction processes and applications[36]

Separation process	Driving force	Separation mechanism	Application	Membrane structure
Dialysis	Concentration gradient	Difference in diffusion rate	Separation of high- and low-molecular-mass compounds	Symmetric, porous/ non-porous
Osmosis	Concentration gradient	Difference in diffusion rate	Water desalination	Symmetric, porous
Hyperfiltration	Pressure gradient (1–10 MPa)	Difference in solubility and diffusion rate	Separation of low-molecular-mass compounds from solution	Asymmetric, non-porous (0.1-1 nm)
Ultrafiltration	Pressure gradient (50 kPa–1 MPa)	Difference in membrane permeation (sieving)	Separation of high- and low-molecular-mass compounds	Asymmetric, porous (1–100 nm)
Microfiltration	Pressure gradient (10–100 kPa)	Sieving	Bacteria filtration	Symmetric, porous (100–1000 nm)
Electrodialysis	Electrical potential	Selective ion transport	Water desalination	Symmetric, ionic
Electro-osmosis	Electrical potential	Difference in diffusion rate	Drying of soil	Symmetric, ionic
Gas separation	Pressure gradient	Difference in solubility and diffusion rate	Separation of gas mixtures	Asymmetric, non-porous
Pervaporation	Pressure gradient	Difference in solubility and diffusion rate	Separation of liquid mixtures	Asymmetric, non-porous

1.3.2.2 Solid Phase Extraction (SPE)

Solid phase extraction gained popularity over liquid-liquid extraction in recent years because it is easily automated and different types of sorbents are available. It is also regarded as more environmental friendly compared to liquid-liquid extraction [37]. SPE is a widely used sample preparation technique for isolation of analytes from a liquid, fluid or gas matrix [10]. It uses the same principle as liquid-liquid extraction because it involves partitioning of solutes between two phases. However, for solid phase extraction partitioning occurs between a liquid phase (sample matrix) and solid phase (sorbent) [38]. The sorbent material is usually packed into a small tube and has a large surface area for extraction of analyte from the liquid matrix as it passes through [39]. The analyte must have greater affinity to the solid phase than the sample matrix for it to be retained [40].

The advantages of SPE over LLE is that there is less solvent usage, it allows parallel extraction, easy of automation, no emulsion formation, higher and more reproducible

recoveries are achieved. Another advantage of SPE is its versatility, a result of the different types of sorbents available [41]. The disadvantage of SPE is the difficulty to master method development because it involves use of a wide range of chemistries and tedious process of optimising solvents and pH.

The main applications of SPE are for:

- clean-up for the removal of matrix interferences
- trace enrichment to give concentrations suitable for analysis
- change of medium to a solvent suitable for the instrumentation to be used [10].

The SPE general procedure is to load the sample solution on the sorbent material, wash away interfering material followed by elution of the analyte into a collection tube [42]. The retention of the analyte depends on the distribution coefficient described by the Nerst distribution law (see Eqn. 1.1). A compound gets distributed between two immiscible solvents according to a constant ratio of the concentrations between the two solvents [40].

$$K_D = \frac{C_S}{C_M} \quad (T = constant) \qquad (1.1)$$

Where C_S= concentration in the upper layer

C_M= concentration in the lower layer

The basic SPE protocol involves:

1. **Conditioning step**

   An appropriate solvent is passed through to activate the sorbent material for its interaction with the analyte. If the sorbent is not adequately activated poor reproducibility and recoveries maybe obtained.

2. **Sample loading step**

   The sample is loaded to the sorbent and sufficient time is allowed for maximum interaction between the analyte and the sorbent.

3. **Washing step**

   Removal of interfering materials retained on the sorbent. The elution strength for the wash solvent should be higher than that of the sample solution but less than that of the elution solvent.

4. **Elution step**

   Application of solvent that is able to elute analyte from the sorbent and collected for later analysis [43]. The elution solvent should be strong enough to disrupt analyte-sorbent interactions.

There are different modes of SPE that are widely used, namely reverse phase solid phase extraction (RP-SPE), normal phase solid phase extraction (NP-SPE), ion exchange solid phase extraction and mixed mode solid phase extraction.

1.3.2.3 Modes of solid phase extraction

The solid phase extraction sorbent material should be chosen carefully so as to control parameters such as selectivity, affinity and capacity. The physical chemical properties of the analyte are the ones that determine the choice of the sorbent material depending on the expected interactions [8]. The different retention or elution mechanisms are due to intermolecular forces between the analyte, the active sites of the surface of the sorbent material and the matrix [43] (see Fig 1.10).

There are two basic approaches to SPE:

- The sorbent material retains the analyte while the interferences are washed off and then analyte eluted with appropriate solvent (RP-SPE).
- The sorbent material retains the interferences while the analyte of interest directly pass through (NP-SPE).

In addition to RP-SPE and NP-SPE, Ion exchange- and mixed mode solid phase extraction are also available. Ion exchange solid phase extraction is where the retention of analyte of interest is based on ionic interactions and for mixed mode solid phase extraction multiple mechanism interactions are combined in the sorbent material.

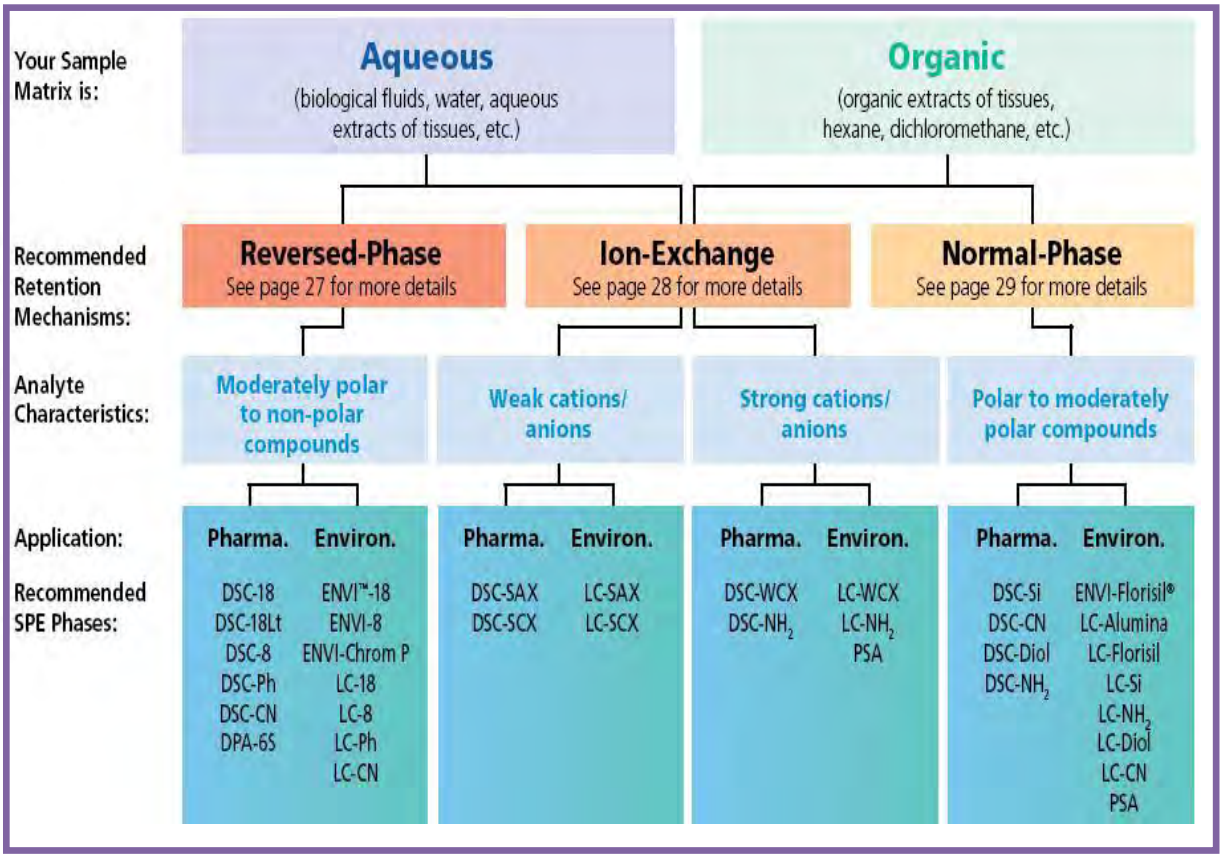


Fig. 1.10: SPE retention mechanisms [122]

1.3.2.3.1 Reverse-phase Solid Phase Extraction (RP-SPE)

In RP-SPE the retention mechanism is based on the interaction between non polar groups of the analyte and the non polar groups of the sorbent through Van der Waals forces. Retention can also occur through secondary interactions such as hydrogen bonding and dipole-dipole interactions [21, 44] (see Fig 1.11). Non polar to moderately polar compounds in a polar matrix are normally retained. The sorbent requires conditioning with an organic solvent followed by an aqueous solvent. Elution is carried out with non polar solvents for non polar analyte and mid-polar solvent for moderately polar analytes (see Fig 2.4). The sorbent materials include C18 as the most popular. The main advantage of the sorbent is the stability of the ≡Si–O–Si≡ bonds formed between the silylating agents and the hydroxyl groups on the silica surface [45]. C8, C4, C2 and phenyl bonded sorbent are also available [123].

RP-SPE is relatively non specific and a wide range of organic compounds are retained therefore it is important to optimize extraction conditions especially the washing step.

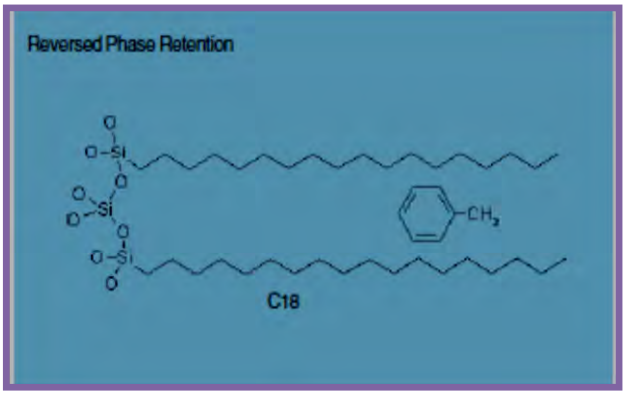


Fig. 1.11: Reverse phase retention mechanism [124]

1.3.2.3.2 Normal Phase Solid Phase Extraction (NP-SPE)

NP-SPE is used for the extraction of polar analytes from non polar matrices. The retention mechanism is based on hydrogen-hydrogen bonding, $\pi - \pi$ interactions and dipole dipole interactions [124]. The sorbent materials commonly used are silica, florisil, amino, cyano, diol and alumina. Non polar solvent is used for conditioning and elution is carried out with polar solvents [123]. The polarity of the conditioning and dilution solvents should be carefully optimized to achieve improved the specificity [124].

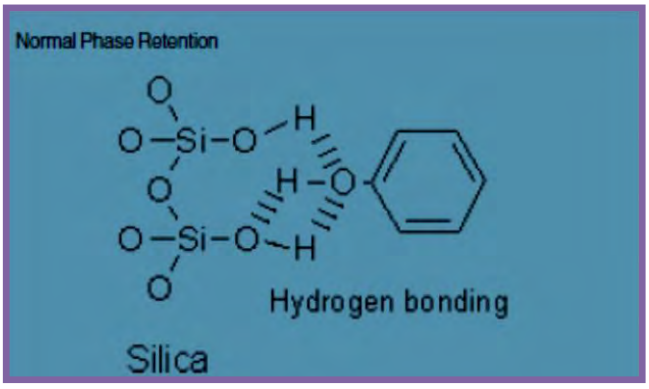


Fig. 1.12 Normal phase retention mechanism [124]

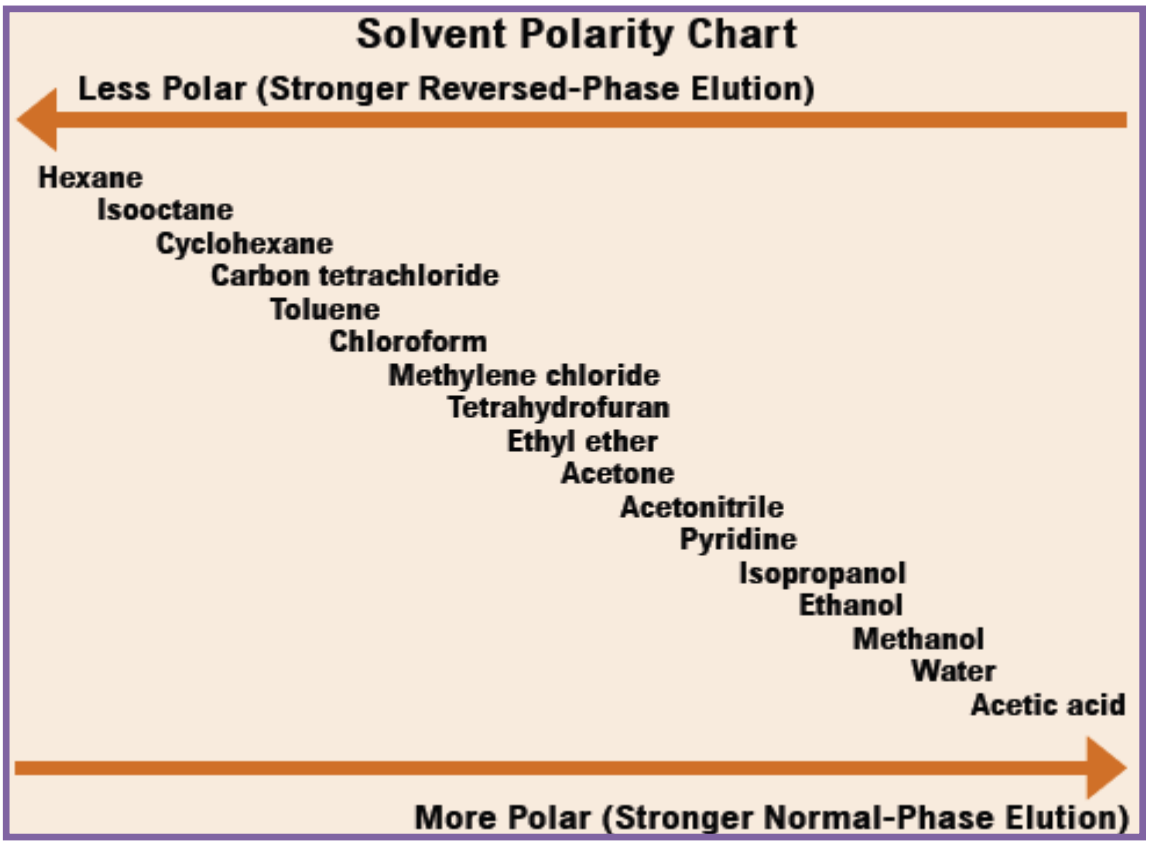


Fig. 1.13 Solvent polarities [132]

1.3.2.3.3 Ion Exchange Solid Phase Extraction

Cation and anion exchange are used for isolation of ionic analytes from aqueous solutions [44]. Both weak and strong ionic functional groups bonded to silica gel or polymers are contained in the ion exchange sorbent [44]. Strong sites are always present as ion exchange sites at any pH, while weak sites are only present as ion exchange sites at pH values greater or less than the pKa. Strong sites are sulfonic acid groups (cation-exchange) and quaternary amines (anion-exchange), while weak sites consist of carboxylic acid groups (cation-exchange) or primary, secondary and tertiary amines (anion-exchange) [38]. Anions and cations are retained on the corresponding sorbent by exchanging the anion or cation in the sample with the anion or cation on the sorbent [124].

Optimal conditions like suitable pH are required. pH is kept at two units lower than the pKa values of the analyte and two units higher than the cation exchange sorbent [40]. Elution is accomplished with high ionic strength buffers (0.1M-0.5M) or by changing the pH of the elution solvent.

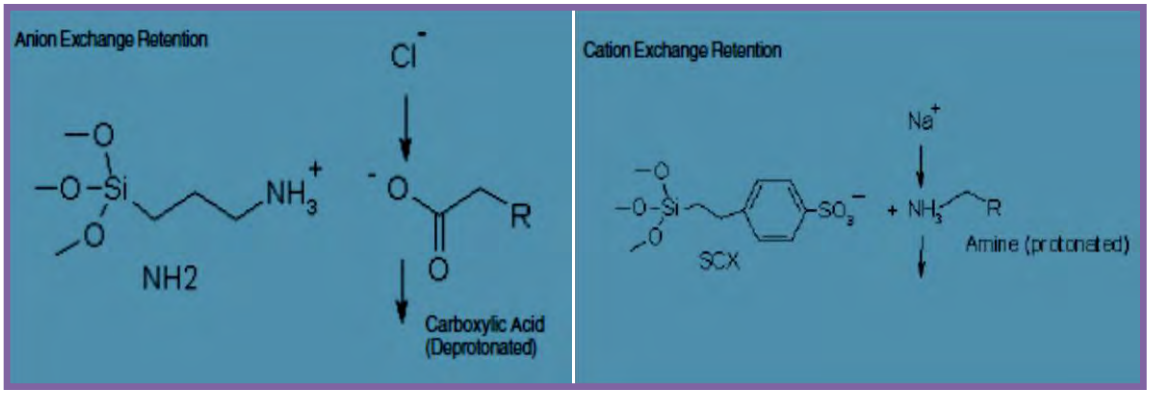


Fig. 1.14 Ion exchange retention mechanism [124]

1.3.2.4 Trends in solid phase extraction

In recent years there have been some developments in SPE regarding formats, phases, automation and high throughput. The developments have led to new sample preparation procedures in environmental analysis. Efforts are towards carrying out multiresidue extraction of many analytes with a wide range of polarities and high throughput [46].

1.3.2.4.1 SPE formats

The development of new formats for SPE has provided high-throughput capabilities and greater convenience for method development. Faster method developments reduced solvent volumes and shorter sample preparation times are achieved. The new formats have also allowed the easy of automation of extraction methods as they are easily removable and flexible [41]. The cartridge format was introduced in the 1980's and it is still popular to date. More recently new formats such as discs, SPE pipette tip and 96-well microtiter plate have been introduced.

1.3.2.4.1.1 SPE cartridges/syringe barrel

Medical grade polypropylene and polyethylene materials have been used to make the SPE reservoir to provide high purity devices [46]. A typical cartridge device consists of a syringe barrel containing a sorbent with particle size ranging from 50–60 μm, with metal or plastic frits at the top and bottom to hold the sorbent in place [10] (see Fig 1.15). The older formats consisted of about 500 mg of sorbent material in syringe barrels of 3 mL and 5 mL volume

however the trend has been towards lowering sorbent mass and volume (100 mg in 1 mL) because improvement in sensitivity of instrumentation has allowed the usage of smaller quantities of samples [47]. Low-volume cartridges and many variations have led to the possibility of on-line hyphenated systems (SPE-LC, SPE-GC) for sample processing resulting in robust analysis of samples [10, 47].

The disadvantages of cartridge/syringe barrel format are:

- clogging of the top frit when handling sample with suspended solids resulting in difficulty of the sample to pass through
- there is restricted flow rate due to smaller cross sectional area resulting in slow processing rates [46, 48].
- channelling reduces the capacity of the sorbent to retain the analyte [48].
- some analytes do not easily desorb from the sorbent [48].

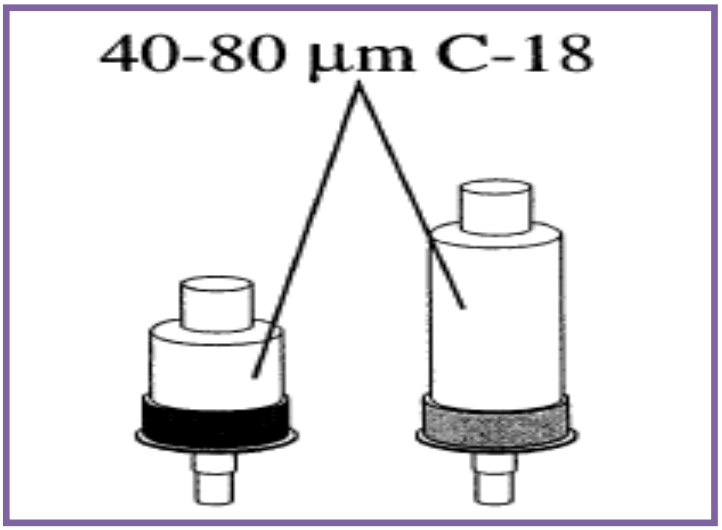


Fig. 1.15: Cartridge\ syringe barrel SPE format [49]

1.3.2.4.1.2 SPE Discs

The SPE disc known as 3M's Empore was the one that made the use of the disc format popular [47]. The similarity between Cartridge and disc devices is that they use the same sorbent chemistry and are distinguished by format only [10]. The difference between the cartridge/syringe barrel and the disc format is that the disc is a membrane loaded with a

sorbent while the cartridge/syringe barrel contains the sorbent [49] (see Fig 1.16). The most common solid supports for the SPE discs include PTFE/Teflon, glass-fiber and paper [124].The disk has a cross sectional area greater than the cartridges therefore high flow rates could be used for large volumes of low concentration samples [47].

Discs are more expensive than cartridges/syringe barrel therefore not convenient for use in routine analysis but on the other hand they offer specific format advantages that favour their use for some applications:

- they function better than cartridges for larger sample volumes containing suspended solids. They are important in surveillance programmes where large volumes are used for analysis of environmental trace contaminants in surface water [43, 49, 50].
- they have smaller particle size and greater mechanical stability [51].
- there is reduced matrix adsorption therefore cleaner background is obtained due to optimised use of bed mass.
- it is also easier to miniaturize discs than cartridges [10].

SPE discs in 96-well plates are popular in the pharmaceutical industry because of their low bed volume that allows good extraction efficiency of biological samples with smaller volumes [124].

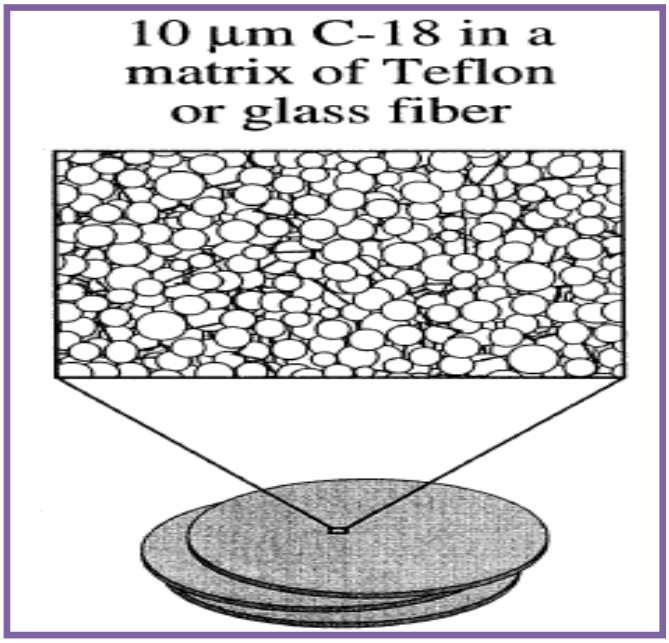


Fig. 1.16: Disc SPE format [49]

1.3.2.4.1.3 SPE pipette tip (PT-SPE)

Micropipette tip based micro-SPE is now an essential tool for purification, concentration, and selective isolation (by affinity and metal chelator) of proteins and peptides in genomics, proteomics, and metabolomics [52]. Several manufacturing companies have developed SPE pipette tips (eg. ZipTip and NuTip) and a number of sample preparations employing the SPE pipette tip have been reported for the extraction of peptides and proteins from biological samples [53].

Pipette-tip extraction is a miniaturized version of the conventional SPE technique. The extraction mechanism is the same as that one of conventional SPE. The sorbent material is packed at the top of a micropipette with a volume of 0.2–1.0 mL. Extraction of analyte is achieved by the repeated aspiration and desorption of the sample solution using a pipette (see Fig.1.17). The SPE process involves the washing of the sorbent with methanol and pre-activation with water [54]. The adsorbed analyte is then eluted with a solvent [54].

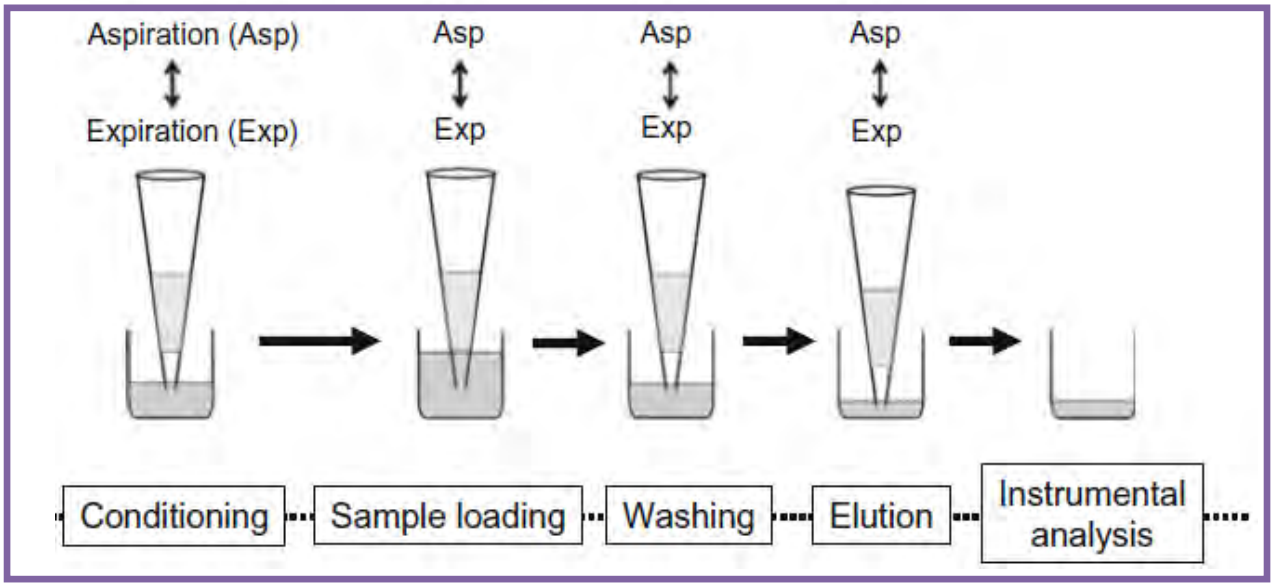


Fig. 1.17: SPE pipette tip extraction process [55]

1.3.2.4.1.4 96-well microtiter plate solid phase extraction

Development of the 96-well microtiter plate SPE format has provided significant advances in high throughput solid phase extraction. The format utilises single blocks or plate having 96

wells containing discs or packed beds of sorbent particles arranged in 8-row by 12-column rectangular matrix (see Fig 1.18). Each of the 96 wells has 1 or 2 ml SPE column with sorbent mass range of 3-10 mg. The packing material is placed between the bottom frit or membrane and the top frit [41]. Bonded silica or copolymer sorbents are highly used in the format for high throughput bioanalytical techniques [56].

The use of 96-well microtiter format configured with multiple sorbents per plate can simplify and speed SPE method development. 96-well plate allows for parallel sample processing in approximately one hour or less. The simultaneous processing of samples reduces handling errors and limits labour-input. The plates are rather costly and, given that a test may use only a few of the wells, laboratories may incur considerable expense in method development experiments [41].

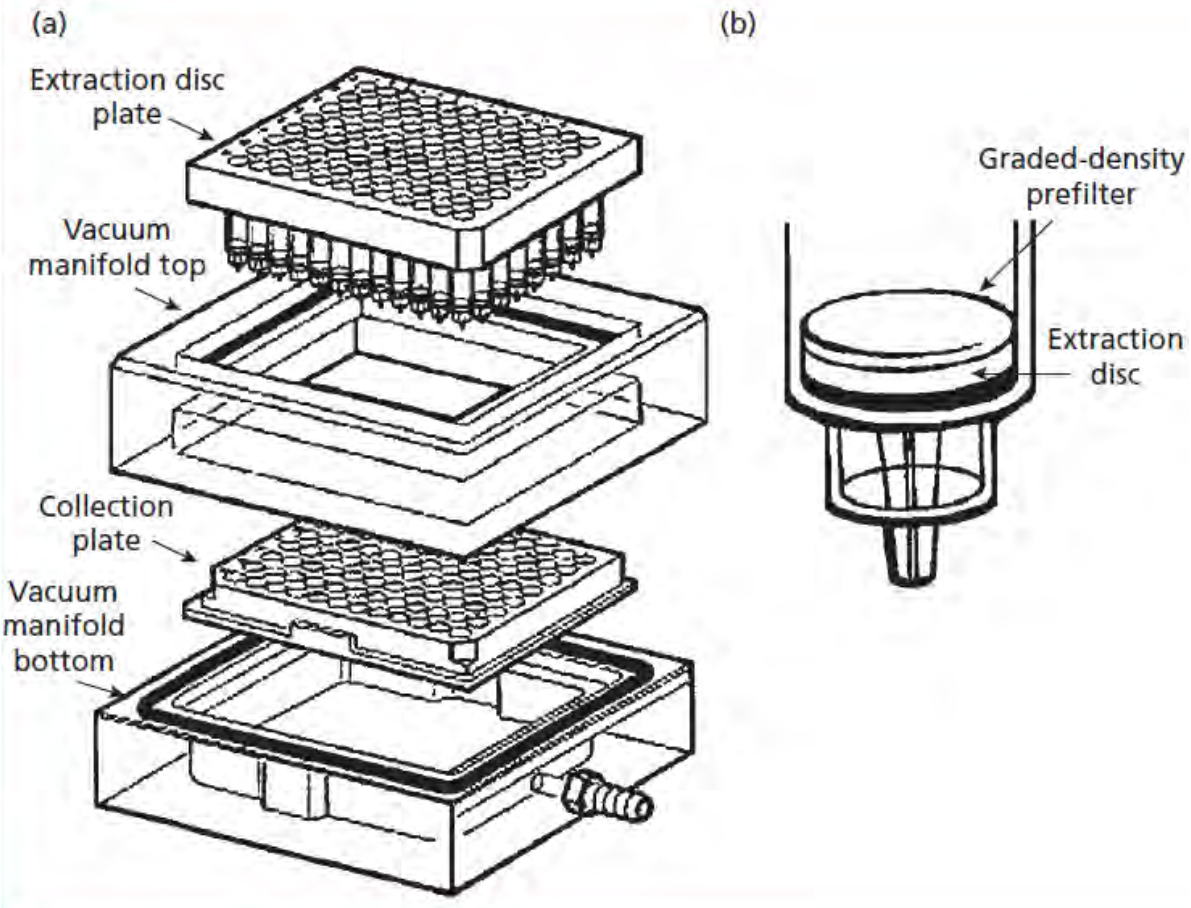


Fig. 1.18: (a) Fixed 96-well SPE plate and (b) a single extraction disc [47]

1.3.2.4.2 Sorbent materials

For many years *n*-alkylsilica has been the universal SPE sorbent. The conventional SPE sorbents presented a lot of challenges that made SPE to be not preferred over other extraction techniques. In order to improve SPE technique, new sorbent materials that overcame challenges presented by conventional sorbents were developed.

1.3.2.4.2 .1 Conventional SPE sorbents

The commercially available sorbents based on silica and bonded silicas have been used for a wide range of analytes. They presented challenges with batch-to-batch variation in analyte recovery therefore inhibited their use and thus prompted manufacturers to develop new sorbent materials [57]. One of the biggest challenges was the presence of residual silanols causing separation to be dependent in more than one mechanism of separation and greater vulnerability to variation between different batches [37]. A need for better understanding of surface chemistry and the complex of interactions involved in analyte retention and elution was therefore required [57].

The packing of the silica cartridges was adapted from the stationary phase of the liquid chromatography columns. The evolution followed those of liquid chromatography with the aim of having more hydrophobic phases with minimum residual silanol groups [46]. The main disadvantages of such sorbents are their limited breakthrough volumes for polar analytes, and their narrow pH stability range.

Reverse phase polymeric sorbents were therefore developed to be used for the trace enrichment of soluble analytes that had poor interactions with reverse-phase C_{18} sorbents [44]. The disadvantage of both the reverse-phase silica sorbent and many of the commercial polymeric sorbents is that they must be conditioned with a wetting sorbent and are not supposed to dry before the loading of aqueous solvent. A better wettability and improved recoveries for polar analytes is achieved by using functionalised polymeric sorbents [46]. The major requirement for a sorbent material is the ability to extract a wide range of analytes over

a wide pH range, fast and quantitative sorption and elution, high capacity, regenerability, and accessibility [58].

Several types of sorbents have been introduced in the market with each one claiming "universality" or a better ability for trapping polar analytes. Examples being highly cross-linked copolymers and their new functionalized form, graphitized carbons, as well as *n*-alkylsilicas which have evolved in the same direction since some of them have been especially designed for the extraction of polar analytes. Mixed mode sorbents, restricted access matrix sorbents, immunosorbent and molecularly imprinted polymers have also been introduced for selective extraction of analytes of interest. The selective sorbents have granted the possibility of obtaining extracts free from matrix interferences in a single or few steps [46].

1.3.2.4.2 .2 Advanced techniques in SPE

1.3.2.4.2 .2.1 Mixed mode SPE

Analytes of interest are bound strongly but reversibly to the sorbent while impurities and other unwanted sample components pass through unretained in an ideal SPE. Unfortunately, this scenario is rarely realized. Typically, conditions strong enough to remove impurities also remove at least some of the analyte, reducing the overall recovery of the method. In an effort to minimise the challenge, mixed-mode SPE phases that take advantage of the differences between the retention mechanisms of analytes and unwanted components of the sample are used [134].

