APPLICATION OF A CUSTOM-SYNTHESIZED MOLECULARLY IMPRINTED POLYMER FOR THE SELECTIVE ISOLATION OF TOTAL GLUCOSE AND FRUCTOSE FROM `100%` FRUIT JUICE PRIOR TO INSTRUMENTAL ANALYSIS

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Master of Science in Analytical Chemistry

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A thesis submitted to Botswana International University of Science and Techonolgy, Palapye, in fulfillment of the requirements of the degree of Master of Science in Analytical Chemistry

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Declaration

I hereby declare that this thesis is solely my work, in fulfilment of the requirements of Master of Science degree in Analytical Chemistry. All the research was carried out under the Department of Chemical and Forensic Sciences, Botswana International University of Science and Technology (BIUST), Botswana.

Hawa Wambui Mukami

4th, October 2018

Date

Dedication

This thesis is dedicated to my mother, Zuhura M. Muturi, and brother, Mohammed M. Mukami whom have been my rock and the reason I push myself to be better.

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There have been many people who have walked alongside me during the undertaking of my Masters. They have encouraged me, guided me, placed opportunities in front of me and showed me the doors that might be useful to open.

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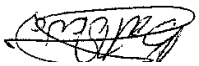
A debt of gratitude is also owed to my family and friends for your continued support and constantly checking up on me. This has taught me the value of belonging. To my mum and brother to whom this thesis is dedicated, thank you for your prayers and for believing in me that one day your daughter/sister would complete and make the world her canvas. I love you both so much.

Finally and without hesitation, I would like to thank God for granting me the opportunity to undertake this Masters degree and for His grace and faithfulness. Without Him none of this would be a reality.

Certification by Supervisor(s)

The undersigned certifies that he/she has read and hereby recommends for acceptance by the College of Science a dissertation titled '**Application of a custom-synthesized molecularly imprinted polymer for the selective isolation of total glucose and fructose from `100%` fruit juice samples prior to instrumental analysis'** in fulfilment of the requirements for the degree of Master of Science in Analytical Chemistry at BIUST.

Supervisor



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Abstract

This thesis presents a novel sample preparation strategy that employed a custom-synthesized glucose – fructose (G-F) specific molecularly imprinted polymer (MIP) powder as an adsorbent for the simultaneous, selective extraction, isolation, pre-concentration of total glucose and