In recent years mixed mode sorbent materials have been developed by blending or copolymerising various phases so that they obtain multiple interaction properties for isolation of analytes [59]. A mixed mode sorbent is designed chemically to have multiple retentive sites on an individual particle. The different retention mechanisms incorporate different ligands on the same sorbent. The sorbents have the potential to retain analytes covering a wide range of polarity by utilizing specific and simultaneous interactions. The sequential cancelling of specific mechanisms of interactions causes elution steps to be selective [59].

1.3.2.4.2.2.2 Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) SPE

QuEChERS stands for (Quick, Easy, Cheap, Effective, Rugged, and Safe) and it is a novel sample preparation method that involves an initial extraction with acetonitrile followed by an extraction/partitioning step after the addition of a salt mixture [60]. The procedure was developed in 2003 for the extraction of pesticides in fresh fruits and vegetables [60]. Good results are achieved for both polar and non polar pesticides. There are three primary QuEChERS methods (see Fig 1.19) which are;

1. the original method that uses acetonitrile as extraction solvent and sodium chloride (NaCl) is used to enhance extraction and to reduce polar interferences.

2. dispersive AOAC 2007.01 method that uses a 1% acetic acid in acetonitrile solution for the extraction and uses anhydrous sodium acetate buffer, to protect base sensitive analytes from degradation and also provides improved recoveries for pH sensitive compounds.
3. European Norm EN 15662 uses acetonitrile for extraction and sodium chloride to limit polar interferences and several citrate buffers to preserve base sensitive analytes [61].

The use of acetonitrile in this procedure has several advantages of;

- extraction of less liphophilic compounds
- extraction of residual water with drying agents
- formation of well differentiated partitioning phases with non polar solvents [60].

The advantages of the QuEChERS method over classical methods of extraction are:

- high recoveries (>85%) are achieved therefore very accurate results are obtained
- high sample throughput
- low solvent usage, low waste generation and chlorinated solvents are not used therefore it is environmental friendly
- easy therefore can be perfommed by a person without much training or technical skill
- method can be carried out in a small mobile laboratory because very little bench space is needed

- acetonitrile is added by a dispenser to an unbreakable vessel that is immediately sealed thus solvent exposure to the worker is minimal
- cheap as inexpensive reagents are used [61]

The main disadvantage of the QuEChERS method is that it requires a highly sensitive and selective analytical instrument because only 1 g mL^{-1} concentration is obtained which is lower than the 2–5 g/mL obtained in most of traditional methods [60].

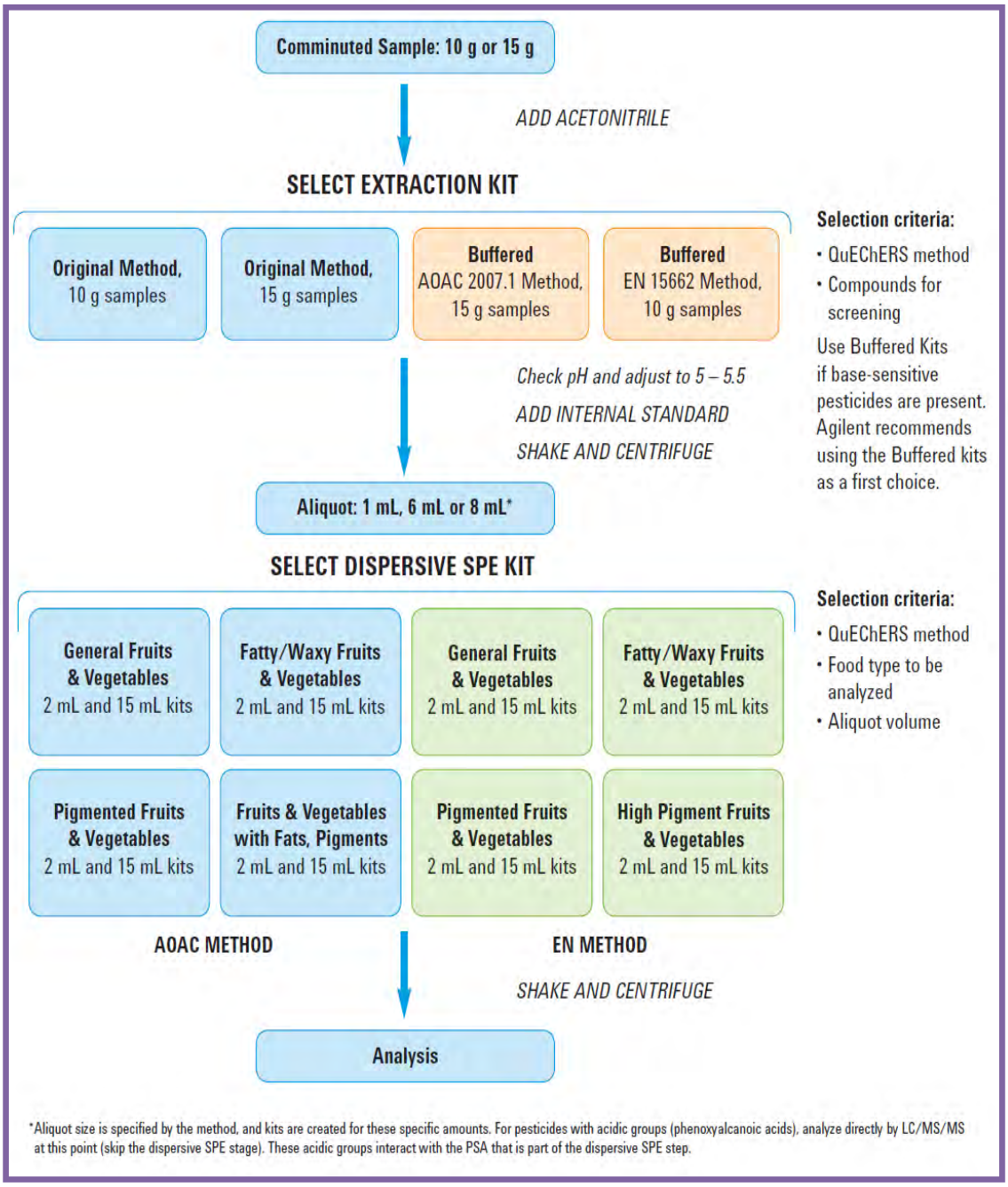


Fig. 1.19: Flow diagram of QuEChERS process [125]

1.3.2.4.2.2.3 Supported liquid-liquid extraction

Supported liquid-liquid extraction (SLE) is an improved liquid-liquid extraction that simplifies and also enables automation of the sample preparation [62]. It differs from traditional liquid-liquid extraction as the sample is totally adsorbed onto a solid support, containing a modified form of diatomaceous earth which acts as stationary phase, on which the extraction occurs. The inert high purity diatomaceous earth is mainly composed of SiO2 which can adsorb large quantities of aqueous samples on its surface [63]. The diatomaceous earth can either be packed in individual cartridges or 96 well-plate to fit any sample size [126].

The sample in aqueous phase is applied to the cartridge and the solution spreads over the surface of the support and gets adsorbed rather than flow through the cartridge. Some time is allowed before extraction for complete adsorption to take place. The diatomaceous earth provides a large surface area for partition into an eluting solvent [64, 65]. The lipophilic substances are extracted from the aqueous into the immiscible organic phase while the aqueous phase remains stationary in the sorbent. The extraction is carried out with gravity and no vacuum is required [127]. Elution with two aliquots (each equal to the original sample volume) instead of one improves extraction efficiency and recovery. The collected eluents could then be analyzed directly or dried and reconstituted [126].

The advantages of SLE are;

- the sorbent maximises extraction of impurities while allowing the organic solvent to pass through the column.
- large surface area of the sorbent allows high loading capacity.
- gravity flow process prevents the formation of emulsion.
- quick as there is no need to wait for phase separation [62].
- easy of automation.

1.3.2.4.2.2.4 Immunoaffinity SPE

Immunochemistry has been used for analysis and for sample pre-treatment in the medical and biological field for a long time, but its application in environmental analysis is relatively recent because of the difficulties encountered in making selective antibodies for small molecules such as many organic pollutants [66]. Immunoaffinity solid-phase extraction (SPE) is based upon molecular recognition using antibodies. The high affinity and high selectivity of the antigen–antibody interaction allows a high degree of molecular selectivity and has proven to be a unique tool in sample preparation development in recent years [67]. Immunoaffinty sorbents are used for targeting single analytes as well as their metabolites because an antibody can bind one or more analytes having structures similar to the ones used for its production [57, 67].

Antibodies are produced with the hope that they would be able to recognise the target analyte but not the whole complex. The most common approach for the production of antibodies is to use sheep, rabbits or mice [57]. A compound capable of producing an immune response is known as an antigen. Analytes of interest with small molecular mass like drugs and pesticides should be coupled with carrier protein in order to initiate immune response. The analyte coupled to the carrier protein is known as a hapten. Coupling is achieved by the introduction of a functional group into the selected molecule, which can be linked to a carrier protein [67, 68]. The hapten designs are based on trial and error because the conclusion of whether the hapten was designed properly is only drawn after producing and characterising the antibodies. However there is a new tool proposed in which a molecular modelling is done first so as to achieve a better design of the hapten according to the desired specificity [68].

Immobilising of antibodies on solid support, (examples being silica and agarose gel or silica beads) results in highly selective SPE and the immobilizing should not disturb the biospecific activity of the antibodies [68, 69]. The immobilization could either be by covalent bonding, adsorption or encapsulation [70]. The solid support is inert (chemically and biologically) and hydrophobic so as to limit the non specific interactions [68, 70]. The support is also easily activated and has good mechanical stability. Uniformity of particle size is also important [71]. Agarose immunosorbents are normally used in offline SPE because of lower back

pressure while silica based immunosorbents are used online coupled with separation techniques [68].

The disadvantage of immunoaffinity SPE is that it takes a long time (several months and even over a year) to produce antibodies [57].

The immunoextraction procedure is shown in Fig. 1.20 with the following steps:

1. Conditioning

   Pure aqueous solution or aqueous solution containing less percentage of organic solvent is used to create a favourable environment for specific interactions between the target analyte and the sorbent. The step allows for the removal of storage solution which is mostly the phosphate buffer containing a small percentage of azide.

2. Percolation of the sample

   Percolation of the sample takes place mostly after pre-treatment of complex samples by centrifugation, filtration, dilution and pH modification. Care is taken that overloading of the immunosorbent does not take place and the affinity of the antibody towards the analyte is not low so as to avoid loss of better recovery. High flow rate may prevent proper binding of the analyte to the immobilized antibodies.

3. Washing

   Removal of interferences is carried out without eluting the analyte

4. Elution of the target analytes

   Elution of target analyte is carried out with the use of displacer agents, chaotropic agents, pH variations and water-organic modifier mixtures to induce disruption of analyte-antibody bonds.

5. Regeneration

   Antibodies may be dissolved in an appropriate solution to regenerate them therefore can be reusable. Reusability may not be recommended if analysis of complex samples is involved.

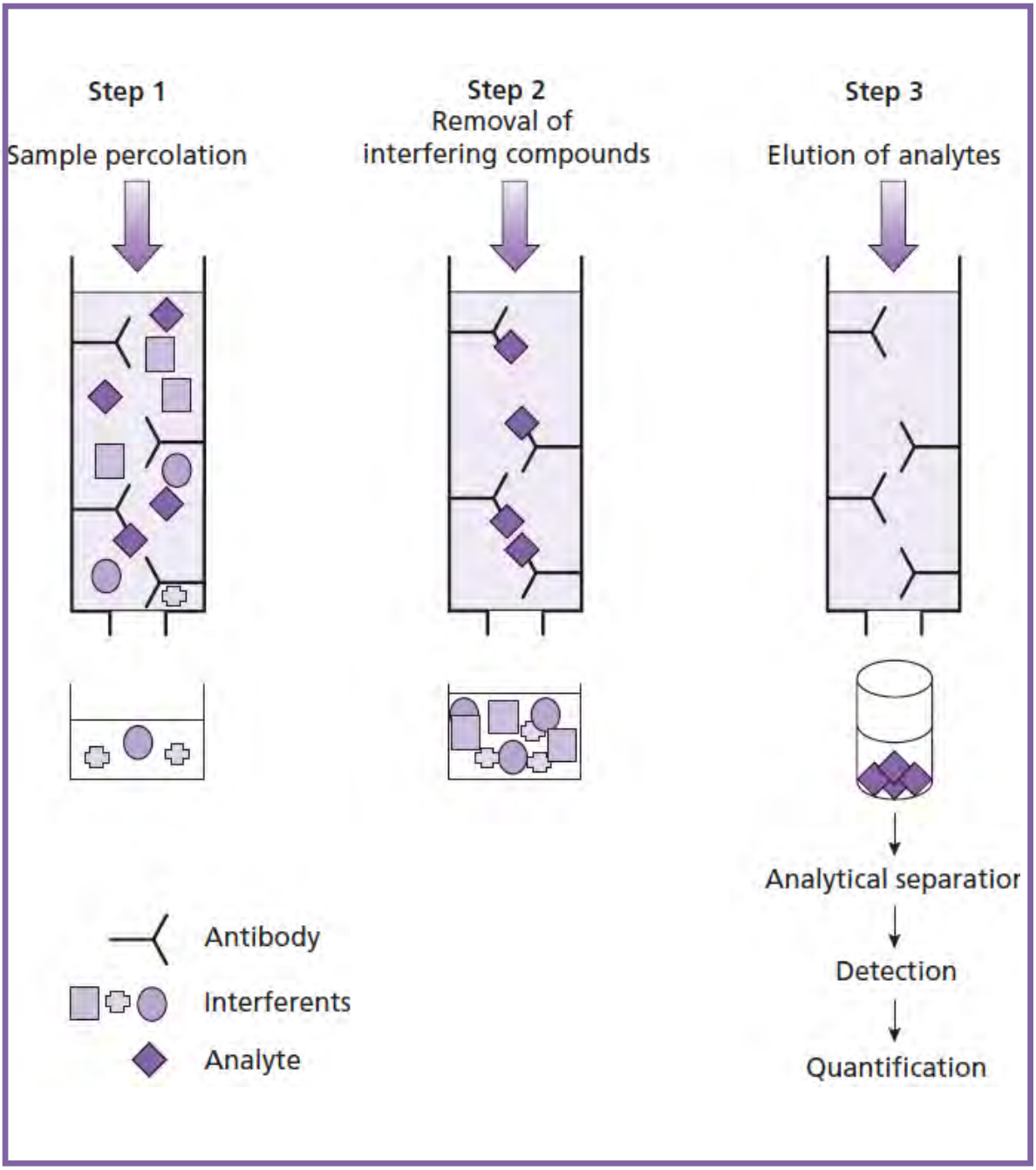


Fig. 1.20: Schematic of an offline immune extraction procedure [70]

1.3.2.4.2.2.5 Molecularly imprinted polymers (MIP)

Molecular imprinted polymers involves formation of host–guest complexes and is driven by intermolecular interactions involving ionic pairing, hydrogen bonding, van der Waals forces, hydrophobic effects and other interactions [72]. They are polymers that are highly stable and possess recognition sites within the polymer matrix that are adapted to the three-dimensional shape and functionalities of an analyte of interest [73]. A typical imprinting consists of a template molecule, at least one type of functional monomer and cross-linker, and a porogenic

solvent [72]. The imprinting is achieved if the target molecule (acting as a molecular template) is present during the polymerization process. The imprinting can be achieved by:

- Covalent imprinting whereby the print molecule and the template are chemically coupled with one of the building blocks of the polymer and the resulting bond is cleaved after polymerisation to obtain free selective binding sites [74, 75]. It provides more homogeneous binding sites but the re-binding is slower due to the necessary formation of the covalent bond between template and MIP, prior derivatization of the template is also required [72]. The other disadvantage is that it is only limited to a number of compounds like alcohols, aldehydes, ketones amines and carboxylic acids [74].

- Non covalent imprinting which is the most common approach. There is self assembly between the template and functional monomers producing highly reticulated polymers that contain binding sites equipped with functional groups in a defined 3 dimensional arrangement [73] (see Fig. 1.21). A non covalent procedure is easier although it may generate heterogeneous binding sites due to the relatively weak interaction utilised [72, 74, 75].

Attempts have been made to combine the advantages of both the covalent and non covalent imprinting. The imprinting is carried out using polymerization of the functional monomer being covalently coupled to a template, and selective rebinding utilizing non-covalent interactions [72]. There are also some functionalities that are introduced into the binding sites by non covalent interaction which establish irreversible covalent bonds with the target analyte in the rebinding step [74].

The template can be removed by solvent, PHWE and soxhlet extraction or chemical cleavage leaving cavities that are complementary to the template in terms of size, shape and arrangement of the functional groups. The resulting polymer has highly specific receptor sites, capable of rebinding the target molecule with high specificity. Molecularly imprinted polymers are therefore comparable to antibodies hence named “antibody mimics” [76]. It has been shown that they can be substituted for biological receptors in certain formats of immunoassays and biosensors [128].

They have potential to be applied in the areas of separation, trace analysis, assays, biomimetic sensors and (bio)-chemical synthesis as they have favourable physical and chemical properties. They therefore can be used under harsher conditions such as in organic solvents, at extreme pH, high pressures and elevated temperatures, where biological macromolecules are often denatured. Recently MIPs have attracted considerable attention as selective SPE sorbents for the cleanup and preconcentration of target analytes prior to determination as they are compatible with organic solvents [77]. The main perceived advantage of MIPs over biological antibodies for SPE is the ease with which they can be obtained and the lower cost and speed.

The main disadvantage of MIP in SPE is the difficulty in removal of the template analyte molecule which persists even after extensive washing. The challenge of template removal leads to leaching of the analyte during application of MIP to actual samples leading to inaccurate results. The challenge occurs because during MIP comparatively large amounts of template (mg levels) are used to prepare the polymer but individual samples may contain a lower concentration of analyte [37].

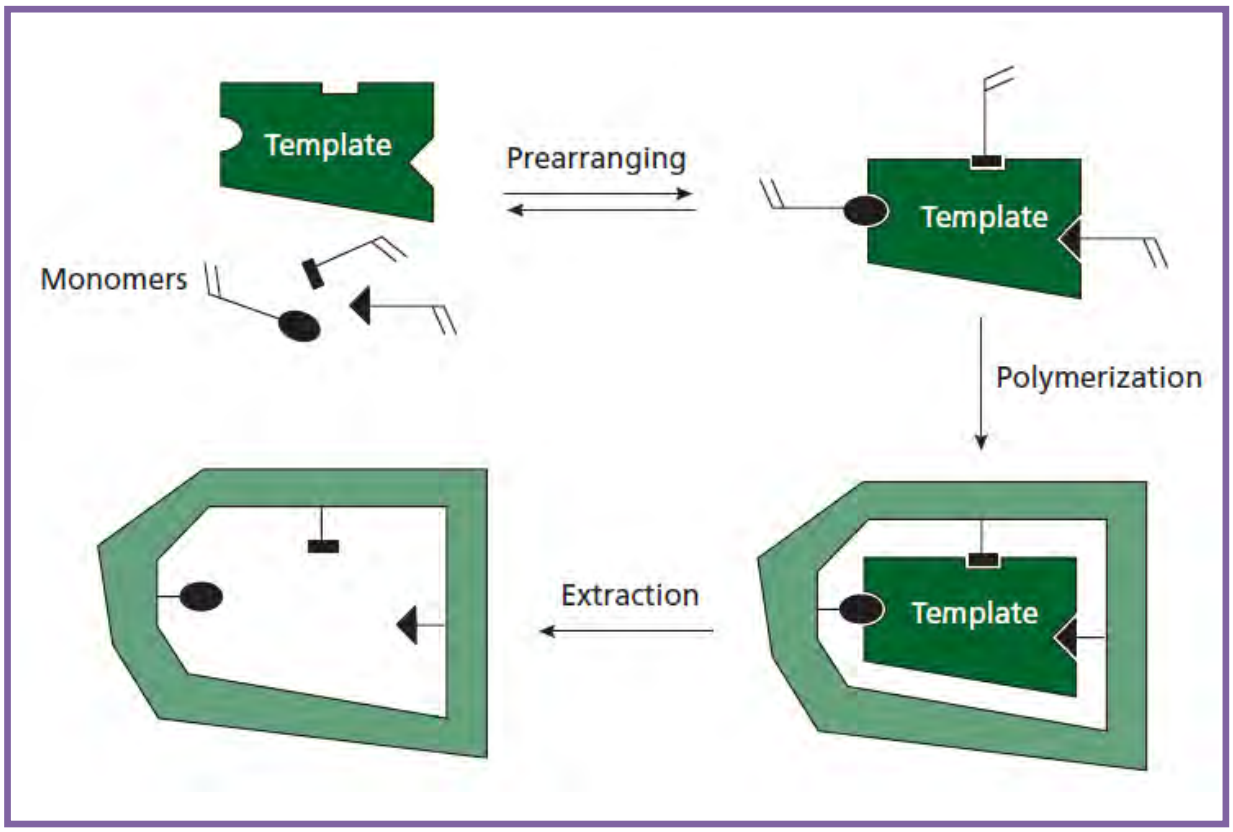


Fig. 1.21: Schematic synthesis process of molecularly imprinted polymer [78]

1.3.2.4.2.2.6 Restricted access media (RAM)

Restricted access media (RAM) are a special class of SPE sorbents used for the direct injection of biological fluids such as plasma, serum or blood. RAM can be applied in SPE to achieve direct analysis. Separation of analytes occurs in an analytical column allowing direct injection of the sample mixture. [79].

RAM is used mostly for the analysis of small molecules, their impurities and metabolites [78]. The RAM supports allows the separation of analytes through a combination of size exclusion and conventional hydrophobic or ion exchange interactions resulting in the passage of micromolecules and restrict the access of macromolecules [80]. The exclusion is as a result of the particles of the packing possessing a surface barrier for large solutes (eg. Proteins) and allows entrance of analyte into the internal surface area and there is a controlled gradient in the chemical surface composition [79].

RAM sorbent material is classified according to the macromolecular exclusion mechanism. The classifications of RAM are, internal surface reverse phases (ISRP), shielded hydrophobic phases (SHP), semi-permeable surfaces (SPS), dual zone phases (DZP) and mixed functional phase (MFP) [79] (see Fig 1.22). Macromolecular exclusions are performed using a physical diffusion barrier based on the size of the pore or by a chemical diffusion barrier created by a network covering the surface of the support, which consists of covalently or adsorptively bonded synthetic/natural polymers or protein at the outer surface of the silica[80]. RAM sorbents can be uni-modal or bimodal. The surface chemistry properties for the inner and outer surfaces are the same for uni-modal and they are different for bimodal [80].

The most popular RAM phase is the dual-mode/bimodal porous packing that is characterized by an outer hydrophilic layer and an inner surface porosity with a hydrophobic bonded phase. The protein is eluted unretained due to the outer hydrophilic surface with minimal interaction. Small pores of the packing excluded proteins, while small molecules and drug metabolites pass into the pores and are retained by hydrophobic interactions with alkyl bonded phases [78]. This separation leads to size-selective disposal of interfering macromolecular matrix constituents.

RAM phases are described as “nonfouling” phases but they have a reputation for eventual fouling with repeated injection of straight biological fluids because protein precipitation can occur causing fouling if the pH and organic solvent composition of the mobile phase are not optimized therefore care should be taken in their use [78].

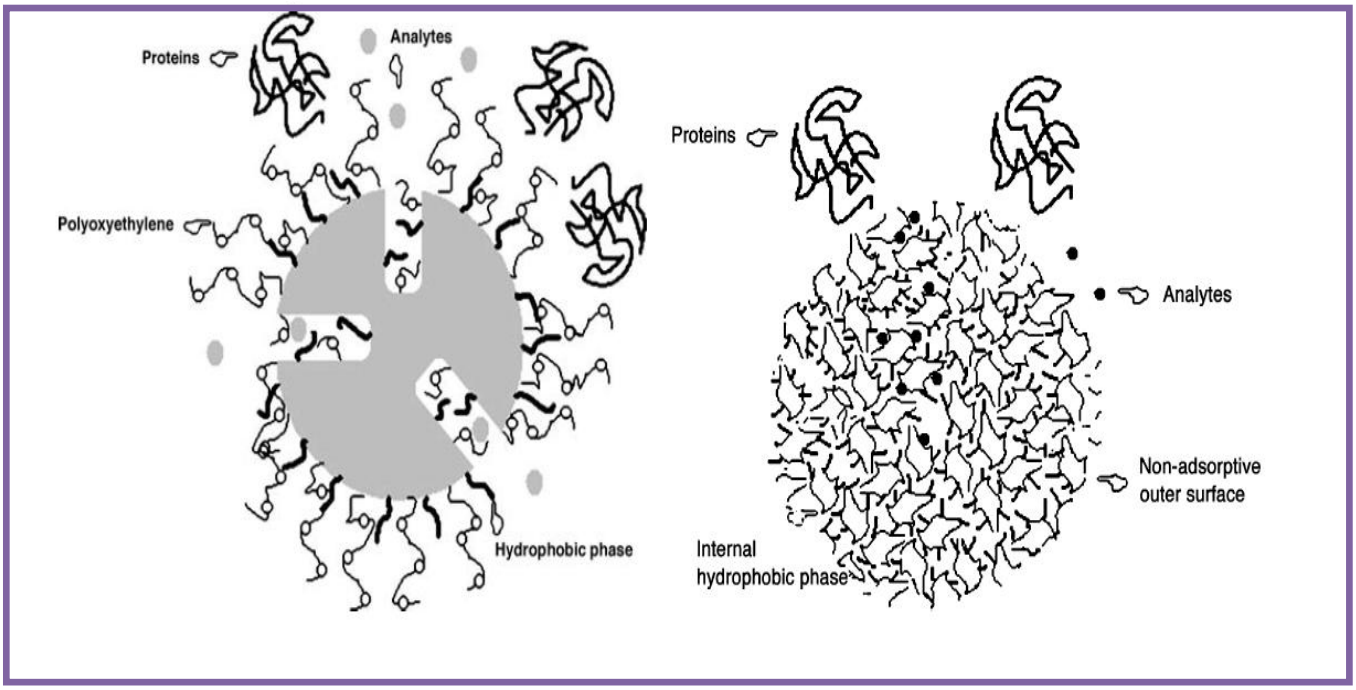


Fig 1.22(a) Schematic of ISRP **Fig 1.22(b) Schematic of MFP [80]**

1.3.2.4.2.2.7 Solid-phase microextraction (SPME)

SPME is a novel sample preparation approach in SPE that uses fused silica fiber that is coated on the outside with an appropriate stationary phase and the analyte is directly extracted and concentrated on the fiber coating [2]. SPME is designed to have the high distribution constants (K) resulting in the coating having a good affinity towards analyte than matrix [81]. It could be used for aqueous or gaseous samples and it completely eliminates the use of organic solvents [82]. SPME addresses the drawbacks of tedious, time consuming methods that are multi step resulting in loss of analyte [2] and also integrates sampling, extraction, pre concentration, and sample introduction into a single uninterrupted step resulting in high sample throughput [83]. It has had a major impact on sampling and sample preparation practices in chemical analysis, bioanalysis, food and environmental sciences [81].

The method has been used routinely with GC, GC-MS, HPLC and LC-MS for the extraction of volatile and semi volatile compounds from environmental, biological and food samples [2]. SPME has gained popularity as an extraction method because of the following advantages:

- selectivity
- good sensitivity: quantitative transfer to GC
- solvent free with GC therefore it is considered green technology
- easy of automation due to flexibility in configuration
- small size therefore suitable for onsite analysis
- intergration of sampling with sample preparation steps
- soft extraction method therefore non exhaustive
- free concentration determinations
- allows binding, speciation studies and reaction monitoring
- unconventional calibration and optimization [81]

SPME is an equilibrium sample preparation technique where only a small amount of extraction phase is used resulting in a small portion of the analyte being removed from the sample. The degree of extraction is dependent on the distribution constant (K_{fs}) between the coating and the sample matrix and the amount extracted at equilibrium is given by Eqn. 1.2 [84]:

$$n = \frac{C_o K_{fs} V_s V_f}{K_{fs} V_f + V_s} \qquad 1.2$$

Where: C_o is the initial sample concentration, n is the concentration of analyte extracted and V_s is the sample volume, V_f is the fiber volume but under conditions of negligible depletion the concentration of analyte extracted is independent of the sample volume Eqn. 1.3 applies [84]:

$$n = C_o K_{fs} V_f \qquad 1.3$$

1.3.2.4.2.2.8 Stir-Bar sorptive extraction (SBSE)

Stir-bar sorptive extraction is a technique that employs polydimethylsiloxane (PDMS) as an extraction medium for extraction of analytes from liquid and gas samples. Stir bars are coated with PDMS followed by introdution to the aqueous sample and allowed to stir extracting the analyte in the process [85]. The analyte partitions into the PDMS phase [86]. The stir bar is then removed from the sample after a certain time and then put in a glass tube followed by transferring to a thermal desorption instrument where the analytes are thermally released or liquid desorption is carried out followed by GC-MS analysis [85, 87].

Sorption using PDMS is a weaker process compared to adsorption therefore:

- degradation of unstable analytes is significantly less or absent compared to adsorbents.
- analytes can be desorbed at lower temperatures because of weaker interactions therefore minimizing the loss of thermolabile analytes.
- all solutes have their own partitioning equilibrium into PDMS therefore retaining capacity of PDMS for a certain analyte is not influenced by the presence of high amounts of water or other analytes.
- degradation fragments of the PDMS sorbent all contain characteristic silicone mass fragments which can easily be discerned with the use of a mass selective detector while organic adsorbents give response that interfere with the elucidation of the unknown [85, 86].

SBSE is an equilibrium technique similar to SPME but SBSE has increased recoveries as compared to SPME due the influence of the distribution constant and phase ratio on extraction efficiency. Extraction is reached at much lower partition coefficient for SBSE compared to SPME [87] (see Fig. 1.23).

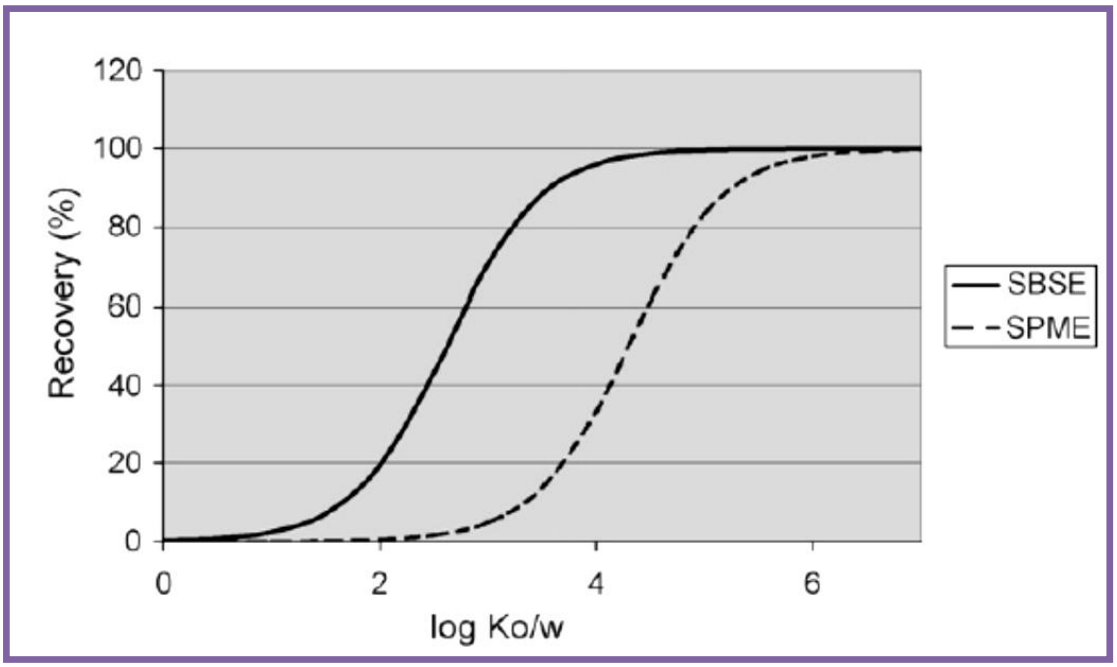


Fig. 1.23: Theoretical percentage recovery as a function of solute log*K*o/w for SPME (100_m fiber, 0.5 µL PDMS) and SBSE (1 cm×0.5 mm *d*f, 25 µL PDMS) and 10mL sample volume. Equilibrium sampling is assumed [87].

CHAPTER 2: COMMERCIAL AND IN-HOUSE MADE POLYMERIC SPE SORBENTS

This chapter gives an overview of trends in the development of new polymeric SPE sorbent materials. The main objective is to describe and highlight the major advantages that the polymeric sorbents brought compared to the previous sorbent materials. Polymeric sorbent materials studied include commercially available Agilent Bond Elut Plexa and in-house made electrospun polymer-silica nanofibers which both possess hydrophobic-hydrophilic interactions. The chapter also discusses the theory associated with breakthrough experiments as they are employed to establish the suitability of electrospun polymer-silica nanofibers as SPE sorbent materials.

2.1 Polymeric sorbents

Polymeric materials were introduced as SPE sorbents so as to overcome previous SPE challenges that were encountered with use of silica based sorbents. The main disadvantages with silica based sorbents are;

- present poor retention of polar compounds
- should remain wetted before sample application
- could not be used over a wide range of pH as silica dissolves at pH above 8 [88].

Modified polymeric sorbents overcame the limitations as they can be used over the whole pH range (resulting in increased method flexibility), less sensitive to drying out after conditioning and are better in the retention of polar compounds [89].

Polymeric sorbents can be made by polymerizing a monomer such as styrene, acrylamide, methacrylic acid or methyl methacrylate by cross linking with another olefinic compound (the cross-linker) like divinylbenzene or ethyleneglycoldimethacrylate [88]. The commonly used polymer is polystyrene-divinylbenzene (PS-DVB) because of its efficiency, ruggedness and wide pH stability [90]. PS-DVB has a hydrophobic structure therefore interacts with analytes through van der Waals forces and $\pi - \pi$ interactions [129]. PS-DVB has greater analyte retention mainly for polar compounds [89].

The unmodified PS-DVB still shows poor selectivity and low breakthrough volumes for highly polar compounds, which leads to their incomplete extraction from predominately aqueous samples[89]. PS-DVB hydrophobic structure is improved with the introduction of hydrophilic functionalities by using a hydrophilic precursor monomer or by chemically modifying the PS-DVB polymer skeleton [129] (see Fig 2.1). A typical example of the modified PS-DVB is Bond Elut Plexa from Agilent.

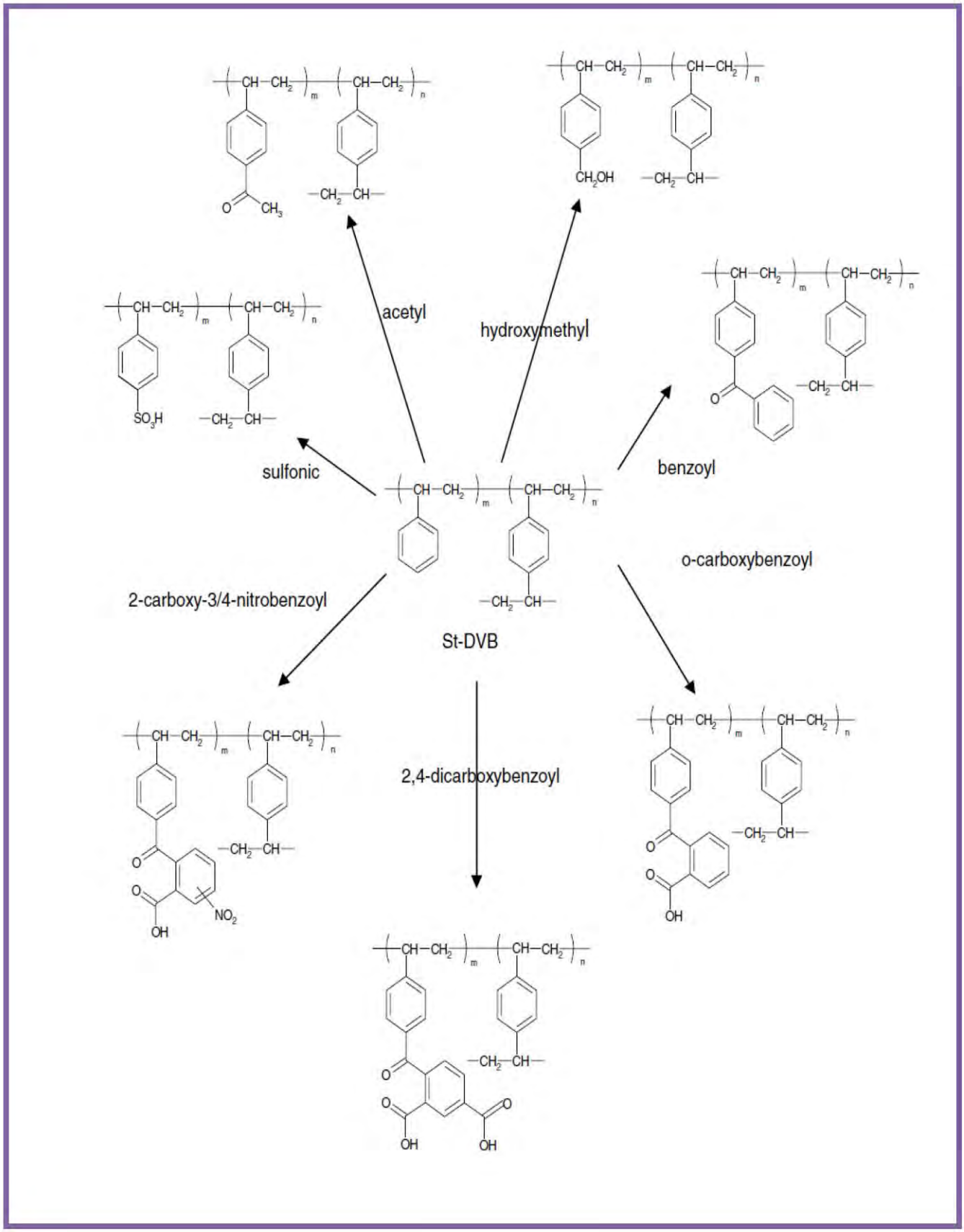


Fig. 2.1: Schematic of chemical modification of PS-DVB [89, 91]

2.1.1 Bond Elut Plexa SPE

Bond Elut Plexa is a commercially available non-polar PS-DVB neutral polymeric sorbent for non-ionic extraction of a wide range of acidic, neutral and basic analytes from different matrices. It is designed for simplicity, improved analytical performance and ease of use [130]. To achieve these properties the SPE mechanism works such that the hydrophobic core strongly retains analyte of interest while the hydrophilic exterior surface keeps the matrix interference out of the pore structure. During the washing step the matrix components are easily washed off while the analyte is still retained strongly in the hydrophobic core (see Fig.2.2). This results in cleaner extract that will give reproducible results and good recoveries. The uniform distribution and narrow size distribution helps in avoiding blockage of the cartridge, it thus improves flow rate and cartridge to cartridge reproducibility [131].

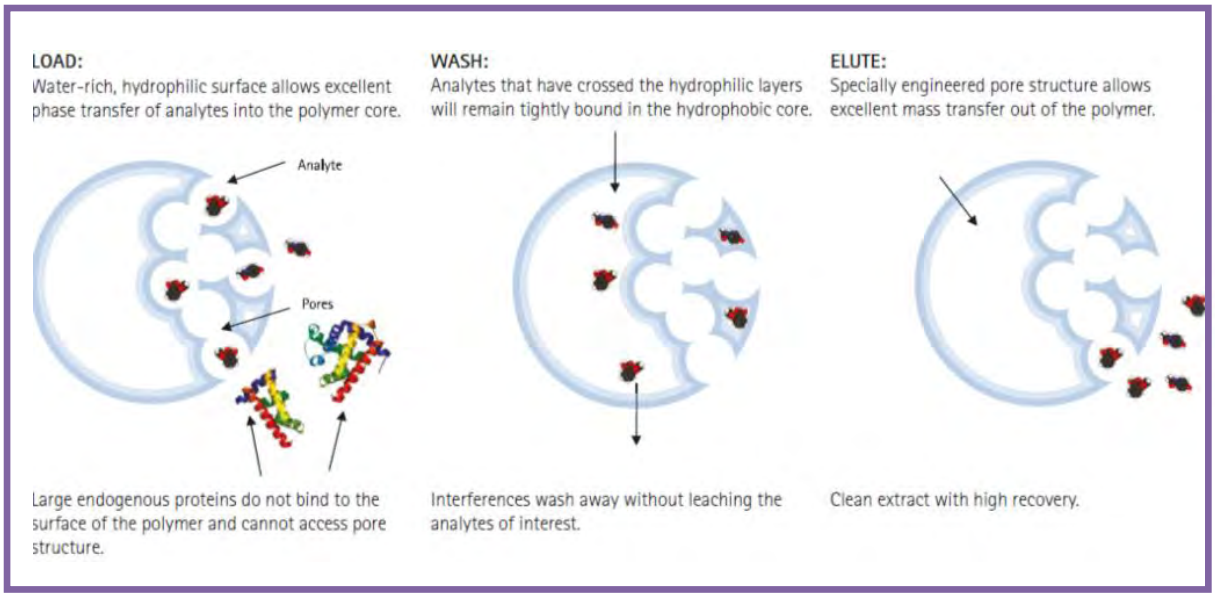


Fig. 2.2: Bond Elut Plexa extraction process [131]

2.2 Electrospun nanofibers SPE

Electrospinning is a process by which a polymer solution or melt can be spun into smaller diameter fibers using a high potential electric field [92, 93]. A typical electrospinning setup in the laboratory consists of a syringe pump, high voltage power supply and a collector.

A syringe pump delivers the solution from the syringe to the spinnerette, the solution forms a drop at the capillary tip. A high voltage power supply with positive or negative polarities induces free charges into the polymer or melt solution and the ions move in response to applied electric field towards the electrode of opposite polarity. The solution drop at the capillary tip takes the form of a taylor cone in the presence of the electric field and when the applied potential reaches a value that overcomes the surface tension of the solution, a jet of liquid is ejected from the tip of the cone. The solvent in the jet then evaporates resulting in a dry fiber formed [93, 94]. The formed fibers may be collected on a rotating drum or a flat surface. The morphology of the fibers is affected by solution flow rate, applied voltage, tip to collector distance, solution concentration, viscosity, surface tension, conductivity and solvent vapour [93-95].

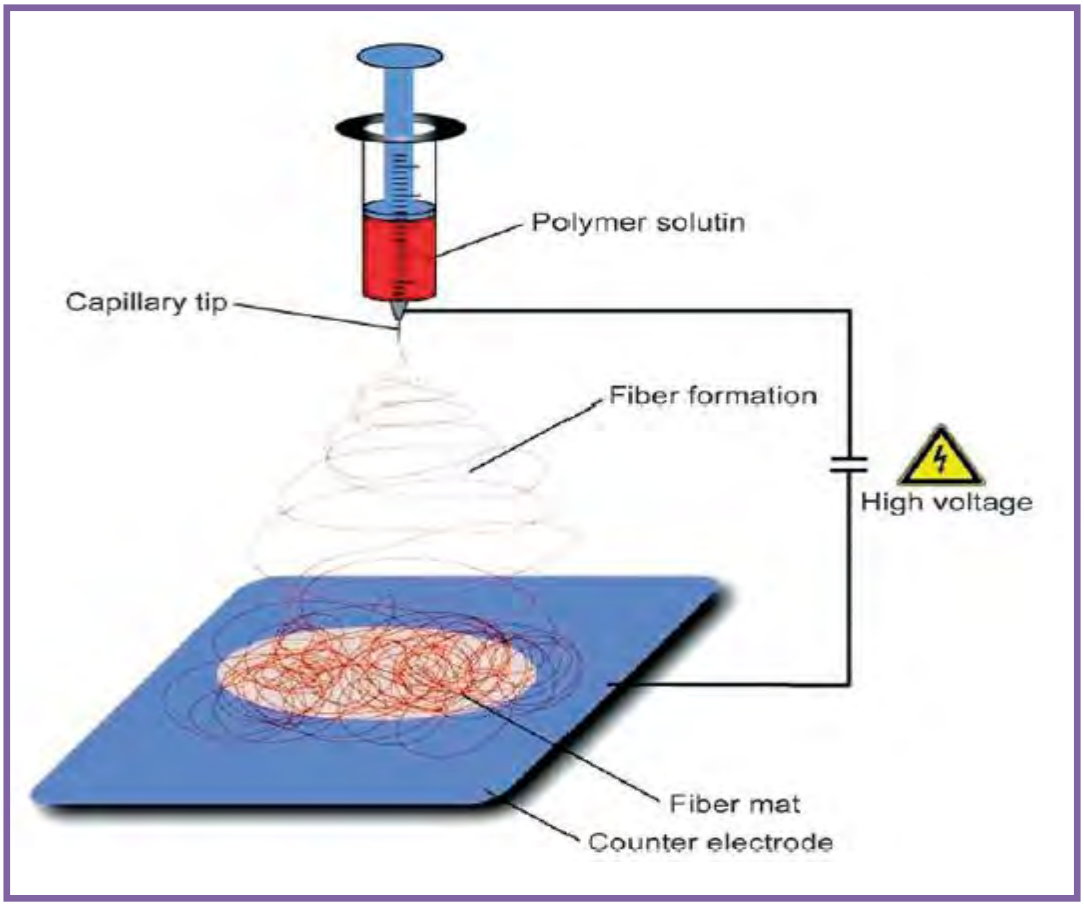


Fig. 2.3: Typical electrospinning setup [92]

Electrospun nanofibers research application has covered a broad range of fields including, but not limited to, protective textiles, tissue engineering, homogeneous catalysis, sensors, and

biomedical and biopolymer applications [96]. They are also gaining recognition as possible SPE sorbent materials as they are believed to offer reduced pressure drop during the extraction and desorption as compared to a conventional particle packed SPE. They are also used on the basis of the hypothesis that they have large surface area and small pore size which facilitates interaction between the sorbent and the target analytes therefore have analytical potential as effective SPE sorbents [93, 97, 98]. Due to large surface area a small amount of the sorbent can be used which results in great reduction of the volume of desorption solvent [99].

Electrospinning has the ability to control the diameter, morphology, secondary structure and special alignment of the nanofibers therefore making electrospun nanofibers good candidates for sorbents used in SPE [97]. It is also used to incorporate the benefits of nanoparticles into nanofibrous form at the same time addressing the limitations of the nanoparticles [98].

2.2.1 Polymer-silica composites

Silica is hydrophilic therefore gives good surface contact with predominantly aqueous samples. Polystyrene has a hydrophobic surface. Silica requires pretreatment with an activating solvent like methanol to obtain better surface contact with the aqueous solution being extracted. However the activation solvent can gradually be leached out of the sorbent thereby causing ineffective extraction. A better strategy is to make the surface of the sorbent permanently hydrophilic [89]. Polyacrylonitrile just like polystyrene has a hydrophobic structure therefore hydrophobicity is introduced by the silica.

The chemical structure of nylon 6 consists of amide groups separated by methylene sequences. The amide group is planar due to the partial double-bond character of the C-N bond [100]. Hydrogen bonding between the amino and carbonyl groups is maximised by the chain orientation therefore non polar interactions expected between methylene chains of nylon 6 and flavonoids [100]. Hydrophilic amide groups are expected to enhance/improve mass transfer resulting in effective extraction. Silica is expected to enhance the hydrophilic character that is already present in the nylon 6. Fig. 2.4 shows the chemical structures of polystyrene, polyacrylonitrile and nylon 6.

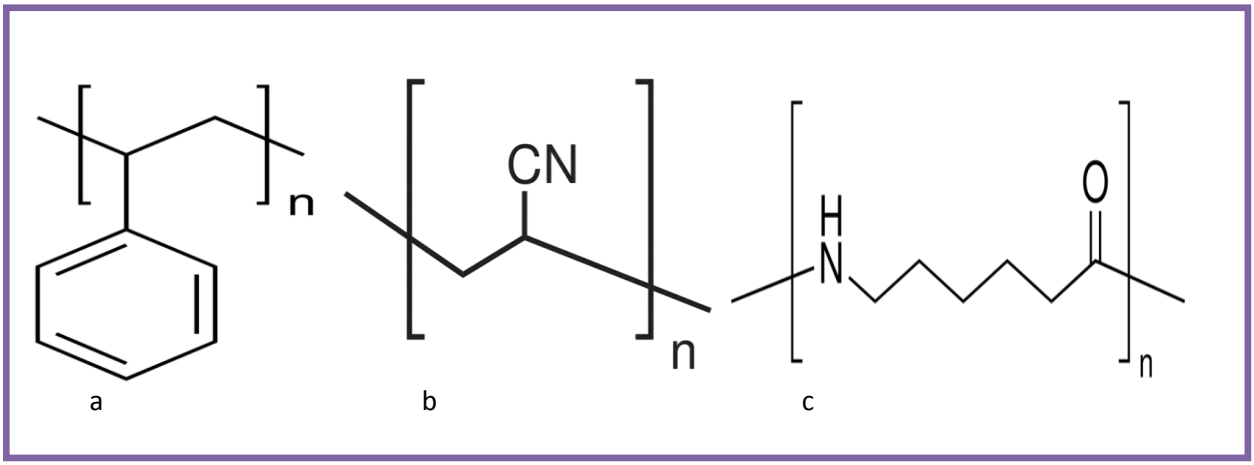


Fig. 2.4: Chemical structures of (a) polystyrene (b) polyacrylonitrile and (c) nylon 6

For the in-house polymer-silica composite, hydrophilicity was introduced by blending polystyrene with hydrophilic silica which contributes to hydrophilic interactions as well as to enhance the movement of the water molecules to the sorbent resulting in improved mass transfer. The sorbent material is compared with the Bond Elut Plexa which also possesses hydrophobic-hydrophilic interactions. The polymer-silica composite combines the advantages of organic material (polymer) which are flexibility, dielectric, ductility and processability and inorganic material (silica) which are rigidity and thermal stability [101].

Generally there are three main preparative methods for polymer-silica composites [101] (see Fig 2.5). Silica nanoparticles are introduced directly in blending and in-situ polymerization methods, while in a sol-gel process silica precursors (e,g silicon alkoxide and tetraethylorthorsilicate (TEOS)) are used [101, 102]. The sol-gel process is mostly preferred as the low temperature processing provides unique opportunity for tailoring well controlled organic-inorganic nanomaterial [102].

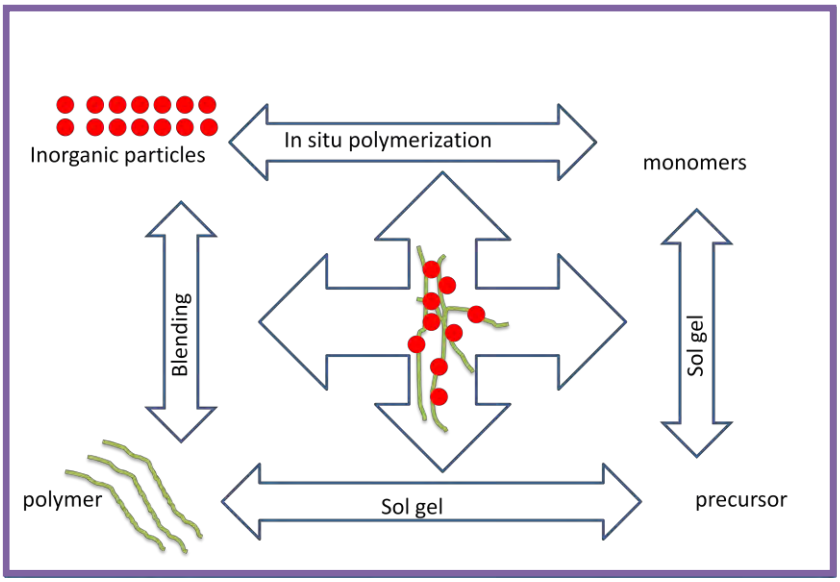


Fig. 2.5: Schematic of preparation methods for polymer-silica composite

2.3 Breakthrough volume (V_B)

One of the most important characteristic parameters in establishing the suitability of a SPE sorbent bed for extracting target analytes is the breakthrough volume as it gives an indication of the sorbent's loading capacity for the target analytes [103]. There are different definitions for breakthrough volume, one of them being the volume of the sample which has to flow through the sorbent to obtain 99-95% retention of analyte. V_B is dependent on the concentration of the analyte, temperature, flow rate and number of theoretical plates [104]. Therefore different parameter settings can be evaluated so as to obtain the ones that give the optimal value. The breakthrough volume provides information about the sorbent loading capacity of the analyte of interest. In addition to the breakthrough volume, two important parameters that are obtained from the breakthrough curve are the holdup volume (V_m) and retention volume (V_R). V_R is the volume at which the adsorption of the analyte is at equilibrium with the desorption of the analyte while V_m is the volume at which the analyte concentration entering the sorbent is almost the same as the one exiting the sorbent. From these parameters chromatographic characteristics of the sorbent bed can then be calculated [105].

Theoretical and experimental methods have been proposed for determining breakthrough curves. Although experimental breakthrough curve determination by frontal analysis is more

tedious, it is more useful for SPE device fabrication. This is due to the fact that it serves as a guide for understanding the effect of sorbent packing format, packing density and sorbent morphology on the flow characteristics of the sample phase. In frontal chromatography the analyte solution is continually fed into the sorbent material until a point where the analyte appears in the eluate, breakthrough is then observed when the sorbent capacity has been reached. The obtained eluate is then analysed using a chromatographic technique like HPLC-DAD [103, 104]. The volume is then plotted against the analyte response to obtain the breakthrough curve. Assuming that there is measurable analyte retention, the breakthrough curve forms a sigmoid shape that gives an indication of the analyte mass transfer kinetics as a function of the sorbent retention characteristics (see Fig. 2.6).

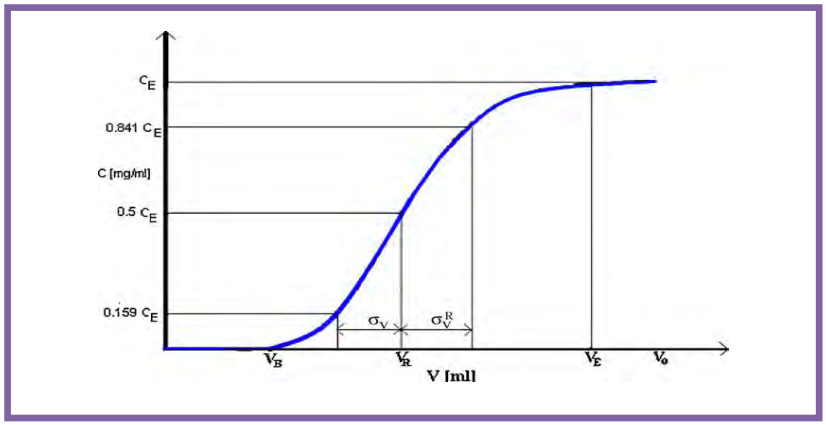


Fig. 2.6: Typical breakthrough curve [104]

The sigmoidal graph is used to calculate the breakthrough volumes (V_B), using the following equations:

$$y = \frac{a}{1+e^{\frac{x-x0}{b}}} \qquad (2.1)$$

Where y represent (C_e/C_i) the ratio of the eluted to the inlet analyte concentration, x is the volume of the sample flowing through the sorbent, a and b are 2 regression parameters. Solving for x would then give equation 2.2:

$$x = x_0 + bln\frac{F}{1-F} \qquad (2.2)$$

V_B and V_m are obtained at F values of 0.01 and 0.99 respectively. V_R is obtained directly from the graph at volumes corresponding to (C_e/C_i) of 0.5. V_R and V_B are then used to calculate the number of theoretical plates (N) using equation 2.3:

$$V_B = V_R \left[\frac{\sqrt{N}-2}{\sqrt{N}}\right] \tag{2.3}$$

The retention factor (k) is calculated using equation 2.4:

$$V_m = V_R (1 + k) \tag{2.4}$$

Evaluation of breakthrough parameters is important because, to achieve a significant concentration of the analyte of interest with minimum further sample manipulation, it is desirable to recover the analytes in a small solvent volume. It is therefore necessary to identify a solvent composition in which analytes have minimal retention factors. Generally the minimum elution volume that can be employed is about 2-3 times the holdup volume for the sampling device with a retention factor of <2 [103].

In this thesis the breakthrough parameters of the new sorbent material were studied using the main flavonoids (quercetin, kaempferol and isorhamnetin) in ginkgo biloba.

CHAPTER 3: LITERATURE REVIEW ON ALKALOIDS, FLAVONOIDS AND BANNED AROMATIC AMINES

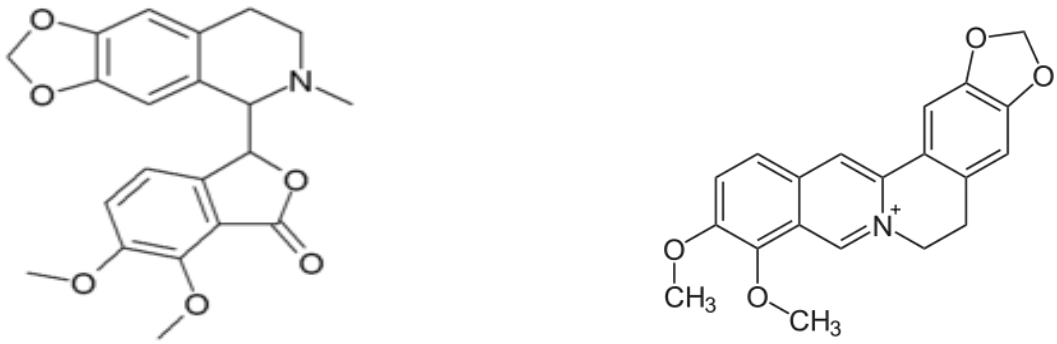
This chapter gives an overview of matrices and analytes relevant to the thesis. It also gives an overview of the scope of thesis.

3.1 Herbal plants

Herbal plants are commonly used for the manufacture of dietary supplements. Their safety to humans is of significant importance therefore studies are carried out to investigate the potential adverse health effects. The U.S. National Toxicology Programme (NTP), which performs research focused on the most critical public health issues, has been conducting a series of long-term studies on the toxicity of herbal medicines and related dietary supplements products nominated by the Public and Federal agencies [106]. New directives and legislation aimed at regulating herbal industries are being stipulated therefore there is a need for simple, reproducible, accurate and easy to use test methods for the detection of active ingredients of herbal plants [107].

3.1.1 Alkaloids in goldenseal (*Hydrastis Canadensis*)

Goldenseal (*Hydrastis Canadensis)* is a small perennial plant indigenous to the hardwood forest of eastern United States of America and Canada. It has been used by Native Americans to treat wounds, ulcers, digestive disorders, skin and eye ailments [108, 109]. The extracts of the tree have been made available as dietary supplements as they are believed to possess antimicrobial, anti-parasitic and anti-viral properties. It has been suggested that goldenseal (*Hydrastis Canadensis)* could be used to obtain false negative results on drug tests after inhaling cocaine or smoking marijuana by either ingesting the goldenseal with water or using it as an adulterant by adding it to the urine[108]. These properties are believed to be due to the plant's alkaloid content. The main alkaloids found in goldenseal (*Hydrastis CanadensiS)* are berberine and hydrastine (see structures in Fig. 3.1) [108]. The minor alkaloids are canadine, hydrastinine, berberastine, and canadaline.



Hydrastine **Berberine**

Fig. 3.1: Structures of hydrastine and berberine

3.1.2 Flavonoids in *Ginkgo biloba*

Ginkgo biloba is an ancient Chinese phytomedicine used to treat various ailments such as circulatory and demential disorders. The extracts of *Ginkgo biloba* are listed as the fifth or sixth most frequently used herbal dietary supplement in the United States, and the third bestselling herbal product in the health food stores in the United States of America [106].The major active ingredients of *Ginkgo biloba* extracts are flavonoids, in particular aglycone derivatives such as quercetin, kaempferol and isorhamnetin [110] (see structures in Fig. 3.2). Flavonoids are a group of polyphenolic compounds which are present in most plants, exhibiting higher concentrations in seeds, fruit skin or peel, bark and flowers. They are used mostly for their antioxidant behaviour for scavenging free radicals as they are known to exhibit vasodilatory, antithrombotic, antineoplastic, antiviral, antimutagenic, antiallergic, anti-inflammatory and antibacterial activities [111]. Therefore flavonoids are very popular as dietary supplements and are in various forms such as tablet, sugar-coated tablet, film-coated tablet, oral solution, drop, and injection [111].

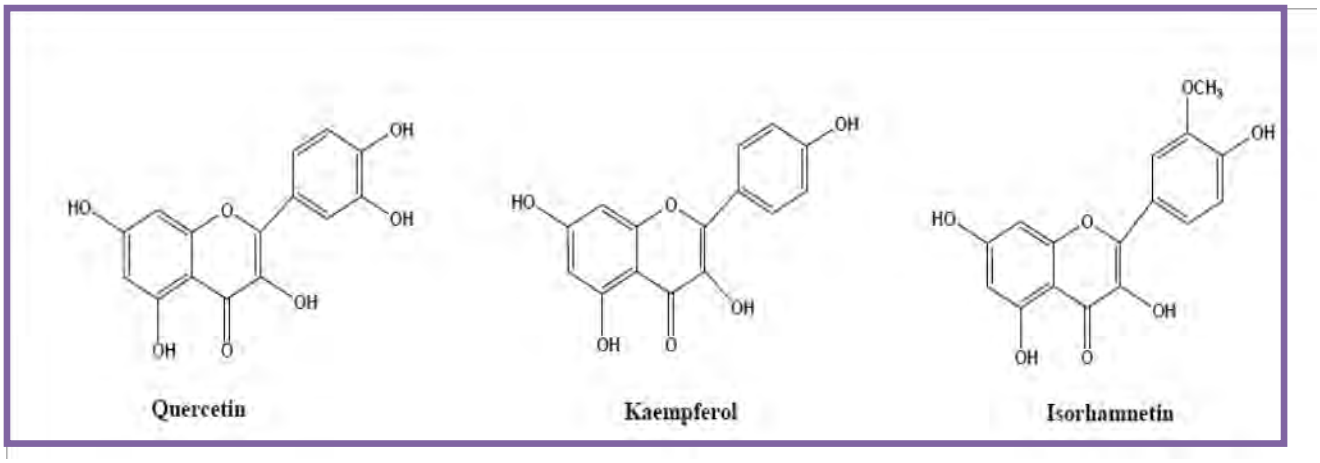


Fig. 3.2: Structures of quercetin, kaempferol and isorhamnetin

3.2 Aromatic amines derived from azo dyes in textile

Azo dyes are an important class of synthetic dyes. They are used for giving a broad spectrum of colour to different materials such as textiles, toys, leather and are used in paints [112]. More than 3,000 azo dyes are used currently and the number represents more than 65% of the global dye market [113]. They possess nitrogen nitrogen double bonds which can undergo reductive cleavage under anaerobic conditions thereby producing aromatic amines some of which are believed to be carcinogenic to humans [114] (see Fig 3.3). Humans are exposed to azo dyes and aromatic amines by various routes such as oral ingestion and dermal absorption through sweat and friction with clothing that is in direct contact with the skin [113]. There are therefore European Union regulations concerning the use of azo dyes that produce aromatic amines as they are carcinogenic.

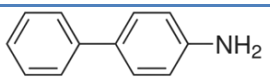
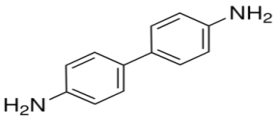
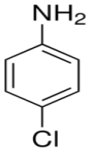
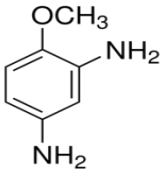
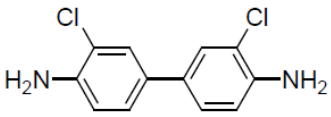
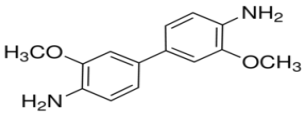
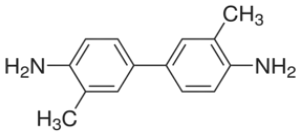
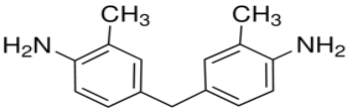
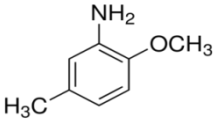
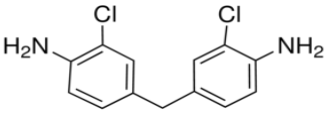
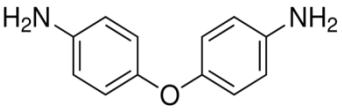
$$A - N = N - A \rightarrow A - NH_2 + B - NH_2$$

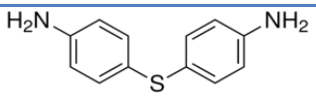
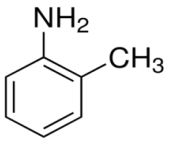
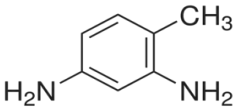
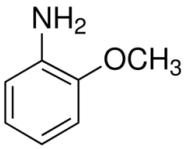
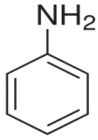
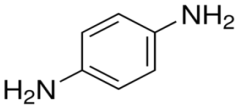
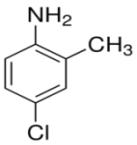
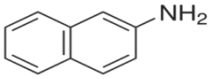
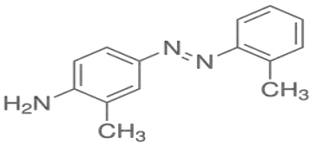
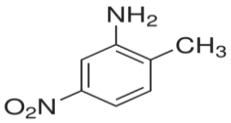
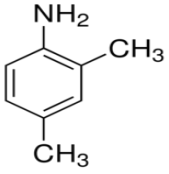
Azo dye aromatic amine aromatic amine

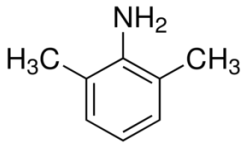
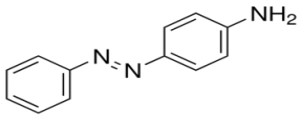
Fig. 3.3: Reductive cleavage of azo dyes to form aromatic amines

A total of 22 banned aromatic amines are included in the European Directive 2002/61/EC [113, 114] (see Table 3.1).

Table 3.1: EU banned aromatic amines including 2,4-dimethylaniline and 2,6-dimethylaniline

No.	Name	CAS No	Chemical structure
1	4-aminobiphenyl	92-67-1	
2	benzidine	92-87-5	
3	4-chloroaniline	106-47-8	
4	4-methoxy-m-phenylenediamine	615-05-4	
5	3,3-dichlorobenzidine	91-94-1	
6	O-dianisidine	119-90-4	
7	O-tolidine	119-93-7	
8	4,4-diamino-3,3-dimethyldiphenyl-methane	838-88-0	
9	2-methoxy-5-methyl aniline	120-71-8	
10	4,4-methylene-Bis(2-chloroaniline)	101-14-4	
11	4,4 oxydianiline	101-80-4	

12	4,4-diaminodiphenylsulfide	139-65-1	
13	O-toluidine	95-53-4	
14	4-methyl-m-phenylenediamine	95-80-7	
15	O-anisidine	90-04-0	
16	Aniline	62-53-3	
17	1,4 diaminobenzene	106-50-3	
18	4-chloro-2-methylaniline	95-69-2	
19	2-naphthylamine	91-59-8	
20	o-aminoazotoluene	97-56-3	
21	5-nitro-o-toluidine	99-55-8	
22	2,4-dimethylaniline	95-68-1	

23	2,6-dimethylaniline	87-62-7	
24	4-aminoazobenzene	60-09-3	

3.3 Scope of the thesis

Chapters 2 and 3 are the main focus of this thesis. Solid phase extraction was employed for development of easy, robust and reproducible sample preparation methods for the determination of flavonoids and alkaloids in herbal medicine and for the determination of banned aromatic amines in textiles.

The SPE applications on alkaloids and flavonoids were previously carried out using Agilent SampliQ SPE. Agilent SampliQ SPE gave way to Agilent Bond Elut plexa which is a similar polymeric material therefore the driving force of the study was to convert SampliQ SPE applications to Bond Elut plexa applications. The matrices were chosen on the basis that they were previously analysed using Agilent SampliQ SPE.

There are standard methods (BS EN 14362-1:2003 and GB/T 17592-2006) that use SLE for extraction of aromatic amines in textiles. The study was conducted to evaluate the performance of Agilent Chem Elut SLE by comparing the recoveries obtained to the ones set in the standard methods. The study also involved the comparison of the performance of several competitive SLE products with Agilent Chem Elut SLE.

There are sorbents materials that possess hydrophobic-hydrophilic characteristics that have been reported but none of them was polymer-silica nanofibers. The main aim of the study was to obtain a better understanding of fabrication of polymer-silica composites and to expand on their usage. The electrospun polymer-silica nanofibers

were therefore investigated to find out if they have the potential to be used as SPE sorbent material that possesses hydrophobic-hydrophilic characteristics.

The objectives of the work were:

- To develop sample preparation methods mainly for chromatographic applications using solid phase extraction (Agilent Bond Elut Plexa) for alkaloids in goldenseal (*Hydrastis Canadensis*) and flavonoids in *Ginkgo biloba.*

- To develop sample preparation methods mainly for chromatographic applications using supported liquid extraction (Chem Elut SLE) for banned aromatic amines exploring the standard methods BS EN 14362-1:2003 and GB/T 17592-2006.

- To apply electrospun polymer-silica composites as SPE sorbents for cleanup of flavonoids in *Ginkgo biloba*.

CHAPTER 4: EXPERIMENTAL

4.1 Materials and Chemicals

Berberine hydrochloride and hydrastine hydrochloride standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Phosphoric and hydrochloric acids were purchased from Merck Chemicals (Gauteng, South Africa) while HPLC grade methanol was purchased from Merck KGaA (Darmstadt, Germany). Ammonia solution (25%) was from Saarchem Analytic (Krugersdorp, South Africa). Goldenseal (*Hydrastis Canadensis)* roots were purchased from a local herbal store in Grahamstown, South Africa. SPE cartridges were Agilent Bond Elut Plexa, 1 ml/30 mg tubes. Supported liquid extraction cartridges used were Agilent Chem Elut, Dikma Pro Elut, Macherey Nagel Chromabond and Agela Isolute with volumes of 50 mL and were all packed with 20 g diatomaceous earth material. Self prepared SLE used diatomaceous earth, frits and 20 mL reservoirs supplied by Agilent Technologies (USA).

Quercetin, kaempferol and isorhamnetin reference standards were purchased from Sigma-Aldrich (Saint Louise MO, USA). Potassium hydroxide pellets were purchased from Merk Chemicals (Johannesburg, South Africa). Solgar *Ginkgo biloba* capsules and tea leaves were purchased from a local herb store (Grahamstown, South Africa).

Aromatic amines reference standards (1,4 diaminobenzene, 4-methyl-m-phenylenediamine, 4-methoxy-m-phenylenediamine, aniline, O-toluidine, O-anisidine, 4-chloroaniline, 2,6-dimethylaniline, 2,4-dimethylaniline, 2-methoxy-5-methyl aniline, 5-nitro-o-toluidine, 4-aminoazobenzene, benzidine, 4-chloro-2-methylaniline, 4,4-oxydianiline, 2-naphthylamine, 4,4-diaminodiphenylsulfide, O-tolidine, O-dianisidine, 4-aminobiphenyl, 4,4-diamino-3,3-dimethyldiphenyl-methane,3,3-dichlorobenzidine, o-aminoazotoluene and 4,4-methylene-Bis (2-chloroaniline) were purchased from Sigma-Aldrich (Saint Louise, MO, USA). The internal standards (naphthalene d8, 2, 4, 5-trichloroaniline, 4-aminoquinaldine and anthraces d10) were purchased from Sigma-Aldrich (Saint Louise, MO, USA). Textile materials (cotton,

wool, polyester/cotton [80%:20%]) were purchased from a local fabric store (Grahamstown, South Africa).

Ammonium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merk Chemicals (Johannesburg, South Africa). Tetraethyl orthosilicate (TEOS), polystyrene, polyacrylonitrile and nylon 6 were purchased from Sigma-Aldrich (Saint Louise MO, USA).

4.2 Instrumentation

Analysis was performed on an Agilent 1200 series HPLC coupled with a diode array detector (DAD). The analytical column was an Agilent ZORBAX Eclipse Plus C_{18} column (4.5 x 75 mm x 3.5 μm) for flavonoids and alkaloids, Agilent Zorbax SB-Phenyl (4.6 mm x 250 mm, 5- Micron) was used for aromatic amines.

Agilent Technologies 7890A GC System coupled with Agilent Technonologies 5975C inert MSD was used for analysis of aromatic amines employing a triple-axis detector. The GC-MSD chromatographic column used was DB-35MS (J & W) 30m × 0.25mm i.d. with 0.25 μm film thickness.

Water was purified using Milli-Q system (Millipore, Bedford, MA, USA). The pH meter used was Jenway 3510 from Bibby Scientific Ltd (Dunmow, Essex, UK).

A Transmission Electron Microscope, a low resolution Vega Texan Scanning Electron Microscope and high resolution Scanning electron microscopy-Energy dispersive X-ray detector were used for the characterization of nanofibers.

4.3 Method development and validation

4.3.1 Preparation of stock and working standards

The stock solution of all the reference standards, 1000 μg mL^{-1} each, were prepared in methanol and stored at 4 ºC. The working mixed standards were prepared from the individual stock standard solutions as required.

4.3.2 Optimization of parameters

4.3.2.1 Separation of peaks for alkaloids using HPLC-DAD

A 5 µL of 80 µg mL^{-1} mixed standard was injected into the HPLC to optimize the separation of peaks. The elution gradient, column temperature, injection volume and flow rate were adjusted to obtain optimal separation of peaks. The column temperature was increased from 25 ^{o}C to 35 ^{o}C, injection volume was reduced from 10 µL to 5 µL and the flow rate was increased from 0.7 mL min^{-1} to 1.00 mL min^{-1}. Berberine and hydrastine were monitored at wavelength of 242 nm. The HPLC optimized conditions were as summarized in Table 4.1 (see Fig 5.1).

Table 4.1: HPLC-DAD conditions for alkaloids

Parameter	Condition
Column	Agilent Zorbax Eclipse Plus C_{18}
	4.6 x 75 mm x3.5 µm
Flow rate	1.00 mL min^{-1}
Injection volume	5 µL
Column temperature	35 ^{o}C
Mobile phase	A:0.1% phosphoric acid
	B:methanol
Run time	6 min
Gradient	Time 0 0.5 3
	%B 25 25 50

4.3.2.2 Separation of peaks for Flavonoids using HPLC-DAD

A 5 µL volume of a mixed standard of 50 µg mL^{-1} of quercetin and kaempferol and 100 µg mL^{-1} of isorhamnetin was injected into the HPLC to optimize their separation. The column temperature was increased from 30 ^{o}C to 35 ^{o}C, injection volume was reduced from 10 µL to 5 µL and the flow rate was increased from 0.7 mL min^{-1} to 1.00 mL min^{-1}. The optimized HPLC conditions used are outlined in Table 4.2 (see Fig 5.2).

Table 4.2: HPLC-DAD conditions flavonoids

Parameter	Condition
Column	Agilent Zorbax Eclipse Plus C18 4.6 mm x 75 mm, 3.5 µm
Flow rate	1 mL min^{-1}
Injection volume	5 µL
Column temperature	35 ^{o}C
Mobile phase	A: 0.5% phosphoric acid B: Methanol
Run time	4 min
Isocratic	40% A 60% B
Detection	370 nm

4.3.2.3a Separation of peaks for aromatic amines using HPLC-DAD

A 10 µL volume of a mixed standard of 35 mg L^{-1} of the aromatic amines was injected into the HPLC to optimize their separation. The parameters in the standard method EN 14362-1:2003 (E) were used as the baseline. The column temperature was set at 30 ^{o}C, injection

volume was reduced from 15 µL to 10 µL and the flow rate was increased from 0.6 mL min^{-1} to 1.00 mL min^{-1}.The HPLC conditions used are outlined in Table 4.3 (see Fig. 5.5).

Table 4.3: HPLC-DAD conditions for separation of aromatic amines

Parameter	Condition
Column	Agilent Zorbax SB-Phenyl 4.6 mm x 250 mm, 5- Micron
Flow rate	1 mL min^{-1}
Injection volume	10 µL
Column temperature	30 ^{o}C
Mobile phase	A: Methanol B: phosphate buffer (pH 6.90)
Run time	70 mins
Gradient	Time %B 0 90 50 50 70 80
Detection	240 nm, 280 nm and 305nm

4.3.2.3b Separation of peaks for aromatic amines using GC-MSD

A 1 µL of a mixed standard of concentrations ranging from 12-35 mg L^{-1} of aromatic amines was injected into the GC-MS to optimize their separation. The parameters in the standard method EN 14362-1:2003 (E) were used as the baseline. The injection temperature was adjusted from 260 °C to250 °C. The GC-MSD conditions used are outlined in Table 4.4 (see

Fig. 5.6). The peaks were identified by retention time and qualifier ions (see Fig 5.6 and Table 5.1).

Table 4.4: GC-MSD conditions for separation of aromatic amines

EQUIUPMENT	**MODEL**
GC-MS information	Agilent Technologies 7890A GC System Agilent Technologies 7693 Autosampler Agilent Technologies 5975C inert MSD with Triple-Axis Detector
Column information	DB-35MS (J & W) 30m × 0.25mm i.d. with 0.25μm film thickness
CONDITIONS	
Injector system	Split Ratio: 15:1
Injection temperature	250 °C
Carrier gas	Helium Total flow: 29.796 mL/min Pressure : 16.465 psi

TEMPARATURE PROGRAMME

	Rate °C/min	Value °C	Hold Time min	Run Time min
Initial		80	1	1
Ramp 1	12	210	1	12.833
Ramp 2	15	230	1	15.167
Ramp 3	3	250	20	41.833

THERMAL AUX 2 (on)

	Rate °C/min	Value °C	Hold Time min	Run Time min
Initial		280		41.833

4.3.3 Calibration curves

4.3.3.1 Alkaloids

A 7 point calibration curve was prepared using concentrations of 0, 10, 20, 40, 60, 80 and 120 μg mL^{-1} for hydrastine and an 8 point calibration curve for berberine was prepared using concentrations of 0, 5, 10, 20, 40, 60, 80 and 100 μg mL^{-1}. The different concentration levels were achieved by diluting the 1000 μg mL^{-1} stock standard solutions into the required concentrations.

4.3.3.2 Flavonoids

Data for calibration curves was obtained by preparing different concentration levels of a standard mixture containing all the three compounds with concentrations of 0, 10, 20, 40, 80 and 120 μg mL^{-1}.

4.3.3.3 Aromatic amines

Different concentration levels of a standard mixture were prepared with concentrations of 0, 5, 10, 20 and 35 mg L^{-1}. The internal standards used were naphthalene d8, 2, 4, 5-trichloroaniline, 4-aminoquinaldine and anthraces d10. Each concentration level was run in triplicates and the resultant calibration curves were found to be linear over the concentration range with regression coefficients ranging above 0.990 (Table 5.2 and Table 5.3). Five points were used for the calibration curves for HPLC-DAD and 6 point for GC-MSD. The internal standard calibration curves for GC-MSD were obtained by plotting $\frac{S_A}{S_B}$ versus concentration where:

S_A is peak area of analyte and S_B is peak area of the internal standard

4.3.4 Limit of Detection and Limit of quantification

The limits of detection (LOD) and limits of quantification (LOQ) were calculated using the standard error of the regression line (S_y) and slope of calibration curve (S). The LOD values were calculated using equations 4.1, 4.2 and 4.3 (see Table 5.4 and 5.5).

$$S_y = \sqrt{\sum \frac{(Y_i - mx_i - b)^2}{n-2}} \quad 4.1$$

where:

x_i: each concentration level used in the calibration curve

Y_i: peak area corresponding to x_i

m: the slope of the calibration curve

b: the y-intercept of the calibration curve

n: number of degrees of freedom

$$LOD = 3.3 * (S_y/S) \quad 4.2$$

$$LOQ = 10 * (S_y/S) \quad 4.3$$

4.3.5 Sample preparation

4.3.5.1 Extraction of alkaloids from goldenseal (*Hydrastis Canadensis)* root using Bond Elut Plexa

200 mg of the goldenseal (*Hydrastis Canadensis)* root was ground and homogenized, then mixed with 200 mL of deionized water. The mixture was refluxed for 1 h with continuous stirring followed by cooling to room temperature. The extracts were filtered using a Whatman filter paper (125 mm diameter) and diluted to 1:3 *(v/v)* with 2% ammonia solution. The pH was adjusted to ~7 with 0.01 M hydrochloric acid and then analysis was conducted by HPLC-DAD.

4.3.5.1.1 SPE procedure

Bond Elut Plexa SPE was used for the cleanup of alkaloids in goldenseal roots following the protocol shown in Fig 4. 1. The collected 1 mL elute was then run in HPLC-DAD.

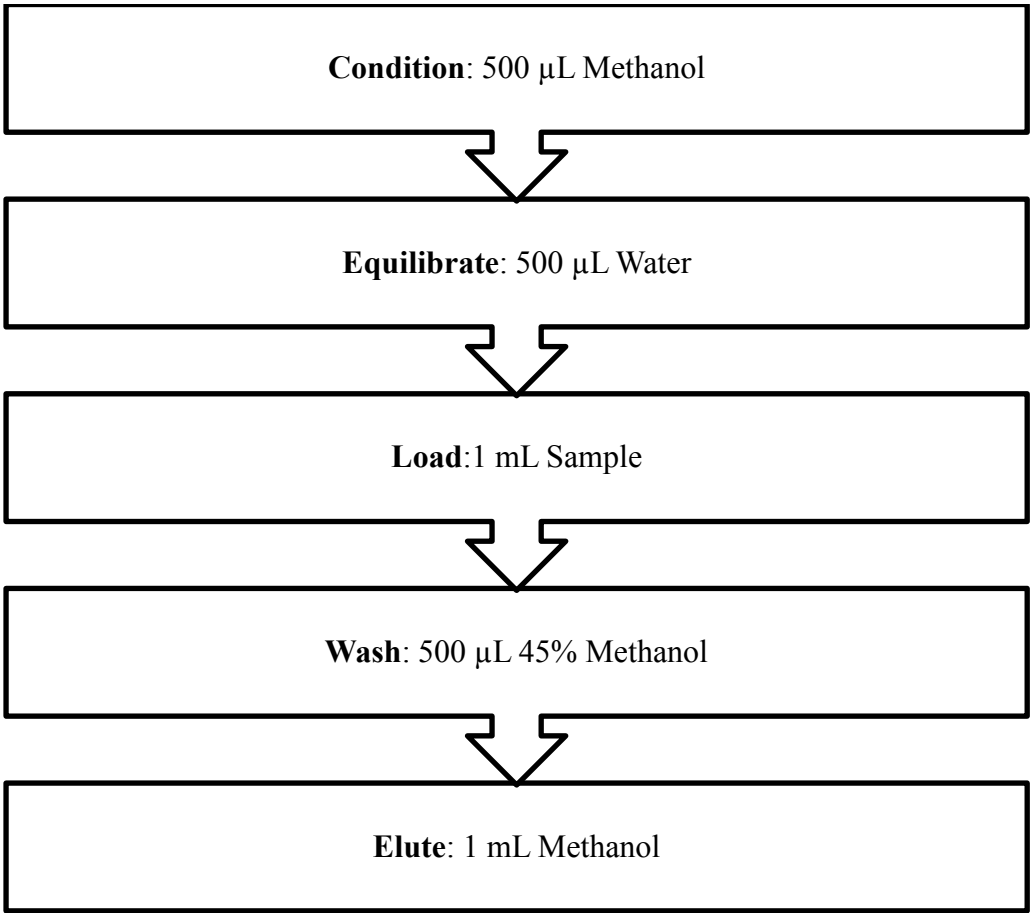


Fig. 4.1: SPE conditions for clean-up of alkaloids

4.3.5.2 Extraction of flavonoids from *Ginkgo biloba* tea leaves and capsules using Bond Elut Plexa

The contents of *Ginkgo biloba* capsules were homoginized. 2 g of the capsules was refluxed with 40 mL methanol and 40 mL 5.5% HCl (*v/v)* while continuously stirring for 1 h. The mixture was left to cool to room temperature and then filtered using a Whatman filter paper (125 mm diameter). The filtrate was diluted to 1:3 (*v/v*) with 2% ammonia solution. The pH was adjusted to ~7 with 1 M KOH. A 1 mL extract was filtered using a 0.45 µm Millipore Millex- HV membrane filter and analyzed with HPLC-DAD and it was compared with the chromatogram of the sample that went through the SPE process (see Fig. 5.4). *Ginkgo biloba* leaves were ground and homogenised using a mortar and a pestle, about 1 g was weighed and the same extraction procedure for ginkgo biloba capsules was followed for *Ginkgo biloba* leaves.

4.3.5.2.1 SPE procedure

The optimal conditions obtained for conditioning, loading, washing and elution steps of the SPE procedure are outlined in Fig. 4.2

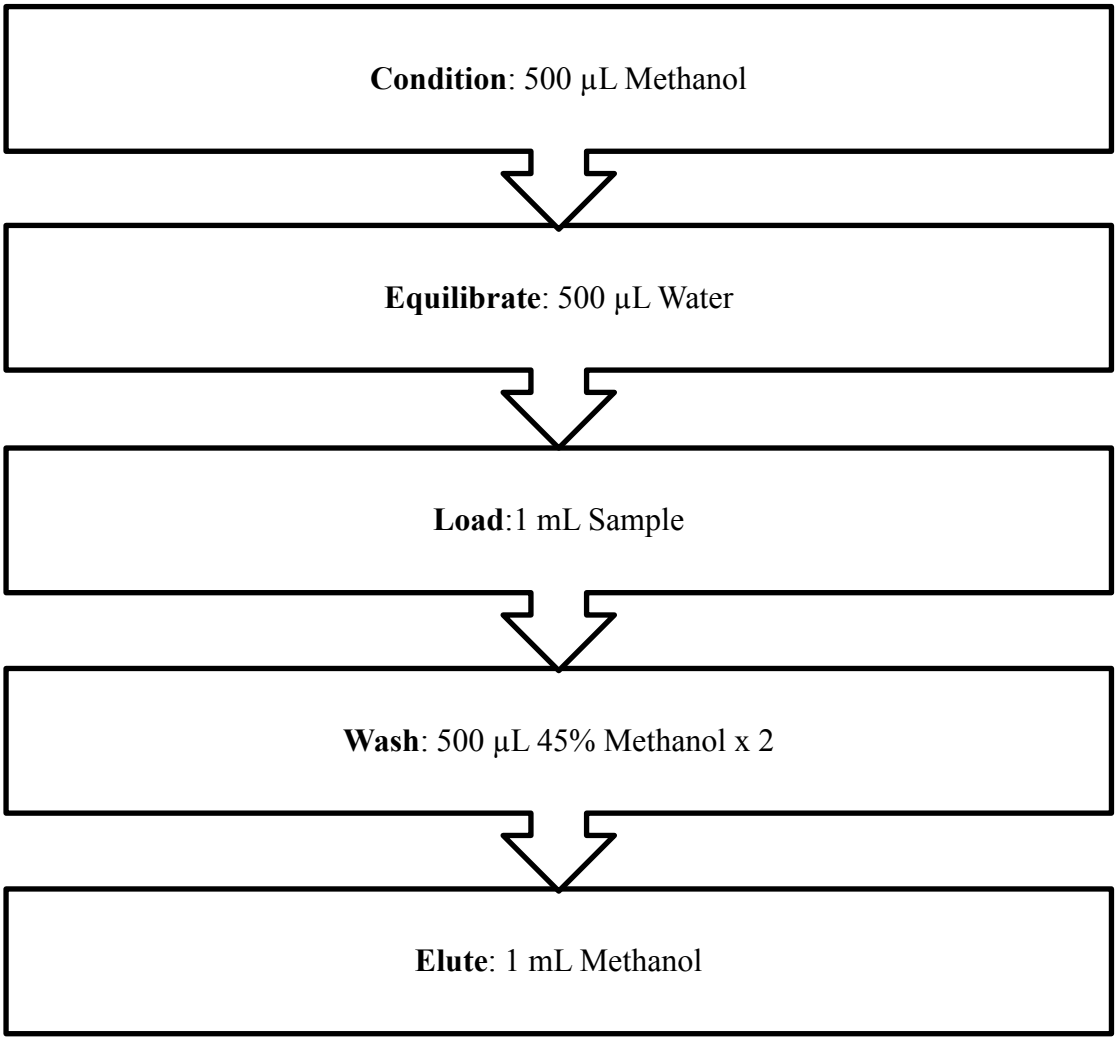


Fig. 4.2: SPE conditions for cleanup of flavonoids

4.3.5.3 Extraction of aromatic amines in textile using SLE

Supported liquid extraction conditions were optimised using Agilent Chem Elut, Pro Elut, Chromabond, Agela Isolute and self prepared cartridges. Packing of self prepared SLE was carried out in-house. A frit was inserted at the bottom of the reservoir followed by packing of about 20g diatomaceous earth material.

4.3.5.3.1 Preparation of test sample

Textile material (cotton, wool, polyester/cotton [80%:20%]) was cut into smaller portions (about 5 mm x 5 mm) and about 1 g textile was weighed into the reaction vessel. About 17 mL of preheated citrate buffer (60 ±2 ^{o}C) was added. The reaction vessel was tightly closed and then was shaken vigorously by hand and placed in an oil bath set at 60 ±2 ^{o}C. Freshly prepared sodium dithionate (600 mg in 3 mL water) was then added to the reaction vessel and was left continuously stirring using a stir bar. The flask was left in the oil bath set at 60 ±2 ^{o}C for about 30 min. The flask was removed from the oil bath and left to cool to room temperature after which the SLE procedure was carried out.

4.3.5.3.2 SLE optimization

The reaction solution was mixed with 0.5 M sodium hydroxide to improve recovery in 1:1 (*v/v*) ratio. The reaction solution was squeezed out of the fibers using a pasteur pipette and decanted onto the SLE column, the solution was allowed to adsorb for about 15 min. The residue fiber was rinsed with 2 x 20 mL aliquots of tert butyl methyl ether for method BS EN 14362-1:2003 and diethyl ether for method GB/T 17592-2006.The total volume of extraction solvent was 80 mL. There was no significant difference between using tert butyl methyl ether and diethyl ether therefore all experiments were carried out using tert butyl methyl ether. The resulting extract was then poured into the SLE column. The eluate was collected in a 100 mL round bottom flask.

The eluate was evaporated to about 1 mL using the rotary evaporator set at temperature of 35 ^{o}C and the remainder was removed by a flow of nitrogen gas. A 2 mL of HPLC grade

methanol was added to the residue, shaken vigorously and then transferred to HPLC vials for analysis.

4.3.6 Recovery and Reproducibility

All the percentage recoveries were calculated using equation 4.4.

$$\%\ Recovery = \frac{concentration\ of\ spiked - concentration\ of\ unspiked}{spiking\ concentration} * 100 \qquad 4.4$$

4.3.6.1 Alkaloids

The recovery and reproducibility of the method was studied by spiking the commercially available goldenseal (*Hydrastis Canadensis)* roots at three different levels. The spiked sample extracts were cleaned up using SPE at each concentration level for 6 replicates.The results obtained are shown in Table 5.6.

4.3.6.2 Flavonoids

Contents of the *Ginkgo biloba* capsules were spiked at three different concentration levels for each analyte. The spiked samples were taken through the whole SPE clean-up procedure. The relative standard deviation (R.S.D) was calculated for all the spiking levels. The results for recoveries and R.S.Ds are summarised in Table 5.7.

4.3.6.3 Aromatic amines

A textile sample was spiked at 3 different concentration levels and was taken through the supported liquid extraction procedure. Six replicates of each level were run. Poor recoveries were obtained. A 0.5 M sodium hydroxide solution was mixed with the sample extract in volume ratio 1:1 (*v/v*) to improve the recoveries. Percentage recoveries were calculated using equation 4.4 for HPLC-DAD. The results were also used to calculate relative standard deviations (see Tables 5.10-5.18). The recoveries obtained for Agilent Chem Elut SLE were

compared with those of other commercially available SLE products and the self prepared SLE (see Fig. 5.9-5.12).

The internal standard method was used to accurately calculate sample concentrations of aromatic amines for GC-MSD analysis using equation 4.5 and 4.6. Equation 4.4 was then used to calculate the percentage recoveries.

$$RRF = \frac{A_{std} * C_{is}}{C_{std} * A_{is}} \quad 4.5$$

$$C_x = \frac{A_x * C_{is}}{RRF_{mean} * A_{iss}} \quad 4.6$$

Where:

RRF: Internal response factor

A_{std}: Peak area of the amine in the standard solution

C_{is}: Concentration of internal standard

C_{std}: Concentration of amine in standard solution

A_{is}: Peak area of internal standard

C_x: Concentration of amine in the sample

A_x: Peak area of amine in the sample

A_{iss}: Peak Area internal standard in the sample

4.4 Application to real samples

The developed test methods were applied to real samples to determine the concentration of alkaloids in goldenseal (*Hydrastis Canadensis)* root, flavonoids in *Ginkgo biloba* tablets and tea leaves and aromatic amines in textile (cotton, wool, polyester/cotton [80%:20%]) . The maximum residue limit for banned aromatic amines is 30 mg kg^{-1}. The obtained results were compared with the maximum residue limit to check compliance (see Table 5.19).

The concentrations obtained were in mass/volume (mg L^{-1}/ µg mL^{-1}). The solid samples were spiked therefore the concentrations obtained were converted to mass/mass (µg g^{-1}/ mg kg^{-1}) using equation 4.5

$$\text{Concentration } (\mu g/g) = \frac{\text{concentration } (\mu g/ml) \times \text{total volume (ml)}}{\text{Nominal mass (g)}} \quad 4.5$$

4.5 Polymer-silica nanofibers as sorbent for extraction of flavonoids in *Ginkgo biloba*

4.5.1 Preparation of silica by sol-gel process

The starting material for silica was tetraethyl orthosilicate (TEOS).TEOS was hydrolysed under acidic conditions to get silica gel using molar ratio 1:2:2:0.01 (TEOS:ethanol:water:HCL). TEOS was mixed with ethanol put on a hot plate set at a temperature of about 80 ^{o}C and a mixture of water and hydrochloric acid was added drop-wise into the TEOS-ethanol mixture. The solution was left on the hot plate for 30 min and was then allowed to cool.

Silica particles were used instead of solution for PAN-silica composite. Sol-gel process was followed to prepare the silica but the solution was left to evaporate until a solid formed then a mortar and pestle was used to grind the solid into powder.

4.5.2 Preparation of polymer solutions

About 2 g of polystyrene was weighed and a molar ratio of 1:1 dimethylformamide (DMF) to tetrahydrofuran (THF) was added to make 20 wt% polystyrene content.

About 1.0 g of PAN was weighed and 10 mL of DMF was added to make 10 wt% polymer solution.

20 wt% nylon was prepared by mixing 2 g nylon with 10 mL mixture of formic acid and acetic acid (1:1 *v/v*). All solutions were left to stir for about 24 h.

4.5.3 Making polymer silica composite

The polymer was left to stir continuously while a silica solution was added dropwise. The addition was quick at the beginning but as white precipitate started forming, silica sol was added slowly allowing the precipitate to dissolve back into the solution before the next addition so as to avoid big lumps forming that could be difficult to dissolve. The silica sol addition was stopped as soon as the polymer solution started to become cloudy so as to avoid separation of the polymer and the liquid. About 5 mL of the silica was able to be added to 10 mL 20 wt% polystyrene and 20 wt% nylon. PAN-silica composite was prepared by adding 5 g of silica particles in 10 mL of 10 wt% PAN solution. The solution was then left to stir for about an hour then it was ready for electrospinning.

4.5.4 Electrospinning

The solution was put in a 25 mL syringe and different electrospinning conditions (see Table 4.5) were explored to obtain fibers collected on an aluminium foil. The polystyrene fibers were then studied under the SEM (see Fig 5.13-5.15). The distribution of silica particles was studied using TEM (see Fig. 5.17 and 5.18).

Table 4.5: Electrospinning conditions

Polymer composite	Concentration (%wt)	Tip to collector distance	Applied voltage	Flow rate
Polystyrene-silica	20	10 cm	+20 kv	0.5 mL hr^{-1}
Nylon 6-silica	20	10 cm	+25 kv	1.0 mL hr^{-1}
PAN-silica	10	10 cm	+20 kv	0.5 mL hr^{-1}

4.5.5 Breakthrough curves

The breakthrough volumes were studied using the frontal chromatography method. The SPE format used for breakthrough studies was the pipette tip. The reference standard solution (20 µg mL^{-1}) of flavonoids (quercetin, kaempferol and isorhamnetin) was passed through the sorbent mass of 40 mg for polystyrene-silica and PAN-silica while 50 mg was used for nylon-silica composite, the flow rate was set at 0.1 mL min^{-1} for polystyrene-silica and PAN-silica while for nylon-silica it was set to 0.5 mL min^{-1}(see Fig. 4.3). The obtained aliquots were run in HPLC-DAD using the developed method for flavonoids. The graph of volume

(mL) versus (C_e/C_i) was plotted using Weibull 5 parameter model (see fig. 5.22-5.24). The obtained graphs were used to calculate the breakthrough volumes (V_B), retention volume (V_R) and equilibrium volume (V_m).

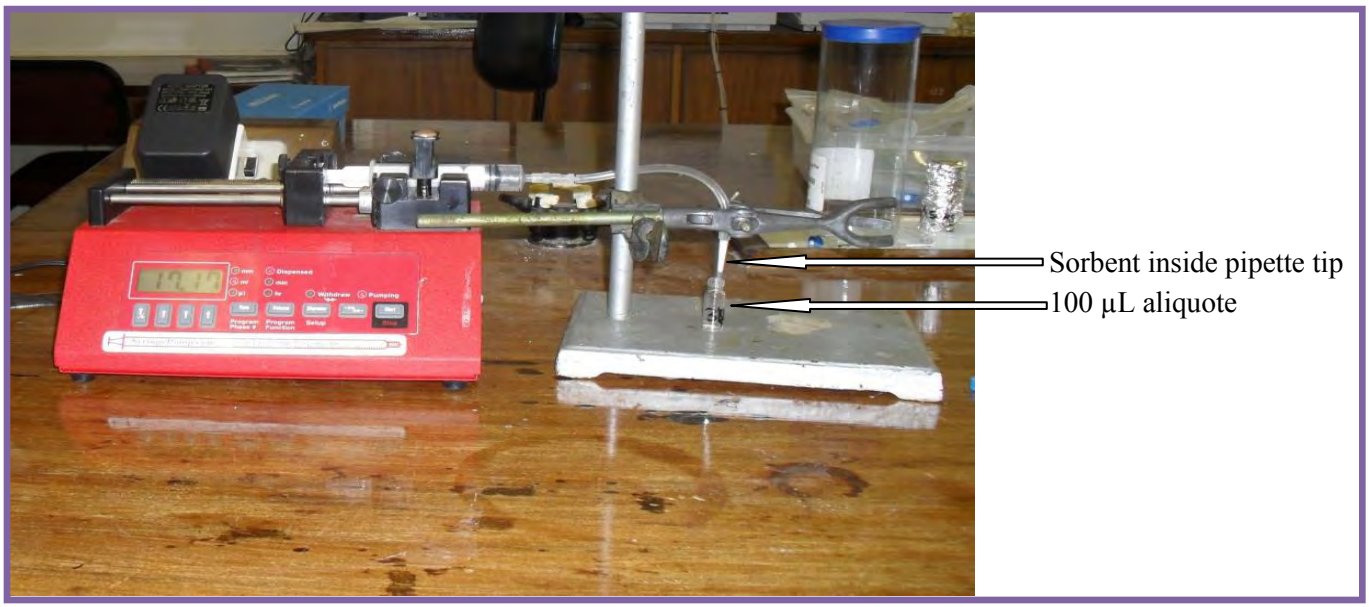


Fig. 4.3: Setup for obtaining aliquots for breakthrough studies

4.5.6 Application to solid phase extraction

The obtained fibers were weighed and packed in 1 mL SPE catridge, the fibers were pushed in between the frits to obtain a length of about 1 cm. The recoveries and effectiveness of clean-up for the new sorbent material were compared with those of commercial Bond Elut Plexa

CHAPTER 5: RESULTS AND DISCUSSIONS

5.1 Separation of peaks

5.1.1 Alkaloids using HPLC-DAD

Good peak separation was achieved for the standard mixture for hydrastine and berberine using HPLC conditions outlined in Table 4.1 (See Fig. 5.1). A goldenseal (*Hydrastis Canadensis)* root sample extract was run with similar HPLC conditions before and after SPE. The results showed a cleaner chromatogram for the SPE extract. There was a decrease in the number of small peaks and an increase in the intensity of the peak height therefore the SPE procedure was able to remove potential interferences (see Fig. 5.2).

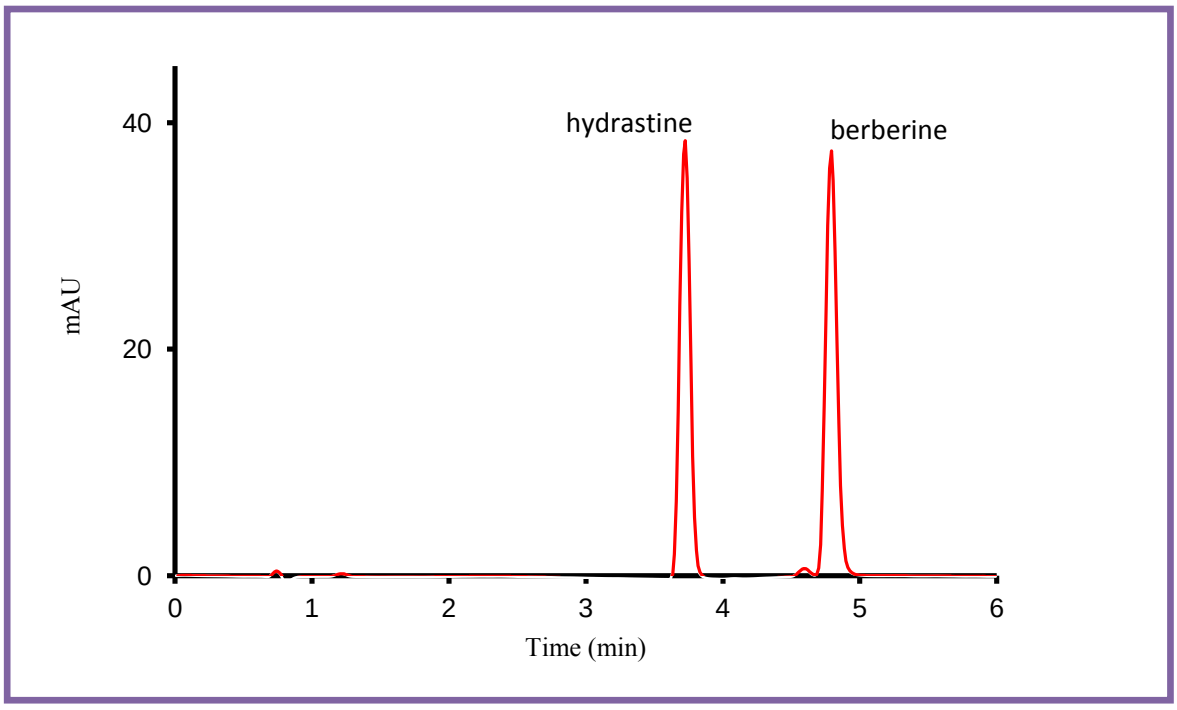


Fig. 5.1: HPLC-DAD Chromatogram of alkaloids mixed standard

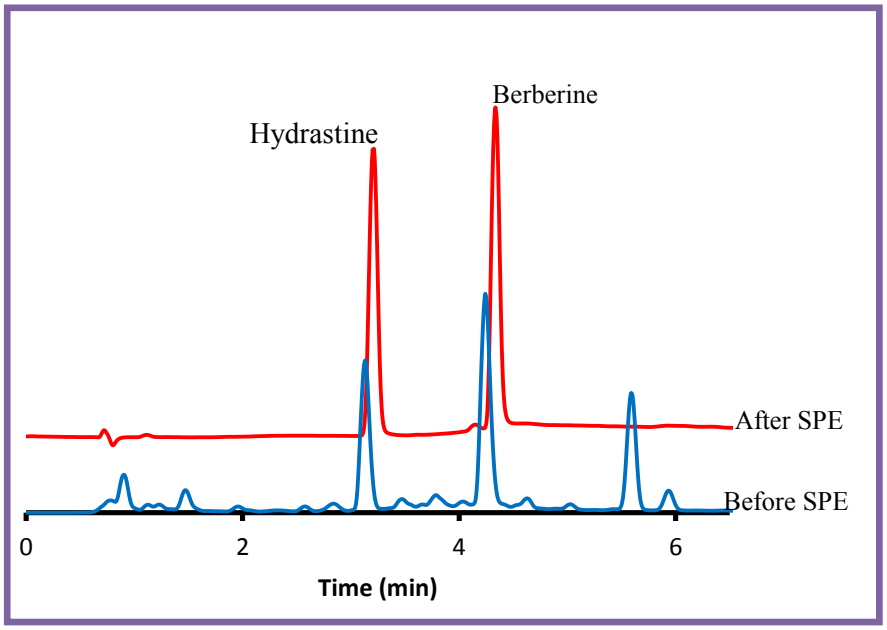


Fig. 5.2: HPLC-DAD chromatograms of alkaloids before and after SPE clean-up

5.1.2 Flavonoids using HPLC-DAD

Good separation of peaks for a standard mixture of quercetin, kaempferol and isorhamnetin was achieved using HPLC conditions outlined in Table 1 (see Fig. 5.3). Extracts from *Ginkgo biloba* capsules were filtered and were injected into the HPLC before and after the SPE procedure. An overlay of chromatograms for the two extracts is shown in Fig. 5.4. The chromatogram for the extract without SPE shows some interfering peaks while they were significantly removed after SPE indicating a successful clean-up by the Bond Elute SPE sorbents.

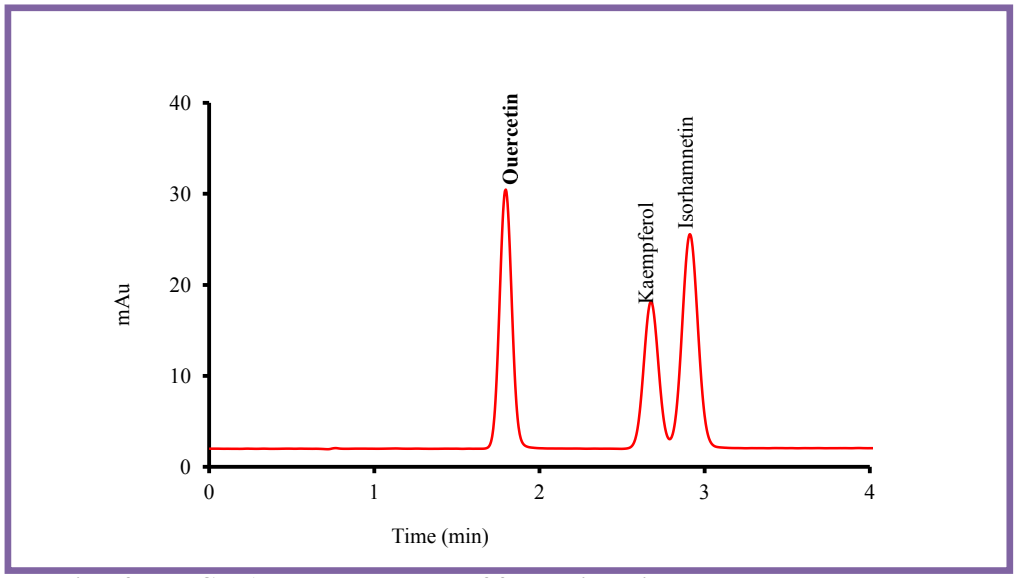


Fig.5.3 HPLC-DAD chromatogram of flavonoids mixed standard

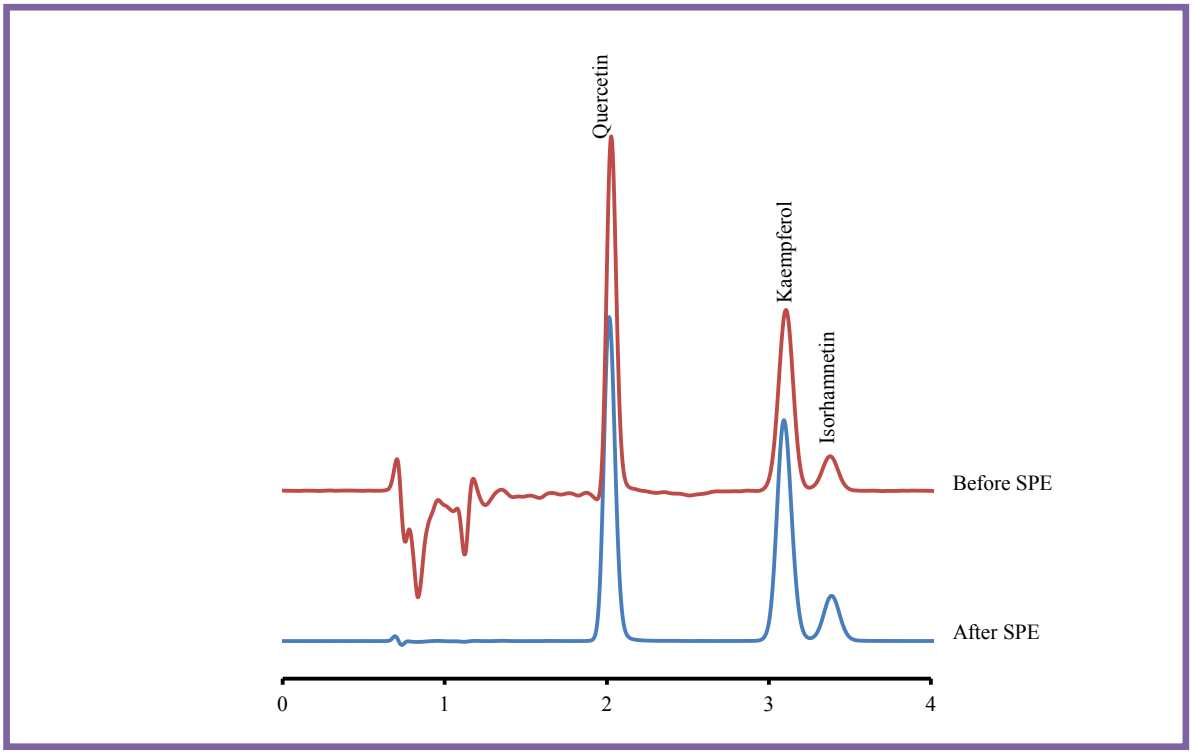


Fig.5.4: HPLC-DAD chromatogram of flavonoids before and after SPE clean-up

5.1.3a Aromatic amines using HPLC-DAD

Good peak separation for a standard mixture of aromatic amines was achieved using optimized HPLC conditions outlined in Table 4.3 (see Fig. 5.5). Peak (11) 4,4-oxydianiline and (19) 2-naphthylamine are coeluting therefore further quantification was not carried out

for these compounds for HPLC-DAD but were able to be separated and quantified in GC-MSD.

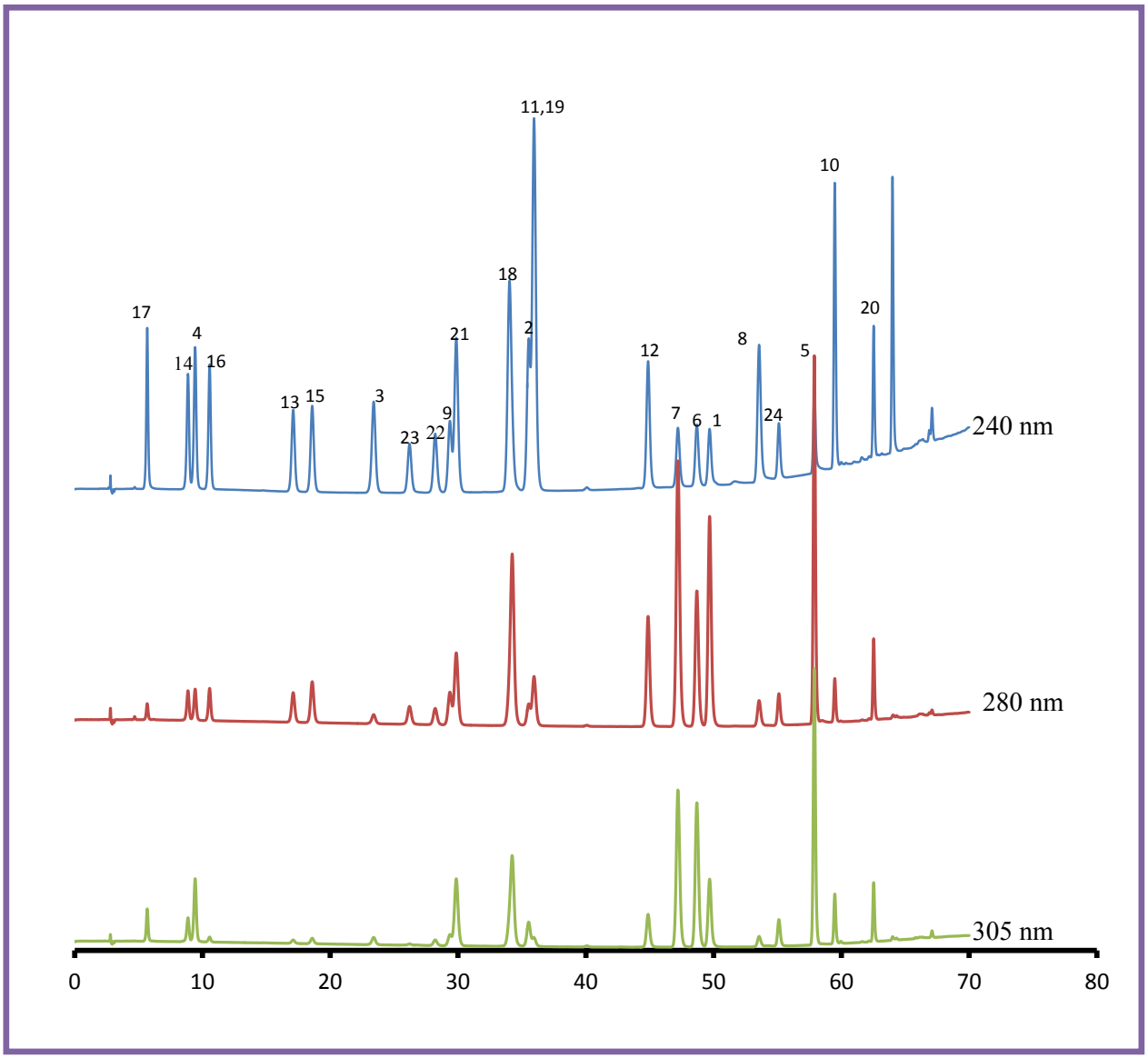


Figure 5.5: HPLC-DAD chromatogram of (17) 1,4 diaminobenzene (14) 4-methyl-m-phenylenediamine, (4) 4-methoxy-m-phenylenediamine,(16) Aniline (13) O-toluidine, (15) O-anisidine, (3) 4-chloroaniline, (23) 2,6-dimethylaniline, (22) 2,4-dimethylaniline (9) 2-methoxy-5-methyl aniline, (21) 5-nitro-o-toluidine, (2) Benzidine, (18) 4-chloro-2-methylaniline, (11) 4,4-oxydianiline, (19) 2-naphthylamine, (12) 4,4-diaminodiphenylsulfide, (7) O-tolidine, (6) O-dianisidine, (1) 4-aminobiphenyl, (8) 4,4-diamino-3,3-dimethyldiphenyl-methane (24) 4-aminoazobenzene, (5) 3,3-dichlorobenzidine, (10) 4,4-methylene-Bis (2-chloroaniline) and (20) o-aminoazotoluene

5.1.3b Aromatic amines using GC-MSD

Good peak separation was obtained within 40 min run time using parameters in Table 4.4. Peaks were identified using retention times and qualifier ions (see Fig. 5.6 and Table 5.1).

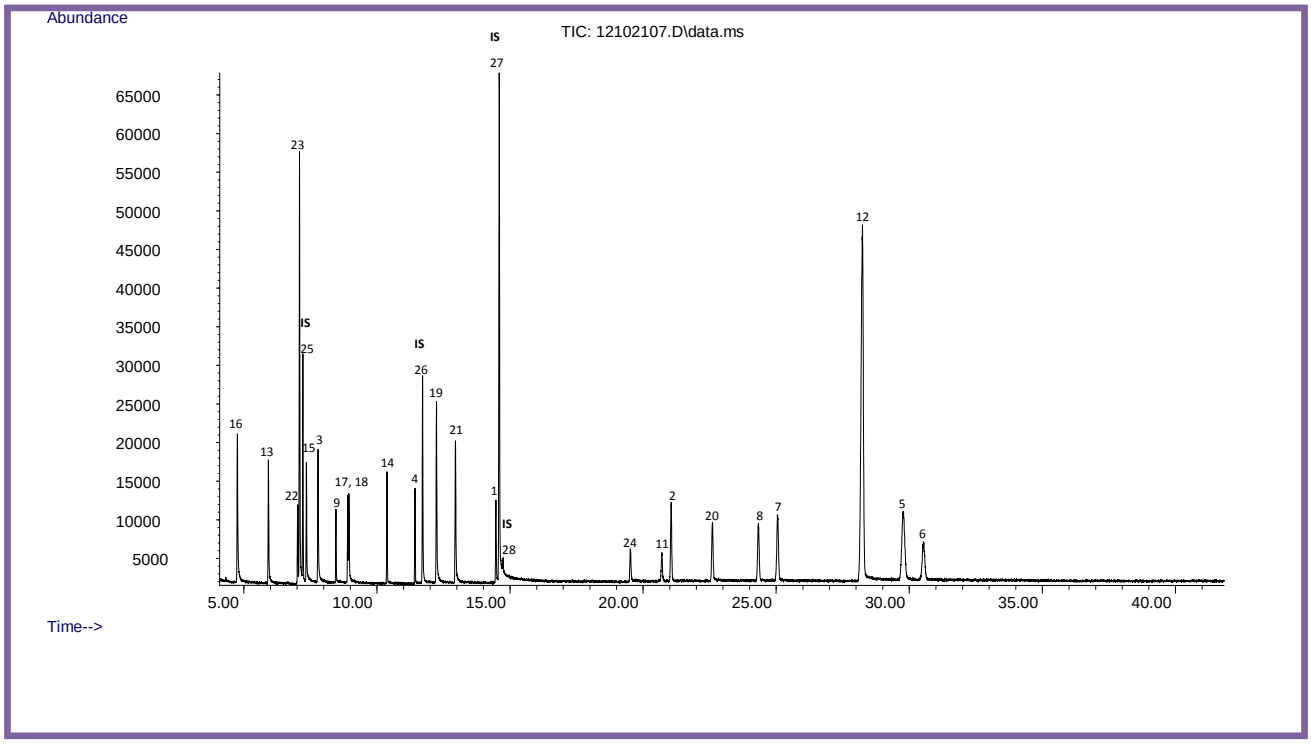


Figure 5.6: GC-MSD chromatogram of (17) 1,4 diaminobenzene (14) 4-methyl-m-phenylenediamine, (4) 4-methoxy-m-phenylenediamine,(16) Aniline (13) O-toluidine, (15) O-anisidine, (3) 4-chloroaniline, (23) 2,6-dimethylaniline, (22) 2,4-dimethylaniline (9) 2-methoxy-5-methyl aniline, (21) 5-nitro-o-toluidine, (24) 4-aminoazobenzene (2) Benzidine, (18) 4-chloro-2-methylaniline, (11) 4,4-oxydianiline, (19) 2-naphthylamine, (12) 4,4-diaminodiphenylsulfide, (7) O-tolidine, (6) O-dianisidine, (1) 4-aminobiphenyl, (8) 4,4-diamino-3,3-dimethyldiphenyl-methane, (5) 3,3-dichlorobenzidine, (20) o-aminoazotoluene with internal standards (25) naphthalene d8, (26) 2,4,5-trichloroaniline, (27) 4-aminoquinaldine and (28) anthraces d10

5.1.3c Identification of peaks by retention times for aromatic amines using GC-MSD

Table 5.1: Aromatic amines with retention times, base peaks and qualifier ions

No.	Name	Molecular weight	Base peak	Qualifier ion	Retention Time
17	1,4 diaminobenzene	108.14	108.1	80.0	8.903
14	4-methyl-m-phenylenediamine	122.17	122.1	94.0	10.378
4	4-methoxy-m-phenylenediamine	138.17	123.0	96.0	11.429
16	Aniline	93.13	93	66.1	4.758
13	O-toluidine	107.15	106	77.0	5.924
15	O-anisidine	123.15	108	80.0	7.349
3	4-chloroaniline	127.57	127	65.0	7.787
9	2-methoxy-5-methyl aniline	134.18	122.0	94.1	8.462
21	5-nitro-o-toluidine	152.15	152.1	77.0	12.951
2	benzidine	184.24	184.1	92.0	21.054
18	4-chloro-2-methylaniline	141.60	106.0	141.0	8.950
11	4,4 oxydianiline	200.24	200.1	108.0	20.702
19	2-naphthylamine	143.19	143.1	115.0	12.237
12	4,4-diaminodiphenylsulfide	216.30	216.1	184.1	28.242
7	O-tolidine	212.29	212.1	106.1	25.055
6	O-dianisidine	244.29	244.1	201.1	30.539
1	4-aminobiphenyl	169.22	169.1	206.9	14.469
8	4,4-diamino-3,3-dimethyldiphenyl-methane	226.32	226.2	120.0	24.334
24	4-aminoazobenzene	197.24	92	197.0	19.522
5	3,3-dichlorobenzidine	253.13	252.0	207.0	29.774
20	o-aminoazotoluene	225.29	106.0	225.1	22.604
23	2,6-dimethylaniline	121.18	121.1	77.0	7.094
22	2,4-dimethylaniline	121.18	120.1	77.0	7.023

5.2 Calibration curves

5.2.1 Alkaloids

Hydrastine and berberine were linear within the range of 0-120 μg mL^{-1} and 0-100 μg mL^{-1} respectively. They both gave regression coefficients of 0.9994 (see Table 5.2)

5.2.2 Flavonoids

The calibration curves were found to be linear over the concentration range of 0-120 μg mL^{-1} with regression coefficients of 0.9989, 0.9992 and 0.9992 for quercetin, kaempferol and isorhamnetin respectively.

5.2.3 Aromatic amines

Calibration curves

Calibration curves were linear within the selected concentration ranges of 0-35 μg mL^{-1} with regression coefficients above 0.990 for both GC-MSD and HPLC-DAD (see Table 5.2 and Table 5.3).

Table 5.2: Table of regression coefficients and equations for calibration curves of aromatic amines using HPLC-DAD

Compound No.	Name	Regression equation	R^2
17	1,4 diaminobenzene	y=21.464x	0.9996
14	4-methyl-m-phenylenediamine	y= 9.6548x	0.9971
4	4-methoxy-m-phenylenediamine	Y=8.0311x	0.9988
16	Aniline	Y= 19.283x	0.9998
13	O-toluidine	Y= 7.4427x	0.9998
15	O-anisidine	Y=9.5238x	0.9981
3	4-chloroaniline	Y=14.544x	0.9992
9	2-methoxy-5-methyl aniline	=6.5032x	0.9993
21	5-nitro-o-toluidine	Y=14.05x	0.9963
2	benzidine	Y=24.951x	0.9980
18	4-chloro-2-methylaniline	Y=14.55x	0.9931
11,19	Coeluting peaks		
12	4,4-diaminodiphenylsulfide	Y= 12.071x	0.9976
7	O-tolidine	Y=20.976x	0.999
6	O-dianisidine	Y= 13.317x	0.9953
1	4-aminobiphenyl	Y= 25.375	0.9925
8	4,4-diamino-3,3-dimethyldiphenyl-methane	Y=13.035x	0.9975
24	4-aminoazobenzene	Y=7.8548x	0.9999
5	3,3-dichlorobenzidine	Y=20.688x	0.999
10	4,4-methylene-Bis(2-chloroaniline)	Y=15.244x	0.9997
20	o-aminoazotoluene	Y=6.9603x	0.9949
23	2,6-dimethylaniline	Y=7.0458x	0.9991
22	2,4-dimethylaniline	Y= 8.5146x	0.9989

Table 5.3: Table of regression coefficients and equations for calibration curves of aromatic amines using GC-MSD

Compound No	Name	Regression equation	R^2
17	1,4 diaminobenzene	y=0.0082x -0.3208	0.9997
14	4-methyl-m-phenylenediamine	y= 0.0032x -0.0734	0.9938
4	4-methoxy-m-phenylenediamine	Y=0.0035x – 0.0895	0.9931
16	Aniline	Y= 0.0731x -0.6178	0.9919
13	O-toluidine	Y= 0.2078x -2.0775	0.9934
15	O-anisidine	Y=0.1464x -4.0852	0.9991
3	4-chloroaniline	Y=0.0053x – 0.0944	0.9924
9	2-methoxy-5-methyl aniline	Y=0.0116x -0.2194	0.9961
21	5-nitro-o-toluidine	Y=0.0055x – 0.1291	0.9964
2	benzidine	Y=0.12x-1.8464	0.9996
18	4-chloro-2-methylaniline	Y=0.0059x – 0.0625	0.9933
19	2-naphthylamine	Y=0.3561x -9.4438	0.9938
12	4,4-diaminodiphenylsulfide	Y= 0.9368x – 19.576	0.9987
7	O-tolidine	Y= 0.1523x – 2.9549	0.9936
6	O-dianisidine	Y= 0.0604x -1.5627	0.9906
1	4-aminobiphenyl	Y= 0.4416 – 5.6684	0.990
8	4,4-diamino-3,3-dimethyldiphenyl-methane	Y=0.0606x -1.3678	0.9982
24	4-aminoazobenzene	Y=0.1543x – 0.6787	0.9967
5	3,3-dichlorobenzidine	Y=0.3883x – 16.152	0.9921
20	o-aminoazotoluene	Y=0.149x -3.659	0.9922
23	2,6-dimethylaniline	Y=0.017x -0.3293	0.9918
22	2,4-dimethylaniline	Y= 0.1136x -1.3833	0.9962
11	4,4-oxydianiline	Y= 0.0836x -0.5936	0.9995

5.3 Limit of Detection and Limit of quantification

5.3.1 Alkaloids and Flavonoids

The limit of detection and quantification were determined using Eqn. 4.1, 4.2 and 4.3. The LOD and LOQ for hydrastine were 2.50 and 8.25 μg g^{-1} respectively while that of berberine were 2.35 and 7.75 μg g^{-1} respectively.

The LOD and LOQ for quercetin, kaempferol and isorhamnetin are as summarized in Table 5.4.

Table 5.4: LODs and LOQs for flavonoids using HPLC-DAD

Name	LOD (μg g^{-1})	LOQ (μg g^{-1})
Quercetin	2.94	9.34
Kaempferol	1.6	5.30
Isorhamnetin	6.50	21.6

5.3.2 Aromatic amines

Table 5.5 shows results for LODs and LOQs obtained using Eqn. 4.1, 4.2 and 4.3. The LODs for aromatic amines from the EN 14362-1:2003(E) standard methods were 5 mg kg^{-1}.

Table 5.5: LODs and LOQs for aromatic amines using HPLC-DAD

Compound #	Name	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
17	1,4 diaminobenzene	2.15	6.51
14	4-methyl-m-phenylenediamine	2.71	8.22
4	4-methoxy-m-phenylenediamine	3.11	9.43
16	Aniline	1.70	5.17
13	O-toluidine	2.77	8.405
15	O-anisidine	1.837	5.56
3	4-chloroaniline	0.25	0.77
9	2-methoxy-5-methyl aniline	1.00	3.03
21	5-nitro-o-toluidine	3.35	10.15
2	benzidine	1.98	6.01
18	4-chloro-2-methylaniline	2.84	8.61
11,19	Coeluting peaks could not be quantified		
12	4,4-diaminodiphenylsulfide	2.22	6.73
7	O-tolidine	2.38	7.21
6	O-dianisidine	1.40	4.23
1	4-aminobiphenyl	0.64	1.95
8	4,4-diamino-3,3-dimethyldiphenyl-methane	2.60	7.86
24	4-aminoazobenzene	1.11	3.37
5	3,3-dichlorobenzidine	0.96	2.90
10	4,4-methylene-Bis (2-chloroaniline)	0.28	0.86
20	o-aminoazotoluene	0.92	5.00
23	2,6-dimethylaniline	0.71	2.15
22	2,4-dimethylaniline	1.20	3.64

5.4 Recovery and Reproducibility

5.4.1 Alkaloids and Flavonoids

Good recoveries were obtained using Bond Elut Plexa SPE with relative standard deviations of less than 5% therefore Bond Elut Plexa demonstrated effective extraction of analytes with good batch to batch reproducibility (see Table 5.6 for alkaloids and 5.7 for flavonoids).

Table 5.6: Recovery and reproducibility data of alkaloids using HPLC-DAD (n=6)

Alakloid	**Spiking level (µg g^{-1})**	**% Recovery**	**%R.S.D.**
Hydrastine	50	76	3.94
	250	83	4.98
Berberine	25	99	4.68
	375	104	3.12

Table 5.7: Recovery and reproducibility data of flavonoids using HPLC-DAD

Analyte	Spiking level (µg g^{-1}) n=6	% Recovery	R.S.D
Quercetin	20	107	4.35
	40	106	3.35
Kaempferol	20	109	2.53
	40	103	1.14
Isorhamnetin	20	88	4.11
	40	73	4.50

5.4.2 Aromatic amines

Most of the recoveries for the aromatic amines calculated from spiked cotton complied with the minimum recovery requirement set in the EN 14362-1:2003(E) standard method except for 4-methyl-m-phenylenediamine, 4-methoxy-m-phenylenediamine and 4,4-methylene-Bis (2-chloroaniline).The recoveries that were not complying were close to the minimum requirement therefore Chem Elut SLE demonstrated potential in effective extraction of aromatic amines. Relative standard deviations are less than 15% therefore the method was reproducible (see Table 5.9). The recoveries and standard deviations for polyester\cotton (80%:20%) are shown in Table 5.10 and Table 5.11 respectively. The recoveries and standard deviations for wool are shown in Table 5.12 and 5.13 respectively. The GC-MSD results for recoveries and standard deviations are shown in Table 5.14-5.16.

Table 5.8: Recoveries for cotton textile using HPLC-DAD

Compound No	Name	Recoveries			Min required % recovery
		10 mg kg^{-1}	15 mg kg^{-1}	20 mg kg^{-1}	
17	1,4 diaminobenzene	6.1	9.8	3.2	Not set
14	4-methyl-m-phenylenediamine	13.0	24.1	30.7	50
4	4-methoxy-m-phenylenediamine	9.9	7.4	19.0	20
16	Aniline	16.0	6.5	17.9	Not set
13	O-toluidine	42.1	29.7	60.4	50
15	O-anisidine	54.5	48.3	91.4	70
3	4-chloroaniline	51.0	43.6	106.9	70
23	2,6-dimethylaniline	31.1	48.7	87.2	Not set
22	2,4-dimethylaniline	39.6	73.5	77.7	Not set
9	2-methoxy-5-methyl aniline	64.4	90.0	74.1	70
21	5-nitro-o-toluidine	66.2	29.9	30.0	Further reduced to (14)
2	benzidine	75.1	51.0	79.7	70
18	4-chloro-2-methylaniline	61.9	54.8	77.3	70
11,19	Coeluting peaks				70
12	4,4-diaminodiphenylsulfide	74.2	43.8	67.1	70
7	O-tolidine	72.6	55.1	85.5	70
6	O-dianisidine	80.9	90.8	72.7	70
1	4-aminobiphenyl	84.6	53.3	65.1	70
24	4-aminoazobenzene	No suitable detection method			
5	3,3-dichlorobenzidine	83.4	42.6	81.3	70
10	4,4-methylene-Bis (2-chloroaniline)	38.2	60.8	67.3	70
8	4,4-diamino-3,3-dimethyldiphenyl-methane	71.4	70.6	87.2	70
20	o-aminoazotoluene	No recoveries			Further reduced to (13)

Table 5.9: Relative standard deviation for cotton textile using HPLC-DAD

Compound No	Name	Relative Standard deviation		
		10 mg kg^{-1}	15 mg kg^{-1}	20 mg kg^{-1}
17	1,4 diaminobenzene	9.4	5.5	7.5
14	4-methyl-m-phenylenediamine	1.1	7.9	6.0
4	4-methoxy-m-phenylenediamine	2.1	5.5	8.6
16	Aniline	9.1	6.7	5.8
13	O-toluidine	11.8	11.1	10.0
15	O-anisidine	0.3	2.9	2.0
3	4-chloroaniline	6.0	0.8	1.6
23	2,6-dimethylaniline	15.7	5.8	5.4
22	2,4-dimethylaniline	2.2	7.9	5.1
9	2-methoxy-5-methyl aniline	0.1	5.2	2.2
21	5-nitro-o-toluidine	1.2	12.0	19.0
2	benzidine	12.7	0.4	0.6
18	4-chloro-2-methylaniline	8.3	0.9	0.5
11,19	Coeluting peaks			
12	4,4-diaminodiphenylsulfide	0.2	11.5	0.5
7	O-tolidine,	0.4	5.0	0.9
6	O-dianisidine,	0.3	1.0	0.8
1	4-aminobiphenyl	0.0	2.2	0.9
24	4-aminoazobenzene	No suitable detection method		
5	3,3-dichlorobenzidine	0.2	0.2	0.5
10	4,4-methylene-Bis (2-chloroaniline)	3.1	1.5	0.3
8	4,4-diamino-3,3-dimethyldiphenyl-methane	6.5	0.2	0.9
20	o-aminoazotoluene	No recoveries		

Table 5.10: Recoveries for polyester\cotton (80%:20%) textile using HPLC-DAD

Compound No	Name	Recoveries			
		10 mg kg^{-1}	15 mg kg^{-1}	20 mg kg^{-1}	
17	1,4 diaminobenzene	5.4	85.0	23.0	Not set
14	4-methyl-m-phenylenediamine	25.6	7.9	35.2	50
4	4-methoxy-m-phenylenediamine	13.6	26.7	5.4	20
16	Aniline	28.7	53.4	36.4	Not set
13	O-toluidine	30.9	20.7	18.2	50
15	O-anisidine	38.6	9.4	33.0	70
3	4-chloroaniline	20.9	76.4	19.7	70
23	2,6-dimethylaniline	22.6	No recovery	12.9	Not set
22	2,4-dimethylaniline	33.3	10.5	16.2	Not set
9	2-methoxy-5-methyl aniline	78.0	81.4	23.5	70
21	5-nitro-o-toluidine	24.5	No recovery	20.0	Further reduced to (14)
2	benzidine	69.0	64.5	89.7	70
18	4-chloro-2-methylaniline	97.8	117.4	96.8	70
11,19	Coeluting peaks				
12	4,4-diaminodiphenylsulfide	72.5	69.5	87.6	70
7	O-tolidine,	75.4	86.6	99.6	70
6	O-dianisidine,	70.9	72.6	89.4	70
1	4-aminobiphenyl	35.1	48.3	58.3	70
24	4-aminoazobenzene	No suitable detection method			
5	3,3-dichlorobenzidine	41.9	81.2	79.3	70
10	4,4-methylene-Bis (2-chloroaniline)	73.3	95.2	100.0	70
8	4,4-diamino-3,3-dimethyldiphenyl-methane	101.3	89.8	110.1	70
20	o-aminoazotoluene	No recoveries	11.0	5.3	Further reduced to (13)

Table 5.11: Relative standard deviation for polyester\cotton (80%:20) textile using HPLC-DAD

Compound No	Name	Relative Standard deviation		
		10 mg kg^{-1}	15 mg kg^{-1}	20 mg kg^{-1}
17	1,4 diaminobenzene	14.7	5.7	6.4
14	4-methyl-m-phenylenediamine	5.9	0.7	4.1
4	4-methoxy-m-phenylenediamine	11.2	1.8	11.4
16	Aniline	4.3	9.1	8.1
13	O-toluidine	15.0	1.7	9.1
15	O-anisidine	5.0	2.3	1.3
3	4-chloroaniline	14.5	1.4	1.8
23	2,6-dimethylaniline	6.2	No recovery	8.3
22	2,4-dimethylaniline	6.8	1.8	3.2
9	2-methoxy-5-methyl aniline	12.9	7.9	0.7
21	5-nitro-o-toluidine	12.4	No recovery	0.8
2	benzidine	1.4	2.7	1.2
18	4-chloro-2-methylaniline	9.7	2.8	2.3
11,19	Coeluting peaks			
12	4,4-diaminodiphenylsulfide	3.0	2.0	1.0
7	O-tolidine	1.7	1.0	1.2
6	O-dianisidine	3.0	2.0	0.9
1	4-aminobiphenyl	9.4	1.3	0.6
24	4-aminoazobenzene	No suitable detection method		
5	3,3-dichlorobenzidine	1.5	5.9	1.8
10	4,4-methylene-Bis (2-chloroaniline)	1.8	1.8	2.2
8	4,4-diamino-3,3-dimethyldiphenyl-methane	3.1	2.6	1.2
20	o-aminoazotoluene	No recoveries	6.0	4.4

Table 5.12: Recoveries for wool textile using HPLC-DAD

Compound No	Name	Recoveries			
		10 mg kg^{-1}	15 mg kg^{-1}	20 mg kg^{-1}	
17	1,4 diaminobenzene	5.4	85.8	23.0	Not set
14	4-methyl-m-phenylenediamine	36.6	50.0	12.6	50
4	4-methoxy-m-phenylenediamine	No recoveries			20
16	Aniline	28.7	53.4	36.4	Not set
13	O-toluidine	73.4	40.0	120.6	50
15	O-anisidine	58.3	58.7	89.9	70
3	4-chloroaniline	42.1	33.5	75.6	70
23	2,6-dimethylaniline	61.1	58.6	87.1	Not set
22	2,4-dimethylaniline	69.6	73.5	77.7	Not set
9	2-methoxy-5-methyl aniline	25.2	56.8	37.0	70
21	5-nitro-o-toluidine	45.0	41.1	75.1	Further reduced to (14)
2	benzidine	23.7	47.2	55.5	70
18	4-chloro-2-methylaniline	37.3	50.2	98.6	70
11,19	Coeluting peaks				70
12	4,4-diaminodiphenylsulfide	23.3	68.8	79.2	70
7	O-tolidine,	26.3	27.4	89.0	70
6	O-dianisidine	32.6	45.6	99.5	70
1	4-aminobiphenyl	27.8	22.4	91.7	70
8	4,4-diamino-3,3-dimethyldiphenyl-methane	86.7	93.2	82.1	70
24	4-aminoazobenzene	No suitable detection method			
5	3,3-dichlorobenzidine	20.7	14.3	39.8	70
10	4,4-methylene-Bis (2-chloroaniline)	10.5	6.4	51.7	70
20	o-aminoazotoluene	No recoveries			Further reduced to (13)

Table 5.13: Relative standard deviation for wool textile using HPLC-DAD

Compound #	Name	Relative Standard deviation		
		10 mg kg^{-1}	15 mg kg^{-1}	20 mg kg^{-1}
17	1,4 diaminobenzene	15.2	5.6	6.4
14	4-methyl-m-phenylenediamine	15.4	9.0	8.0
4	4-methoxy-m-phenylenediamine	No recoveries		
6	Aniline	4.3	9.1	8.1
13	O-toluidine	14.0	12.6	2.8
15	O-anisidine	11.6	7.9	7.3
3	4-chloroaniline	8.1	1.7	8.4
23	2,6-dimethylaniline	5.0	5.7	5.4
22	2,4-dimethylaniline	2.2	7.9	5.1
9	2-methoxy-5-methyl aniline,	7.7	6.7	7.8
21	*5-nitro-o-toluidine	10.0	10.5	7.1
2	benzidine	10.3	15.6	6.1
18	4-chloro-2-methylaniline	11.9	6.3	9.2
11,19	Coeluting peaks			
12	4,4-diaminodiphenylsulfide	10.6	8.3	5.9
7	O-tolidine,	10.1	6.8	5.1
6	O-dianisidine	12.1	8.9	4.7
1	4-aminobiphenyl	1.6	5.5	6.8
8	4,4-diamino-3,3-dimethyldiphenyl-methane	10.8	3.1	12.4
24	4-aminoazobenzene	No suitable detection method		
5	3,3-dichlorobenzidine	12.3	15.3	11.5
10	4,4-methylene-Bis (2-chloroaniline)	15.4	14.5	3.9
20	o-aminoazotoluene	No recoveries		

Table5.14: Recoveries and R.S.Ds for cotton using GC-MSD

Compound No	Name	Spike 1	RSD	Spike 2	RSD	Spike 3	RSD	Min % recovery
17	1,4 diaminobenzene	17.7	6.1	9.1	2.0	20.6	2.3	Not set
14	4-methyl-m-phenylenediamine	22.4	0.1	12.7	0.4	24.2	7.6	50
4	4-methoxy-m-phenylenediamine	22.9	2.1	4.5	5.2	8.1	4.0	20
16	Aniline	17.0	1.2	25.9	5.3	No recovery		Not set
13	O-toluidine	53.4	8.0	23.4	9.9	27.3	0.8	50
15	O-anisidine	51.9	3.5	87.5	8.7	98.6	6.6	70
3	4-chloroaniline	75.4	2.9	67.1	7.7	66.3	5.8	70
9	2-methoxy-5-methyl aniline	49.4	8.9	7.1	3.0	13.3	8.3	70
21	5-nitro-o-toluidine	8.7	9.2	6.8	5.8	25.2	9.2	Further reduced to (14)
2	benzidine	86.7	1.4	60.1	5.2	64.3	3.8	70
18	4-chloro-2-methylaniline	76.6	9.6	72.1	6.9	69.6	4.5	70
19	2-naphthylamine	84.0	8.6	78.9	2.3	93.3	5.4	70
12	4,4-diaminodiphenylsulfide	81.5	7.8	74.7	5.6	90.4	6.8	70
7	O-tolidine	94.2	2.5	71.4	1.4	98.2	2.7	70
6	O-dianisidine	20.2	1.2	70.6	9.2	64.2	6.7	70
1	4-aminobiphenyl	80.4	1.7	107.9	5.0	94.6	3.3	70
8	4,4-diamino-3,3-dimethyldiphenyl-methane	87.3	0.1	67.7	1.8	108.9	0.7	70
24	4-aminoazobenzene	0.6	6.1	No recovery		0.07	7.0	
5	3,3-dichlorobenzidine	77.2	7.2	77.9	0.4	93.9	7.4	70
20	o-aminoazotoluene	0.6	6.1	0.7	3.0	0.1	7.0	Further reduced to (13)
23	2,6-dimethylaniline	28.6	4.5	80.4	8.7	87.3	3.9	Not set
22	2,4-dimethylaniline	28.6	3.7	29.3	2.3	72.3	9.2	Not set
11	4,4-oxydianiline	25.8	3.8	87.8	2.3	62.0	7.7	70

Table 5.15: Recoveries and R.S.Ds for wool using GC-MSD

Compound No	Name	Spike 1	RSD	Spike 2	RSD	Spike 3	RSD	Min % recovery
17	1,4 diaminobenzene	32.9	6.7	7.7	6.8	17.9	9.4	Not set
14	4-methyl-m-phenylenediamine	43.5	0.4	32.4	7.1	22.4	0.5	50
4	4-methoxy-m-phenylenediamine	13.3	8.4	4.4	3.8	5.0	2.2	20
16	Aniline	13.6	9.3	No recovery				Not set
13	O-toluidine	No recovery		5.5	1.5	0.4	0.2	50
15	O-anisidine	27.5	1.6	112.1	9.5	89.6	6.9	70
3	4-chloroaniline	56.5	0.1	70.0	2.9	34.0	4.8	70
9	2-methoxy-5-methyl aniline	4.6	8.3	8.4	6.8	67.5	1.2	70
21	*5-nitro-o-toluidine	6.5	8.8	9.7	3.0	9.1	8.8	Further reduced to (14)
2	benzidine	54.5	4.8	91.0	8.2	114.1	6.1	70
18	4-chloro-2-methylaniline	70.0	5.2	60.8	0.1	62.8	4.8	70
19	2-naphthylamine	94.7	8.5	94.7	4.6	76.8	8.3	70
12	4,4-diaminodiphenylsulfide	98.7	3.1	98.4	6.8	63.0	8.9	70
7	O-tolidine	50.6	1.3	76.3	1.6	66.9	7.7	70
6	O-dianisidine	52.4	1.4	45.1	9.5	76.2	6.0	70
1	4-aminobiphenyl	26.4	8.7	35.3	1.8	58.1	9.6	70
8	4,4-diamino-3,3-dimethyldiphenyl-methane	78.2	7.3	98.9	6.7	62.9	0.3	70
24	4-aminoazobenzene	0.5	9.3	0.1	9.7	0.1	2.2	No suitable method
5	3,3-dichlorobenzidine	66.8	4.3	54.6	1.2	60.7	8.5	70
20	o-aminoazotoluene	No recovery						Further reduced to (13)
23	2,6-dimethylaniline	38.8	2.3	80.5	2.1	61.3	3.1	Not set
22	2,4-dimethylaniline	53.9	5.8	82.6	0.3	96.7	8.8	Not set
11	4,4-oxydianiline	23.8	4.7	83.0	0.3	62.8	2.4	70

Table 5.16: Recoveries and R.S.Ds for polyester\cotton (80%:20%) using GC-MSD

Compound No.	Name	Spike 1	RSD	Spike 2	RSD	Spike 3	RSD	Min % recovery
17	1,4 diaminobenzene	23.8	1.9	20.8	9.1	15.6	7.1	Not set
14	4-methyl-m-phenylenediamine	23.6	8.7	9.7	3.0	25.6	5.0	50
4	4-methoxy-m-phenylenediamine	30.3	9.7	2.8	4.9	5.4	4.2	20
16	Aniline	No recovery						
13	O-toluidine	15.7	0.6	10.3	9.8	26.1	4.9	50
15	O-anisidine	62.5	4.0	66.8	9.3	87.6	2.3	70
3	4-chloroaniline	75.3	9.2	63.7	9.9	64.1	1.7	70
9	2-methoxy-5-methyl aniline	2.9	2.3	4.5	8.5	6.2	0.6	70
21	5-nitro-o-toluidine	20.5	5.0	42.0	3.5	33.6	9.3	Further reduced to (14)
2	benzidine	89.9	4.7	61.7	5.0	103.6	8.4	70
18	4-chloro-2-methylaniline	91.5	1.6	82.1	3.0	89.2	6.7	70
19	2-naphthylamine	58.5	1.4	88.3	7.8	71.1	6.6	70
12	4,4-diaminodiphenylsulfide	59.6	2.2	29.2	4.1	71.5	9.8	70
7	O-tolidine	81.5	3.3	89.3	4.8	70.8	0.2	70
6	O-dianisidine	14.3	8.2	18.8	0.5	47.8	5.0	70
1	4-aminobiphenyl	104.2	6.7	75.0	4.1	95.2	0.1	70
8	4,4-diamino-3,3-dimethyldiphenyl-methane	75.5	4.8	72.3	8.2	83.0	5.4	70
24	4-aminoazobenzene	15.1	8.0	1.5	7.3	2.5	9.5	No suitable method
5	3,3-dichlorobenzidine	57.9	9.3	73.7	9.4	68.2	2.2	70
20	o-aminoazotoluene	No recoveries						Further reduced to (13)
23	2,6-dimethylaniline	65.9	4.9	81.3	1.0	92.4	0.8	Not set
22	2,4-dimethylaniline	67.4	2.1	88.2	5.0	63.9	9.3	Not set
11	4,4-oxydianiline	61.4	3.2	87.6	8.0	91.0	9.1	70

5.4.3 Comparison of Chem elut SLE with self prepared SLE and other commercially available products

Chem Elut SLE was found to perform better than the self prepared SLE (see Fig 5.7). There was no significant difference between Chem Elut and other commercially available products (Chromabond, ProElute and Agela) (see Fig 5.8, 5.9 and 5.10).

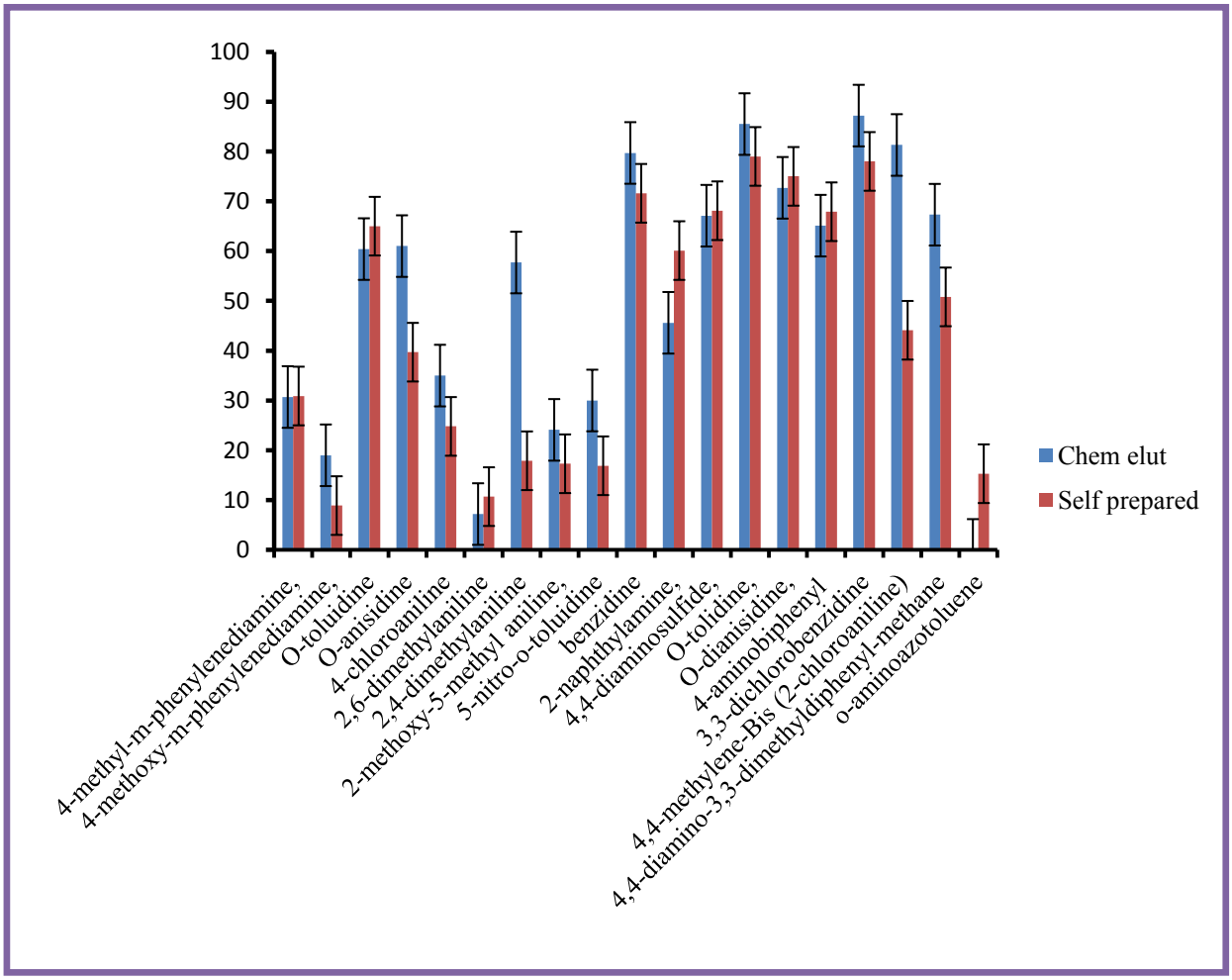


Fig. 5.7: Comparison of Chem Elut and self prepared SLE

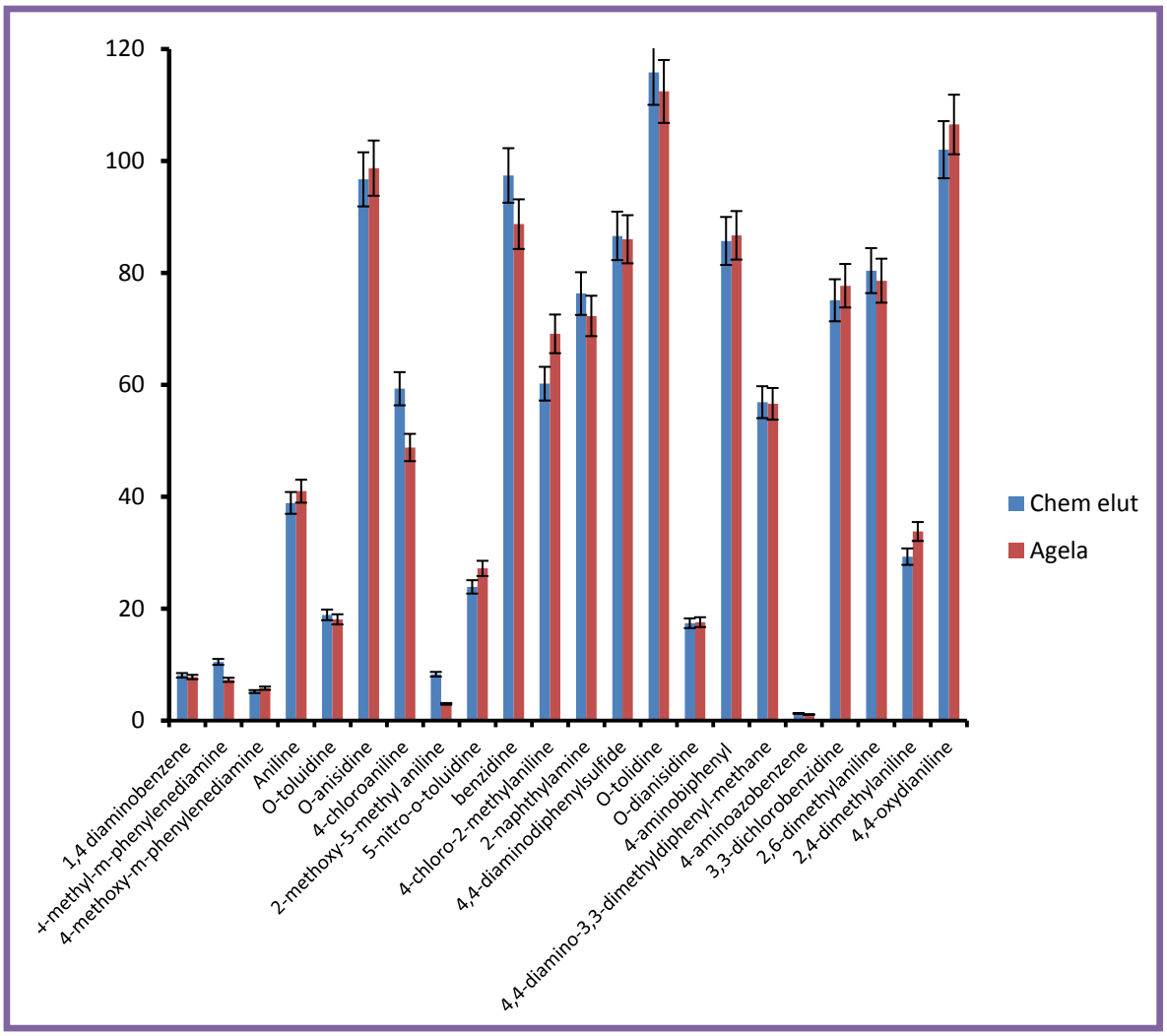


Fig. 5.8: Comparison of Chem Elut and Agela SLE

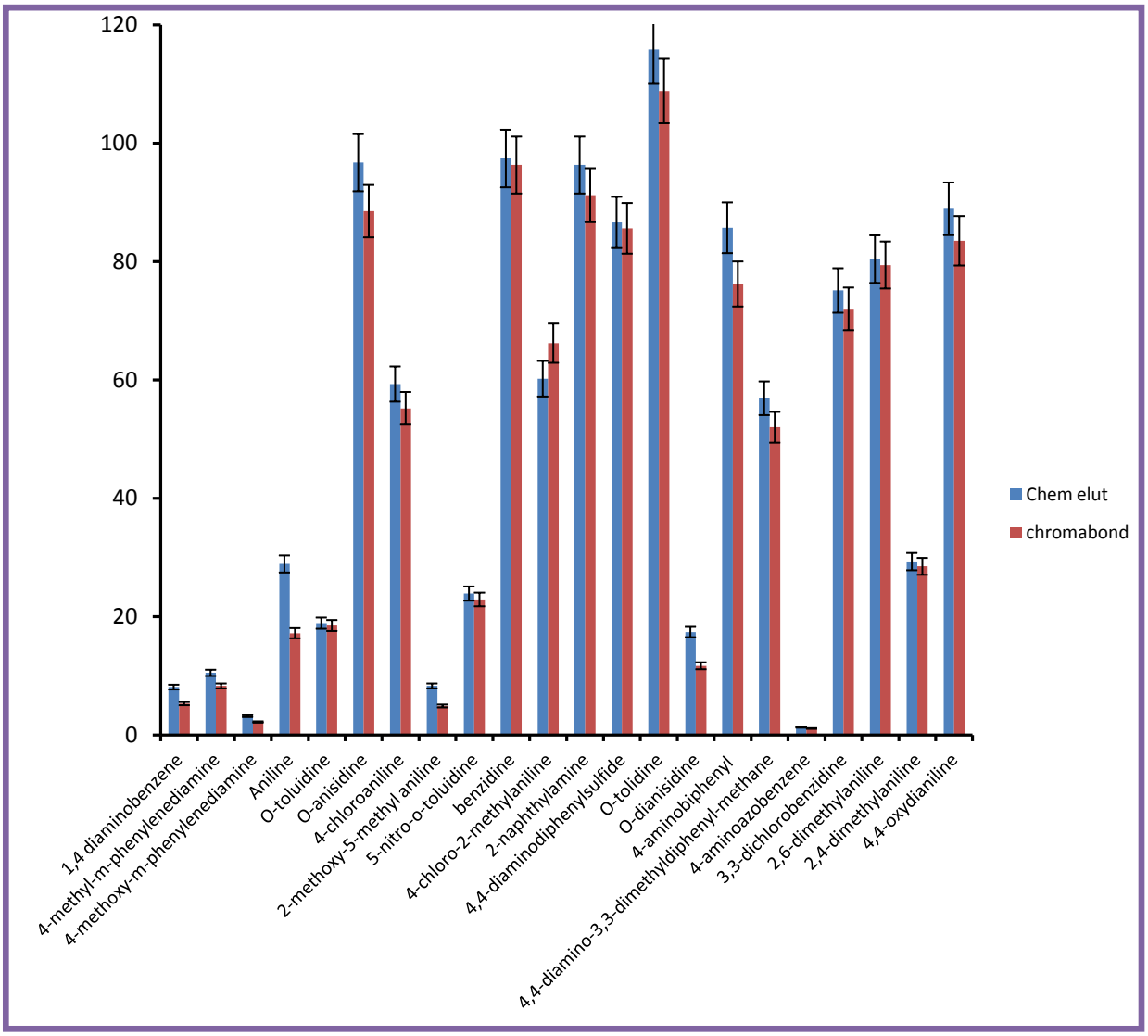


Fig. 5.9: Comparison of Chem Elut and chromabond SLE

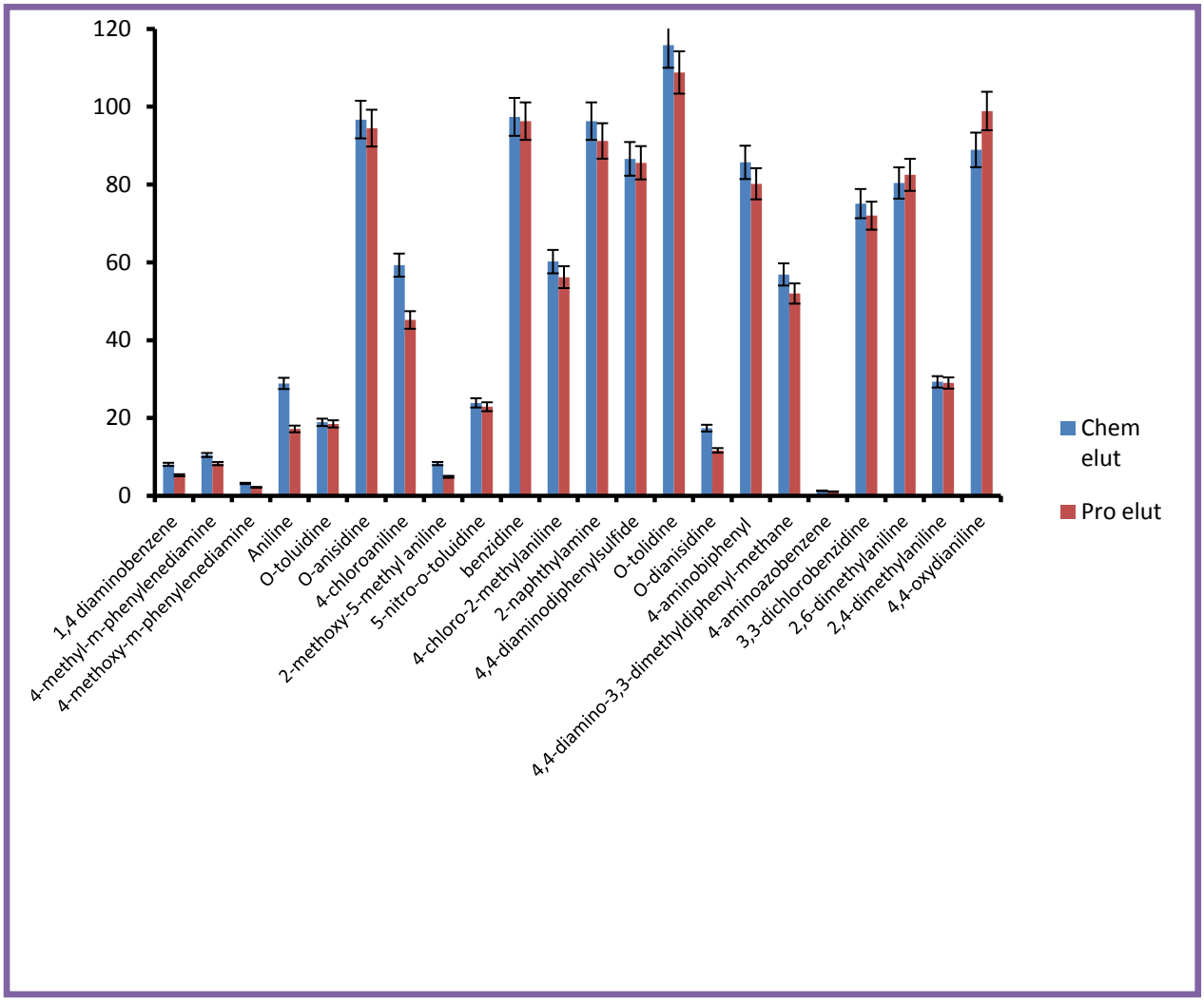


Fig. 5.10: Comparison of Chem Elut and Pro Elut SLE

5.5 Application to real sample

5.5.1 Alkaloids and flavonoids

Goldenseal (*Hydrastis Canadensis)* root extracts were analysed using the developed method. The roots contained 111.2 and 340.6 µg g^{-1} hydrastine and berberine respectively.

Ginkgo biloba tablets were analysed using the developed method and the concentrations were found to be 29.08 µg g^{-1}, 40.4 µg g^{-1} and 6.16 µg g^{-1} for quercetin, kaempferol and isorhamnetin respectively. The concentrations for flavonoids *Ginkgo biloba* leaves were not detected.

5.5.2 Concentration of aromatic amines in textile material

There was no detection of amines using HPLC-DAD while for GC-MS the concentrations were not detected except for aniline and 4,4-oxydianiline. Aniline and 4,4-oxydianiline gave concentration values above the limit of detection for polyester/cotton (80%:20%) but were below 30 mg kg^{-1} which is the maximum residue limit set by EU therefore were still compliant (see Table 5.17).

Table 5.17: Concentrations of amines in textile using GC-MSD

No	Name	cotton	RSD	wool	RSD	Poly\cot	RSD	Result
17	1,4 diaminobenzene	<LOD		<LOD		<LOD		Not detected
14	4-methyl-m-phenylenediamine	<LOD		<LOD		<LOD		Not detected
4	4-methoxy-m-phenylenediamine	<LOD		<LOD		<LOD		Not detected
16	Aniline	<LOD		<LOD		7.3	14.0	<30 mg kg-1 therefore compliant
13	O-toluidine	<LOD		<LOD		<LOD		Not detected
15	O-anisidine	<LOD		<LOD		<LOD		Not detected
3	4-chloroaniline	<LOD		<LOD		<LOD		Not detected
9	2-methoxy-5-methyl aniline	<LOD		<LOD		<LOD		Not detected
21	5-nitro-o-toluidine	<LOD		<LOD		<LOD		Not detected
2	benzidine	<LOD		<LOD		<LOD		Not detected
18	4-chloro-2-methylaniline	<LOD		<LOD		<LOD		Not detected
19	2-naphthylamine	<LOD		<LOD		<LOD		Not detected
12	4,4-diaminodiphenylsulfide	<LOD		<LOD		<LOD		Not detected
7	O-tolidine	<LOD		<LOD		<LOD		Not detected
6	O-dianisidine	<LOD		<LOD		<LOD		Not detected
1	4-aminobiphenyl	<LOD		<LOD		<LOD		Not detected
8	4,4-diamino-3,3-dimethyldiphenyl-methane	<LOD		<LOD		<LOD		Not detected
24	4-aminoazobenzene	<LOD		<LOD		<LOD		Not detected
5	3,3-dichlorobenzidine	<LOD		<LOD		<LOD		Not detected
20	o-aminoazotoluene	<LOD		<LOD		<LOD		Not detected
23	2,6-dimethylaniline	<LOD		<LOD		<LOD		Not detected
22	2,4-dimethylaniline	<LOD		<LOD		<LOD		Not detected
11	4,4-oxydianiline	<LOD		<LOD		5.4	10.1	<30 mg kg-1 therefore compliant

5.6 Polymer-silica nanofibers as sorbent for extraction of Flavonoids in *Ginkgo biloba*

5.6.1 Characterization

The morphology of fibers was characterised using a scanning electron microscope (SEM) and the obtained results are shown in Fig 5.11-5.13. Fig.5.14-5.16 presents the transmission electron microscope (TEM) images showing the distribution of silica within the polymer and Fig. 5.17-5.19 presents information about the surface composition of the fibers obtained from high resolution SEM-EDX. The SEM images showed that the fibers are flexible enough not to break during sample preparation. The SEM images also showed that a smooth surface was continuously produced with considerable ease under the optimised electrospinning conditions. Smooth fibers are charactirised by high specific surface area preferred for an SPE sorbent material. The continuously long fibrous morphology and formation of interconnected voids formed from the entanglement of the fibers may contribute to the high retention capacity of the sorbent material [133] (see Fig 5.11-5.13). Large interconnected voids make the contact between the sorbent and the analyte of interest easier and the flow of the solution smoother resulting in improved sorption kinetics [133]. The TEM images show that silica particles were monodispersed within the polymer when surfactant triton-X100 was added. The images show very good distribution of silica particles therefore contributing to good mechanical stability of the fibers. The images also showed that the particles have a smooth surface. Both the SEM and the TEM images indicated that the fibers possess smooth morphology. The TEM images only show small sample fractions that are hardly representative therefore SEM-EDX was also used to characterise the fibers.

The SEM-EDX results show the composition of the surface of the fibers indicating the presence of elements that have possible interactions with analytes of interest. The possible interactions with polystyrene-silica composite are polar interactions from the silica and non polar interaction from the polystyrene and they are the same interactions that are possible with PAN-silica composite. The interactions with nylon-silica composite are mostly polar due to silica and the presence of nitrogen in nylon 6. The presence of aluminium in the results for nylon-silica is due to the use of aluminium foil for collection of fibers during electrospinning process.

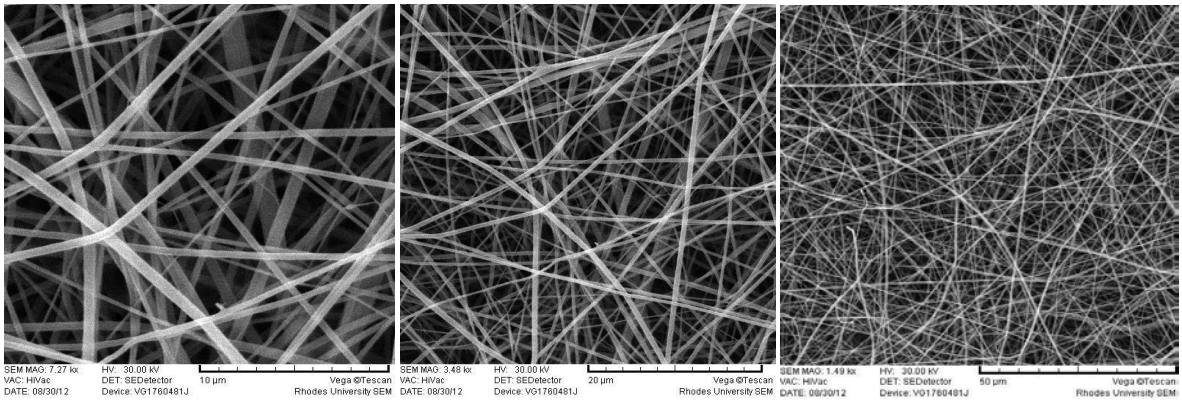


Fig. 5.11: SEM micrographs of polystyrene (20%)/silica composite 2:1 at x10, x20 and x50 magnifications

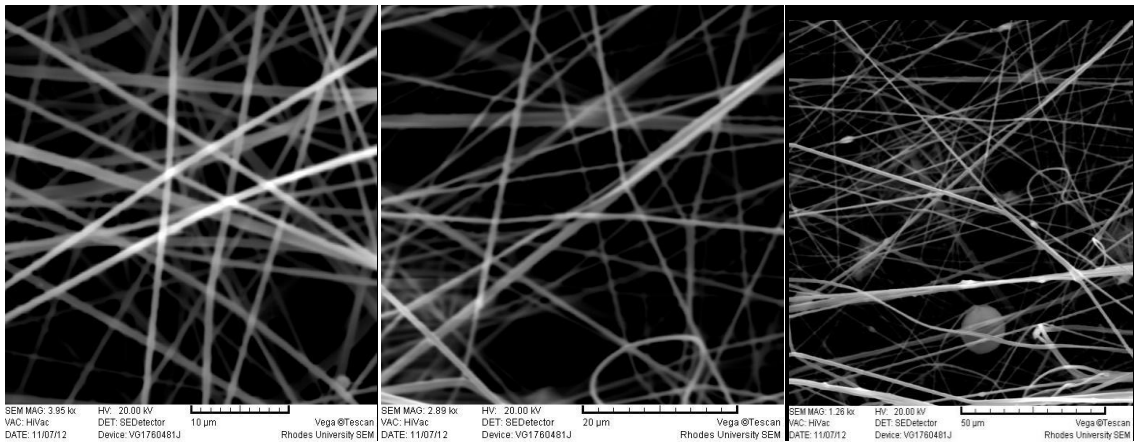


Fig. 5.12: SEM micrographs of PAN (10%)/silica composite 2:1 at x10, x20 and x50 magnifications

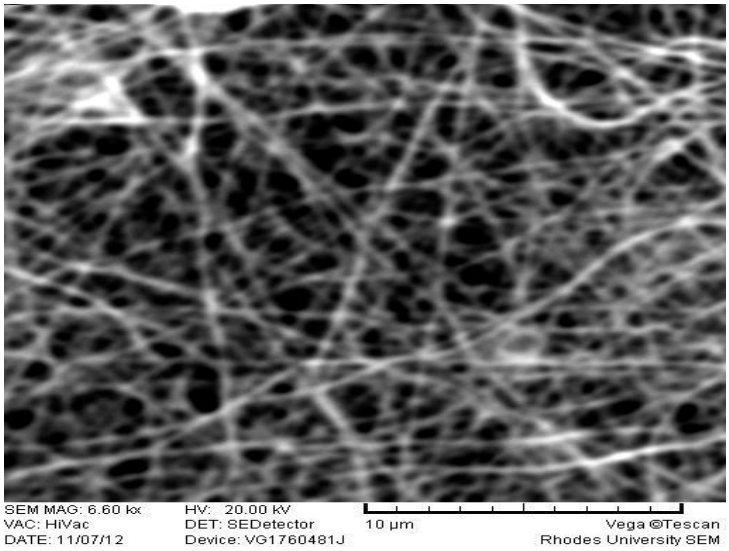


Fig. 5.13: SEM micrographs of nylon (20%)/silica composite 2:1 at x10 magnifications

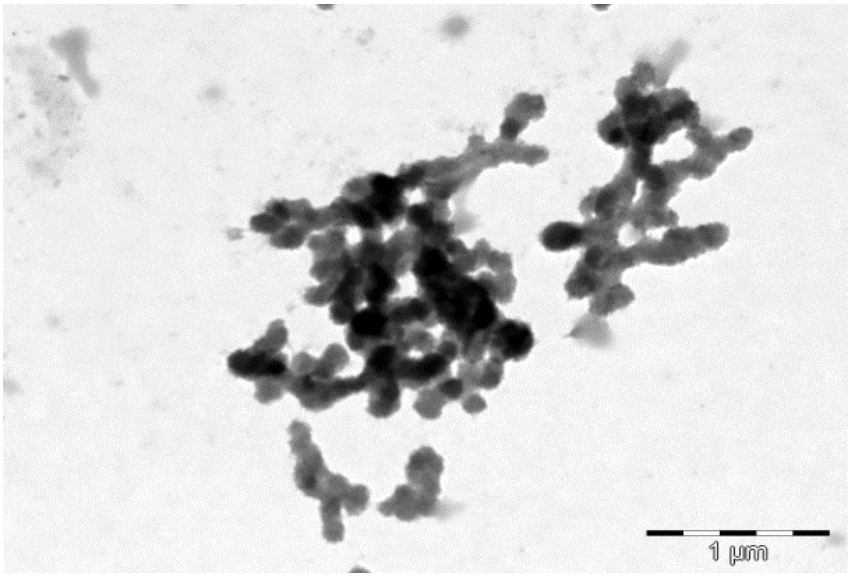


Fig. 5.14: TEM micrographs of silica from sol gel process

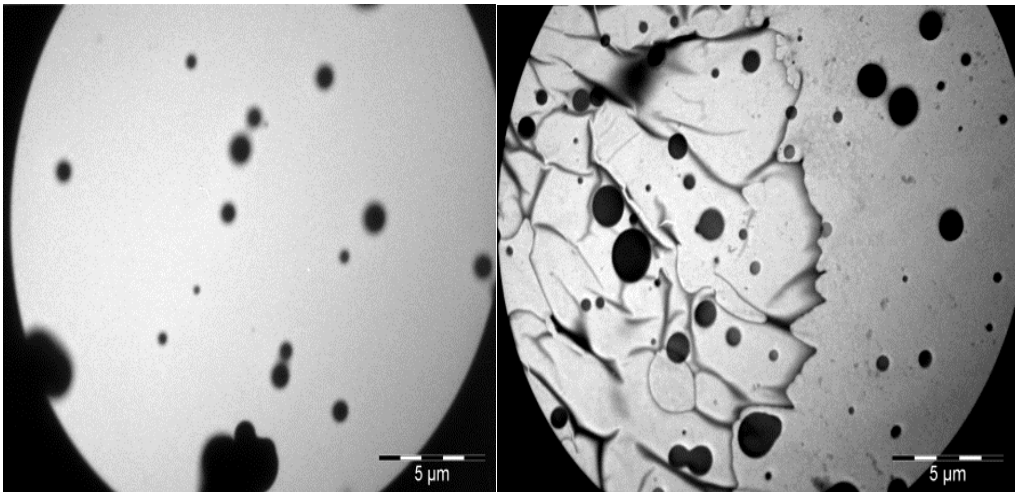


Fig. 5.15: TEM micrographs of polystyrene (20%)/silica composite ratio 2:1 before addition of surfactant (triton X-100)

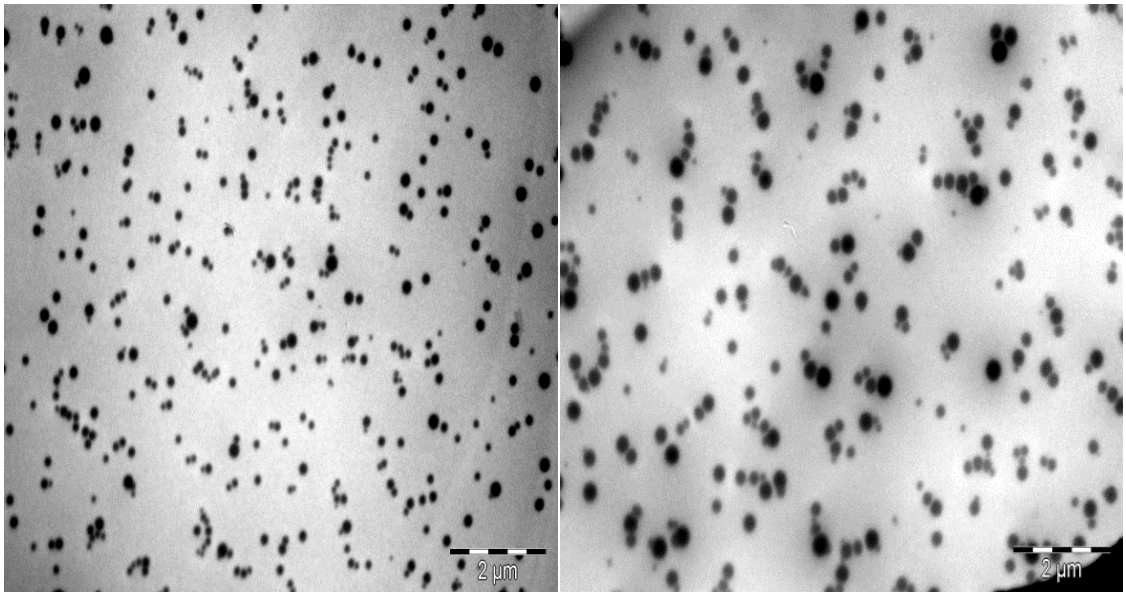


Fig. 5.16: TEM micrographs of polymer-silica composites after addition of surfactant

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Label:

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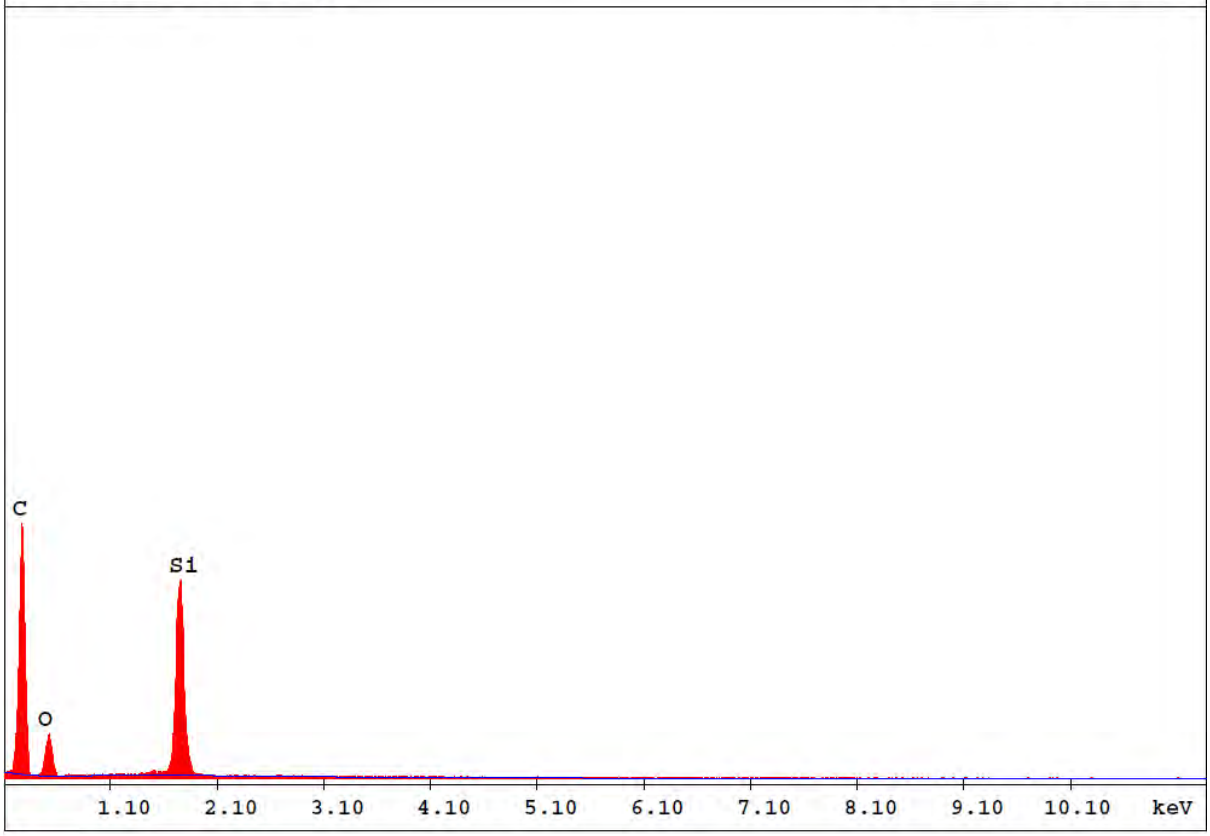
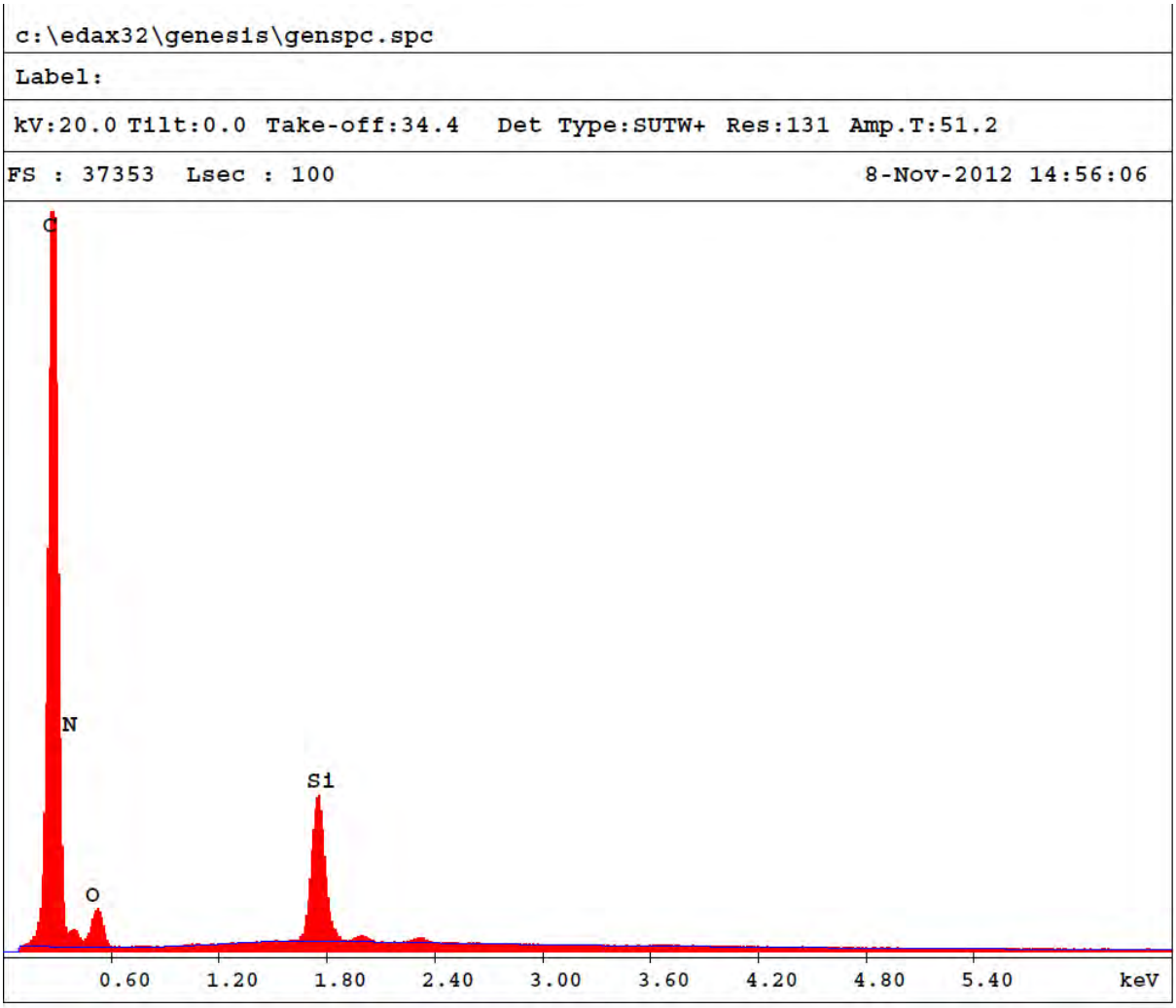


EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : User c:\edax32\eds\genuser.sec

Element	Wt %	At %	K-Ratio	Z	A	F
C K	76.93	84.75	0.2958	1.0080	0.3814	1.0001
O K	12.31	10.18	0.0195	0.9913	0.1596	1.0001
SiK	10.75	5.07	0.0942	0.9509	0.9216	1.0000
Total	100.00	100.00				

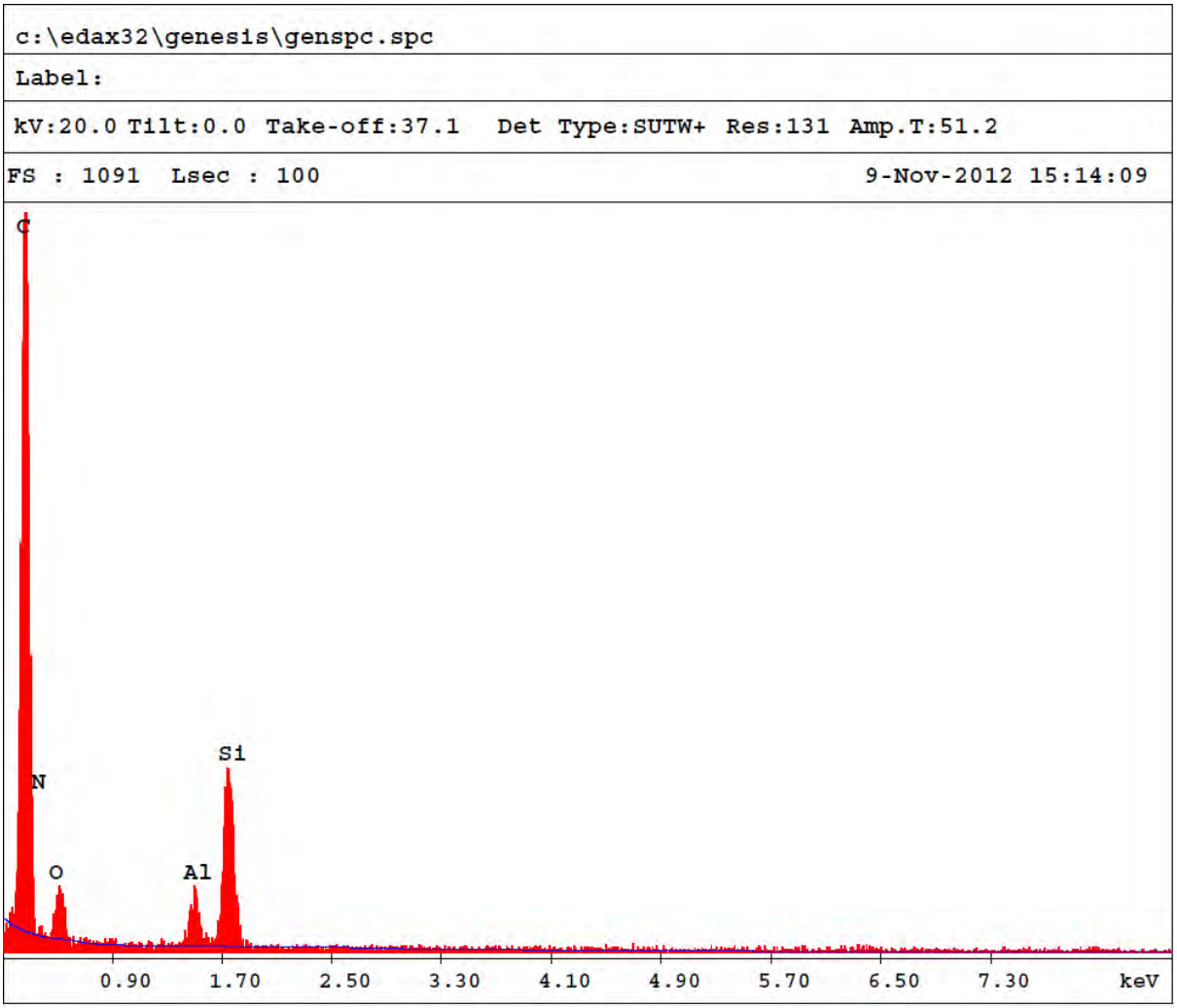
Fig. 5.17: SEM-EDX results for polystyrene-silica composite



EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : User c:\edax32\eds\genuser.sec

Element	Wt %	At %	K-Ratio	Z	A	F
C K	79.13	83.68	0.4692	1.0042	0.5903	1.0001
N K	9.98	9.05	0.0092	0.9955	0.0928	1.0001
O K	6.87	5.46	0.0093	0.9876	0.1376	1.0000
SiK	4.01	1.82	0.0349	0.9474	0.9171	1.0000
Total	100.00	100.00				

Fig. 5.18: SEM-EDX results for PAN-silica composite



EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : User c:\edax32\eds\genuser.sec

Element	Wt %	At %	K-Ratio	Z	A	F
C K	83.31	88.41	0.4463	1.0052	0.5329	1.0001
N K	3.15	2.87	0.0028	0.9964	0.0895	1.0001
O K	7.46	5.94	0.0109	0.9885	0.1483	1.0001
AlK	1.29	0.61	0.0100	0.9212	0.8387	1.0030
SiK	4.79	2.17	0.0414	0.9483	0.9122	1.0000
Total	100.00	100.00				

Fig. 5.19: SEM-EDX results for nylon-silica composite

Polystyrene, PAN and nylon 6 have 10.8, 4.0 and 4.8% silica content respectively. In summary polystyrene had higher silica content compared to other polymers therefore has enhanced analyte mass transfer compared to the other polymers.

5.6.2 Breakthrough curves

The suitability of the sorbents materials to isolate target analytes was evaluated using breakthrough curves. The data point for the breakthrough curves were fitted using Weibull five-parameter model (see Fig. 5.20-5.22).The number of theoretical plates (N) were calculated to determine the SPE column efficiency (see Table 5.18-5.20). Typical cartridges provide about 5 to 15 theoretical plates per cm of bed height[115]. The obtained results were within the range except for isorhamnetin which was slightly higher than 15 for polystyrene-silica composite, the cause maybe due to fewer experimental points. Retention factor (k) was also calculated because it has an influence on the recovery.

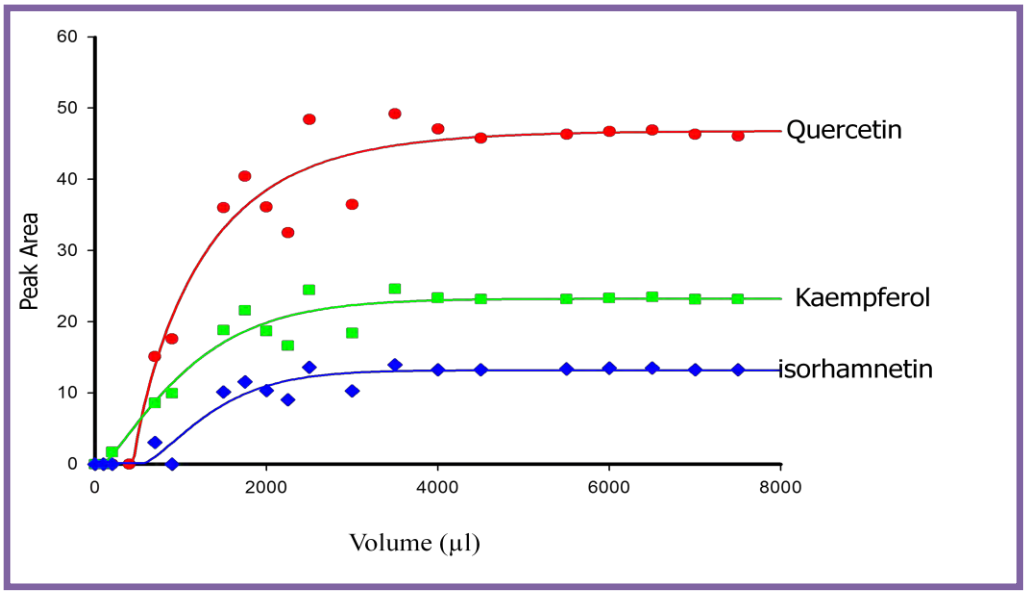


Figure 5.20: Breakthrough curves for flavonoids obtained using polystyrene-silica composite

Table 5.18: Breakthrough parameters for polystyrene-silica composite

Analyte	V_b (µL)	V_R (µL)	V_m (µL)	N	K
Quercetin	320	998	5320	8.67	4.33
Kaempferol	114	920.97	3866	5.21	3.20
Isorhamnetin	650	12778	3215	16.58	1.52

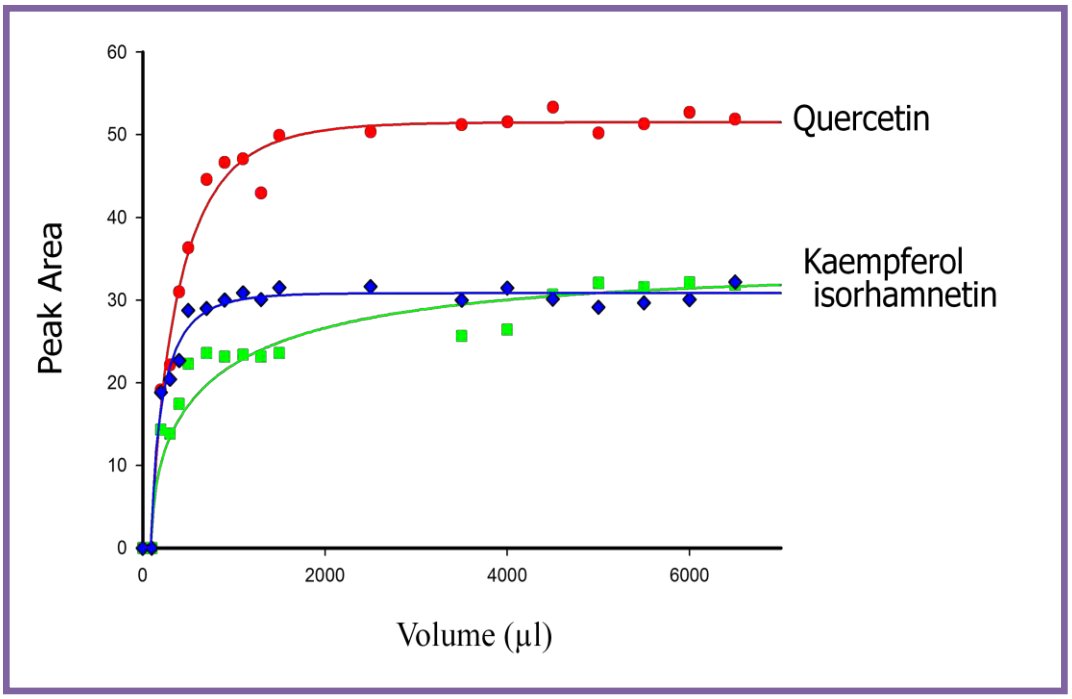


Fig. 5.21: Breakthrough curves for flavonoids using PAN-silica composite

Table 5.19: Breakthrough parameters for PAN-silica composite

Analyte	V_b (µL)	V_R (µL)	V_m (µL)	N	K
Quercetin	117	302	2340	10.66	6.75
Kaempferol	27	462	6907	4.51	13.94
Isorhamnetin	53	184	1384	7.87	6.50

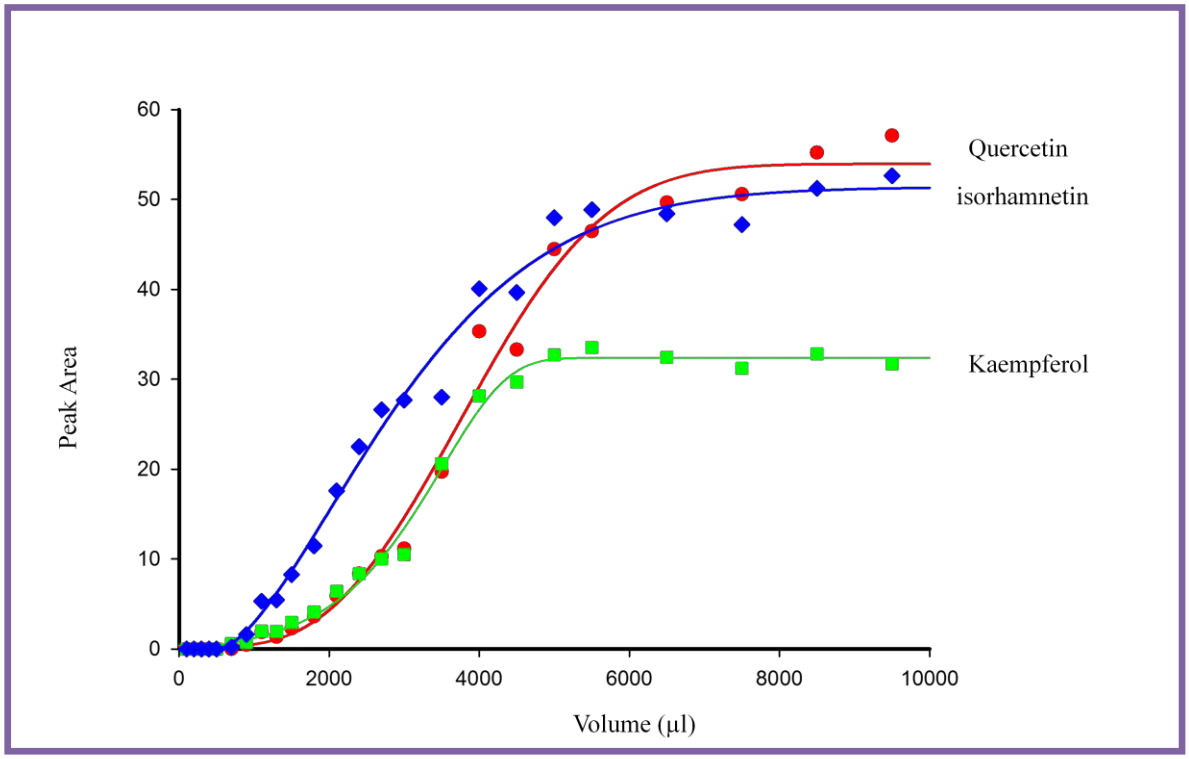


Fig. 5.22: Breakthrough curves for flavonoids using nylon-silica composite

Table 5.20: Breakthrough parameters for nylon-silica composite

Analyte	V_b (µL)	V_R (µL)	V_m (µL)	N	K
Quercetin	1012	3862	6958	7.34	0.80
Kaempferol	366	3196	5564	5.10	0.74
Isorhamnetin	689	2785	8341	7.06	1.99

5.7 Application of polymer-silica sorbents to real samples

5.7.1 Optimization of SPE

Preliminary evaluation was conducted using SPE conditions as shown in Table 5.21.The chromatogram overlay in Fig. 5.23 show that almost all the analytes were lost during the loading step therefore a need to optimise the sorbent mass and load volume. Reducing the load volume from 1 mL to 0.5 mL resulted in reduced loss of analytes during the loading step. Analyte was also lost during the washing step when using 30% methanol. Loss of analyte during washing step was also observed when using 5% methanol therefore the washing step was carried out using deionised water. Good percentage recoveries were obtained with 0.5 mL load volume and the optimal sorbent mass for polystyrene-silica was found to be 40 mg. During the loading step the analyte was lost only in 30 mg sorbent mass (the highest concentration lost being 0.6 µg mL^{-1}). About 7 µg mL^{-1} was lost during washing step in 30 mg sorbent while less than 3 µg mL^{-1} was lost for both 40 mg and 50 mg. The best sorbent mass for PAN- silica was 50 mg while for nylon-silica was 50 mg as well, however recoveries for nylon-silica were very low (less than 50%) because analyte was lost during loading step and washing step. This may be due to a lower retention factor for the sorbent as most of the analyte was lost during the loading step. There was also a need to apply pressure for the sample to pass through the sorbent hence it was not easy to regulate the flow rate (see Fig 5.25-5.27).

Table 5.21 SPE conditions

Composite	Polystyrene-silica
Load volume	1 mL
Sorbent mass	30 mg
Wash solvent	30% methanol

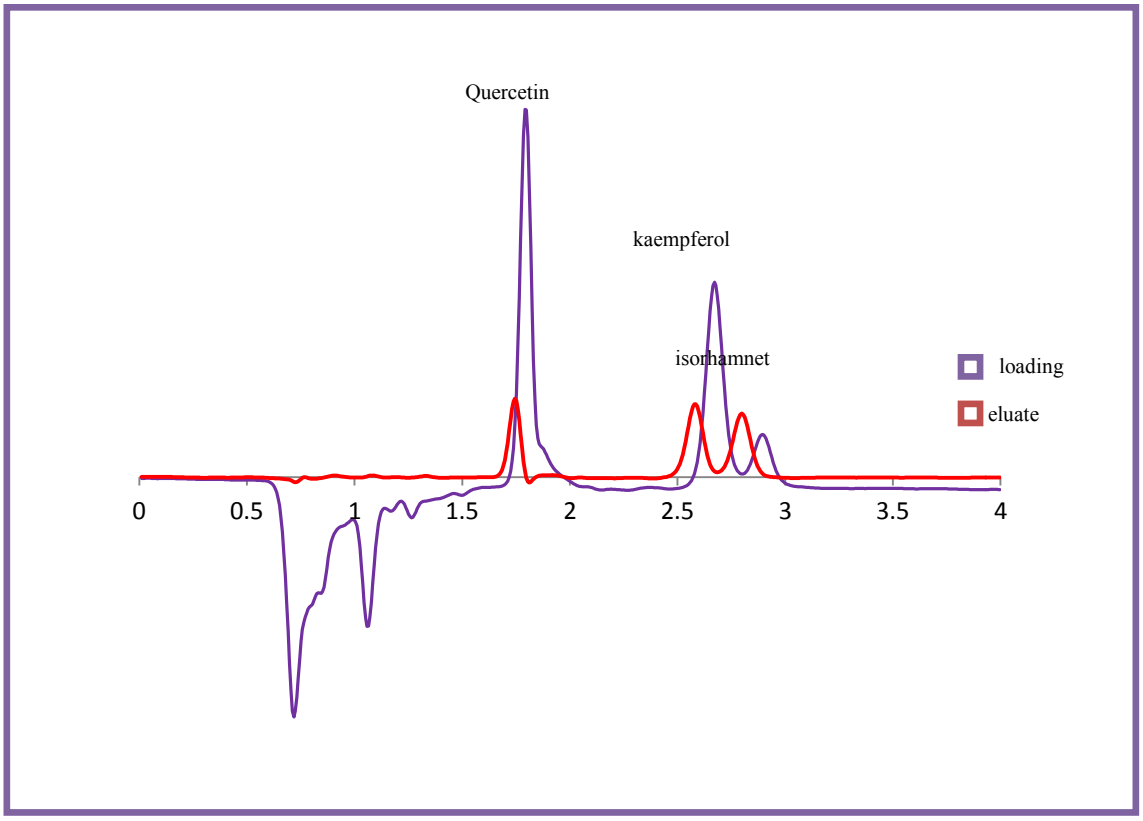


Fig. 5.23: HPLC-DAD chromatogram overlay of the collected load and elute solutions

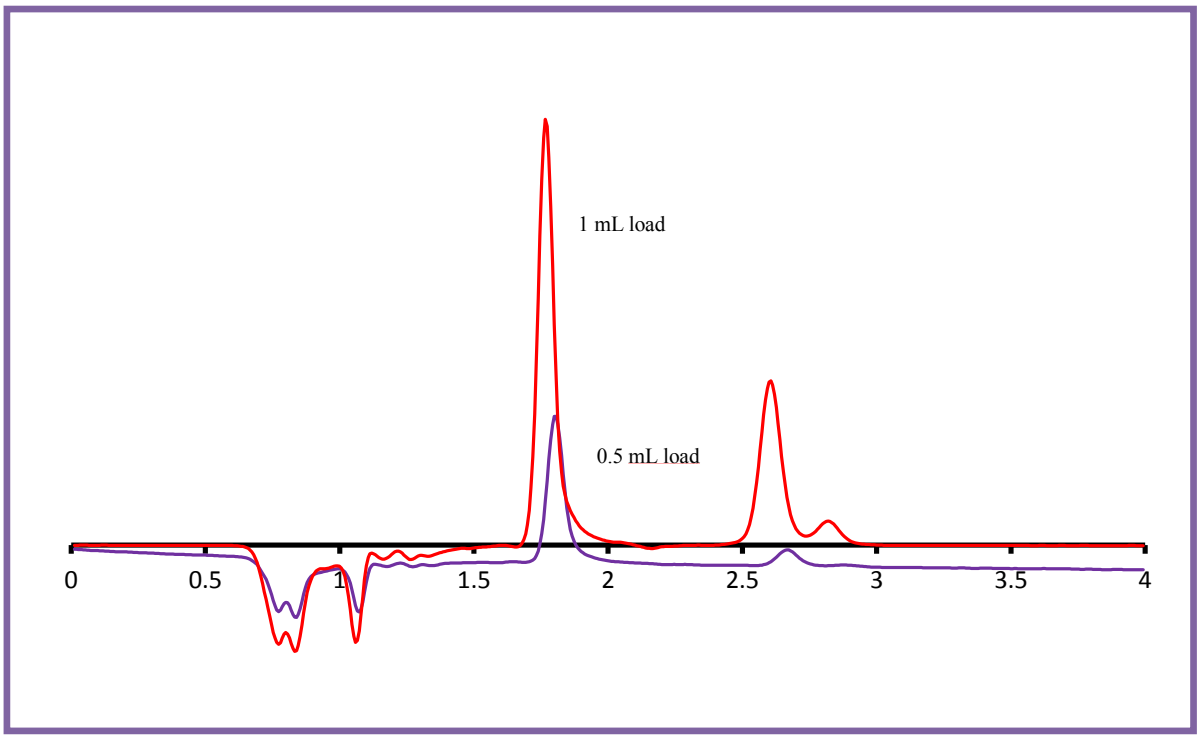


Fig. 5.24: HPLC-DAD chromatogram overlay for optimization of volume for loading

5.7.2 Optimization of sorbent mass

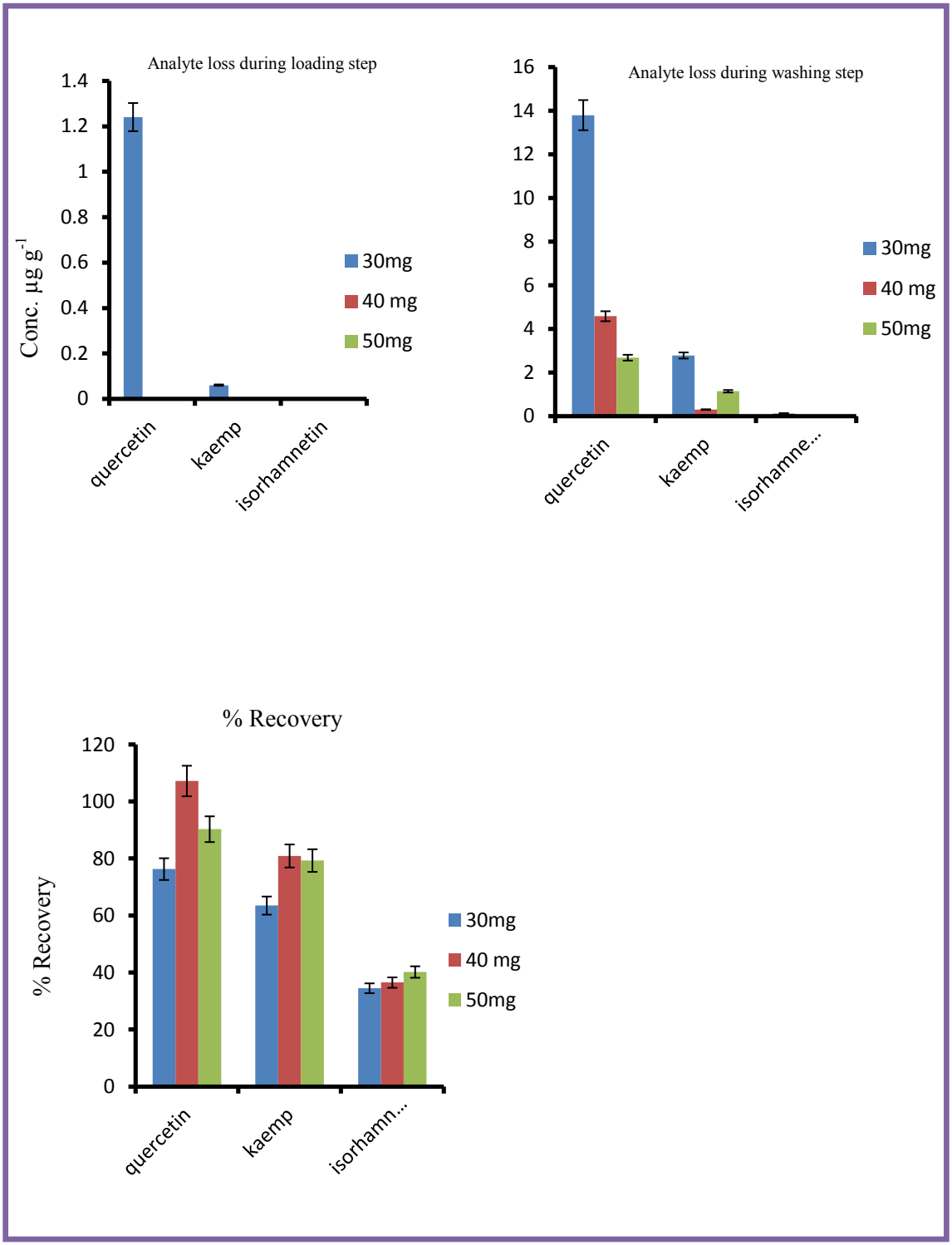


Fig. 5.25: Optimization of sorbent mass for polystyrene-silica composite

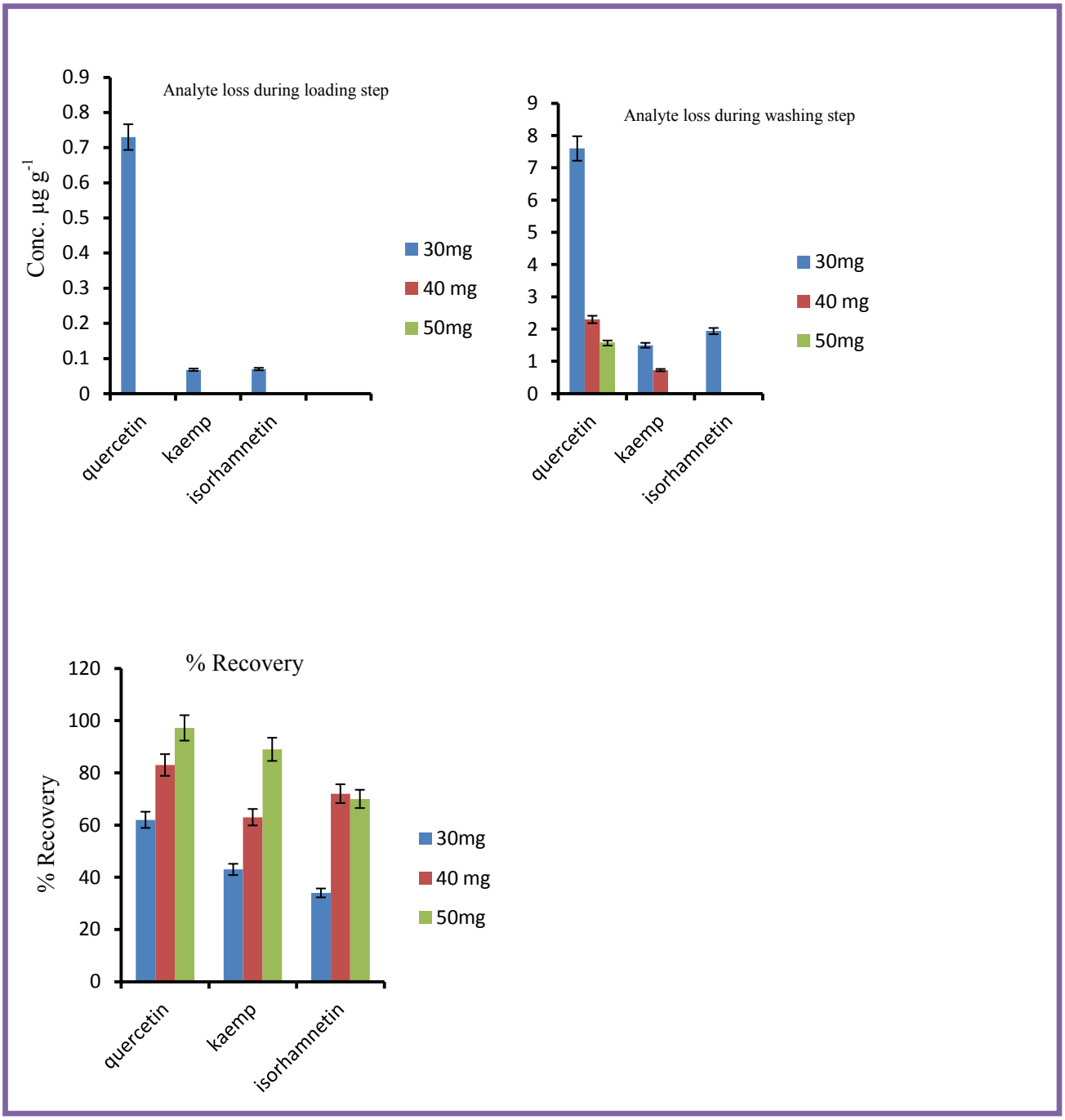


Fig. 5.26: Optimization of sorbent mass for PAN-silica composite

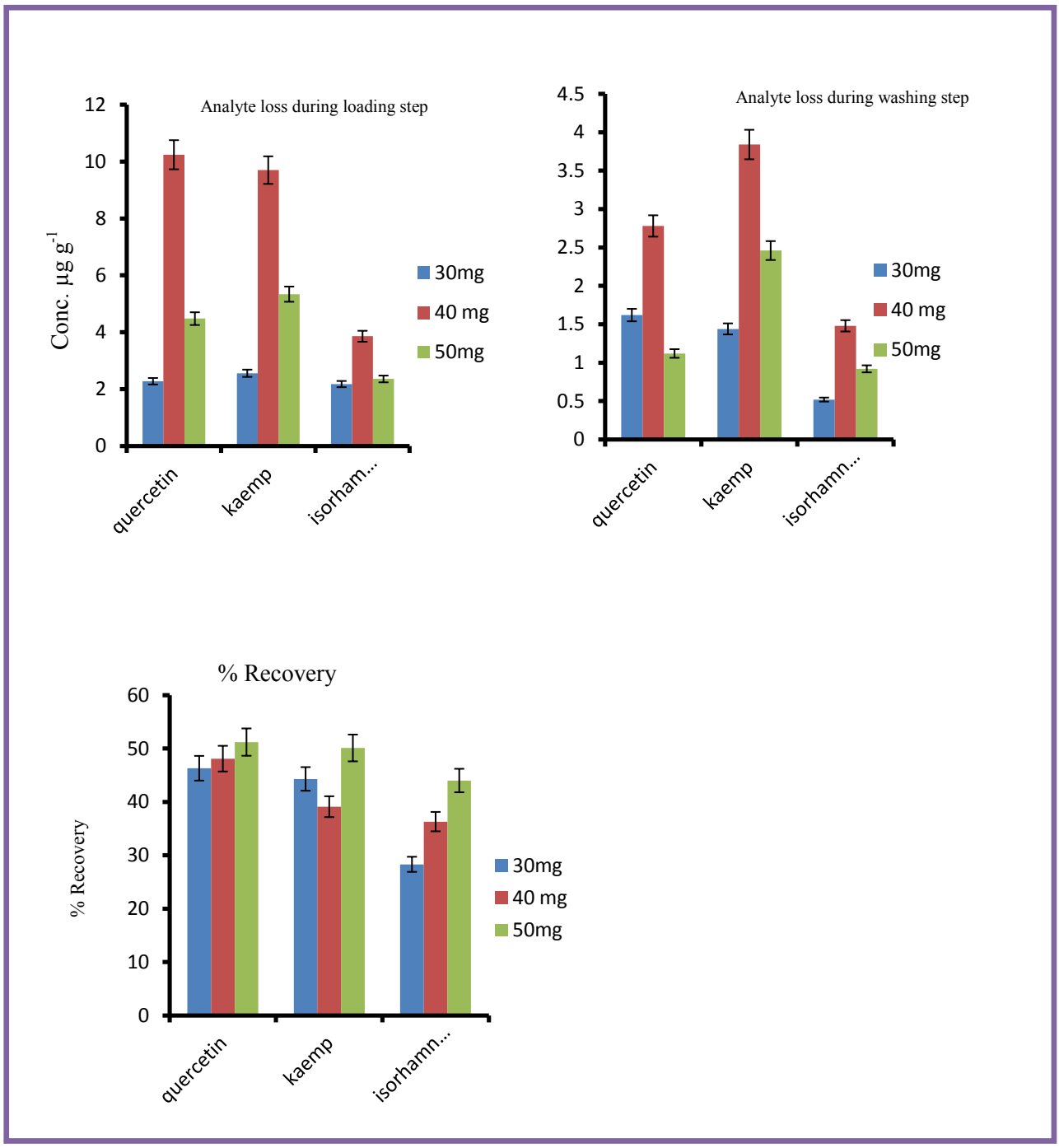


Fig. 5.27: Optimization of sorbent mass for nylon-silica composite

The sorbent mass plays an important role in the retention of the analyte of interest. An increase in the sorbent mass increases the total surface area for the retention of the analyte of interest. Results in Fig. 5.25-5.27 showed that increase in sorbent mass resulted in increase in the retention of the analytes as shown by reduced sample loss during loading and washing steps and improved percentage recoveries. Fig. 5.25 also showed that if the sorbent mass is too large there is strong retention and incomplete elution resulting in lower recoveries shown by quercetin and kaempferol for the 50 mg being lower than for the 40 mg.

5.7.3 Comparison of recoveries with Bond Elut Plexa

Bond Elut Plexa presented better recoveries than the electrospun sorbents. However polystyrene-silica and PAN-silica sorbents showed good recoveries therefore have the potential to be employed as sorbent materials. Nylon-silica showed lower recoveries and it might due to the low retention factor and also poor regulation of the flow rate.

Table 5.22: Comparison of recoveries of Bond Elute Plexa with elestrospun sorbents

Sorbent	Analyte	Spiking level ($\mu g\ g^{-1}$) n=6	% Recovery	R.S.D
Bond Elut Plexa	Quercetin	40	107	4.35
	Kaempferol	40	109	2.53
	Isorhamnetin	40	88	4.11
Polystyrene-Silica	Quercetin	40	90.3	3.3
	Kaempferol	40	79.3	0.7
	Isorhamnetin	40	40.2	5.0
PAN-silica	Quercetin	40	83.1	2.0
	Kaempferol	40	46.5	3.5
	Isorhamnetin	40	72.3	1.6
Nylon-silica	Quercetin	40	51.1	2.3
	Kaempferol	40	50.1	3.9
	Isorhamnetin	40	44.0	5.3

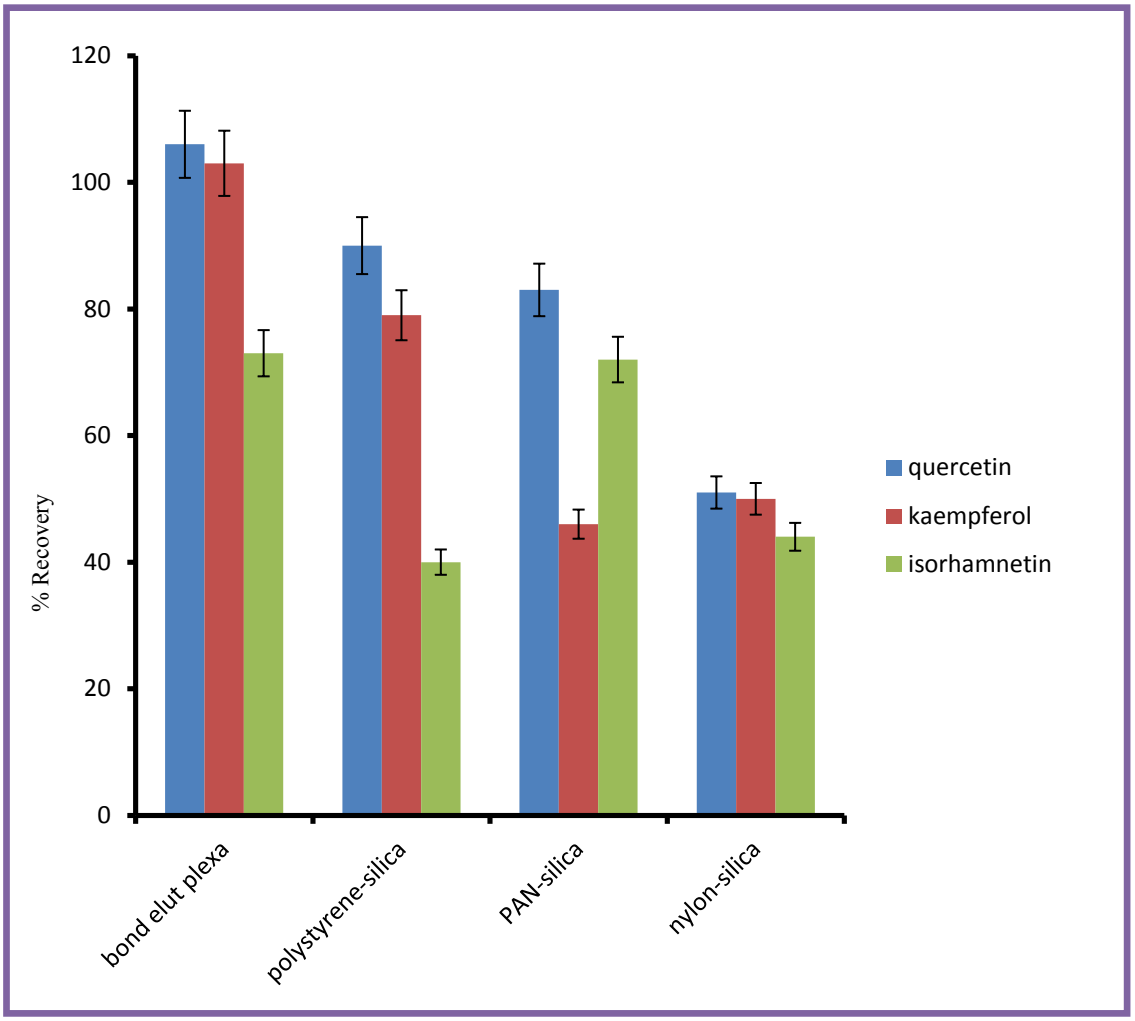


Fig. 5.28 Comparison of recoveries of Bond Elut Plexa with electrospun sorbents

The trend showed bond Elut plexa> polystyrene-silica> PAN-silica> nylon-silica in order of decreasing percentage recoveries. The trend may be due to consistency with the decrease in π electrons. Therefore it is proposed that the PS-DVB for bond Elut Plexa and polystyrene had higher percentage recoveries because of the presence of benzene rings characterised strong $\pi - \pi$ interactions (see Figs. 2.1 and 2.4).

5.7.4 Evaluation of the effectiveness of clean-up using electrospun sorbents

Washing interferences using 5% methanol caused significant loss of analyte. Nylon-silica sorbent showed effective clean-up but poor recoveries were obtained. Polystyrene-silica sorbent still showed ion suppression but it was less than the chromatogram in which the sample was not passed through SPE clean-up. PAN-silica behaved the same way as polystyrene-silica composite (see Figs. 5.30 and 5.31). Effective cleanup and good recoveries were obtained using the SPE conditions outlined in Fig. 5.32.

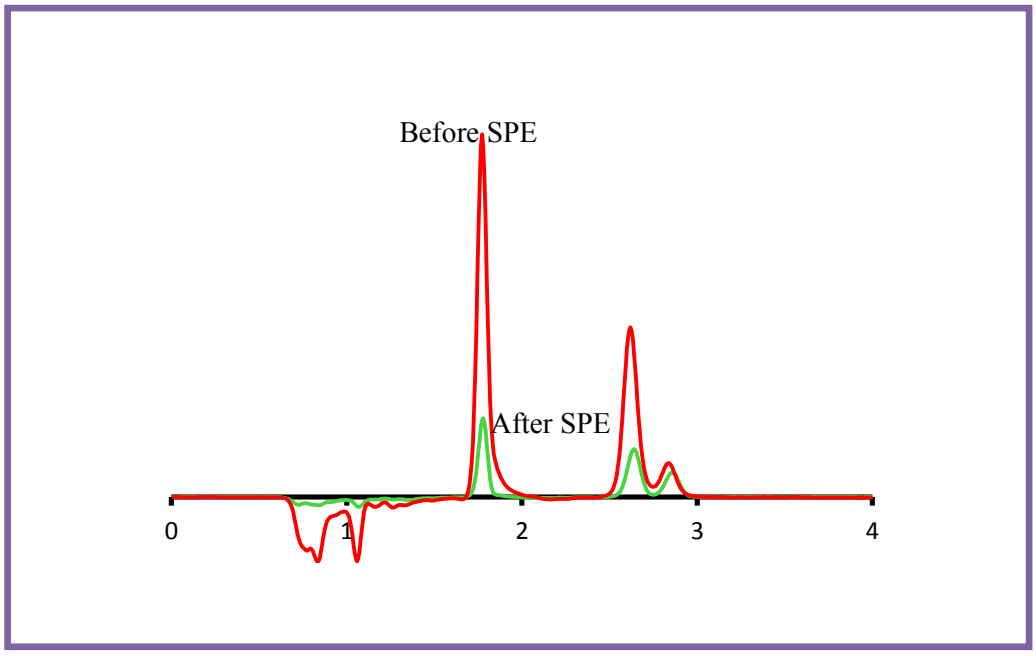


Fig. 5.29: Comparison of chromatograms before and after SPE with nylon-silica sorbent

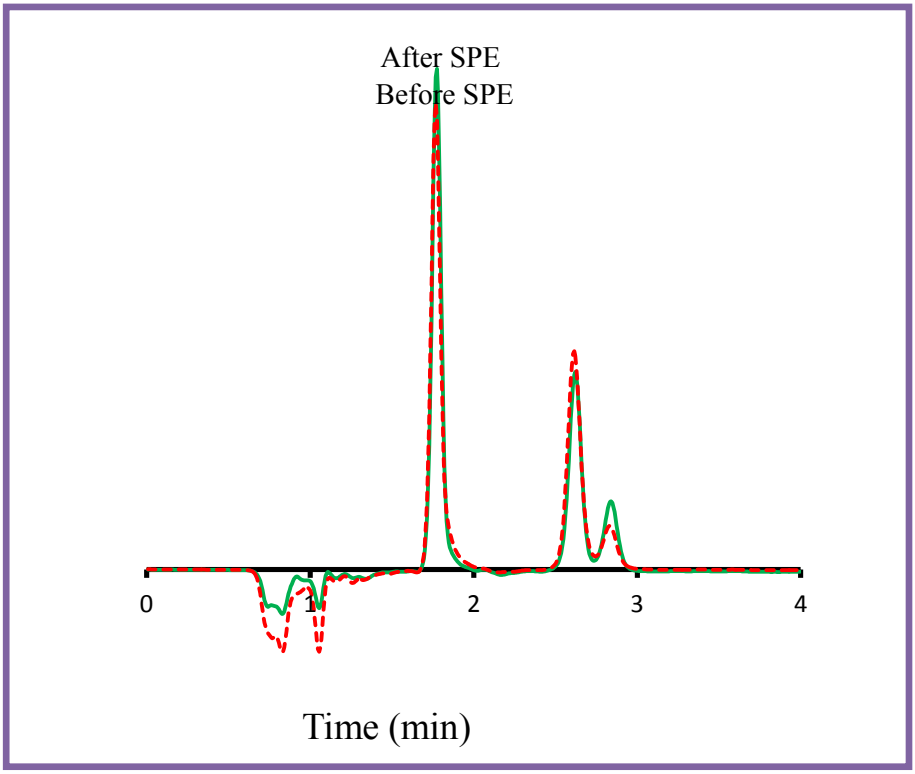


Fig. 5.30: Comparison of chromatograms before SPE and after SPE with polystyrene-silica sorbent

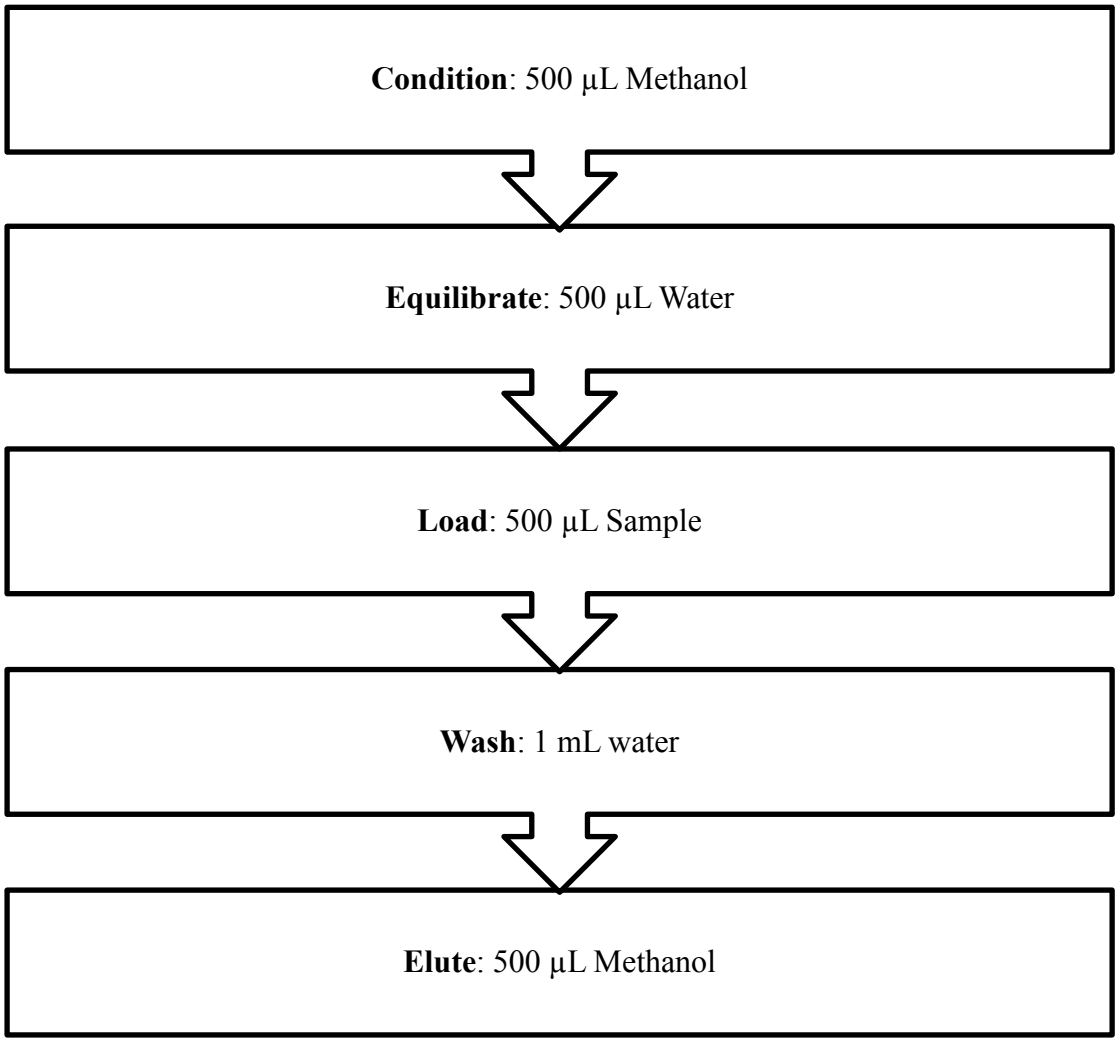


Fig. 5.31: SPE protocol for polymer-silica electrospun sorbents

CHAPTER 6: CONCLUSION

The experimental results obtained demonstrated that Agilent Bond Elut Plexa SPE was effective in clean-up of flavonoids in *Ginkgo biloba* and alkaloids in goldenseal (*Hydrastis Canadensis)*. The method developed using the SPE was fast, accurate and reproducible with good recoveries and R.S.D. of less than 5%. The recoveries of hydrastine ranged from 76-83% while those of berberine ranged from 99-104%. The limits of detection and quantification for hydrastine were 2.50 and 8.25 μg g^{-1} respectively while that of berberine was 2.35 and 7.75 μg g^{-1} respectively. The recoveries of quercetin, kaempferol and isorhamnetin ranged from 100-107%, 103-109% and 73-88% respectively. The limits of detection of quercetin, kaempferol and isorhamnetin were 2.94, 1.6 and 6.50 μg g^{-1} respectively while the limits of quantification were 9.34, 5.30 and 21.6 μg g^{-1} respectively.

A simple, accurate and reproducible method for the determination of banned aromatic amines in textile was developed using Chem Elut supported liquid extraction. The supported liquid extraction method can be a good alternative to use instead of traditional liquid-liquid extraction as it is faster because there is no waiting time for phase separation. Cleaner extracts were obtained and there was no vigorous shaking that is required in traditional liquid-liquid extraction.

Electrospun polymer-silica fibers demonstrated the potential to be used as sorbent materials for SPE as shown by the breakthrough results and recoveries. Good recoveries were obtained for both polystyrene-silica and PAN-silica sorbents while poor recoveries were obtained for nylon-silica sorbent. The calculated retention factor showed that nylon-silica sorbent had poor retention for flavonoids. The polystyrene-silica and PAN-silica sorbents were found to have comparable results to those of commercially available Agilent Bond Elut Plexa therefore they can be used as efficient SPE sorbents for the flavonoids studied. The fibers possess continuous long morphology as shown by SEM images which could result in large specific surface area and therefore the fibers are qualified as efficient sorbents for SPE.

Only SPE performance was studied for this work, therefore for future work there is a need to conduct tests for thermal stability and mechanical strength that are expected to be contributed by silica.

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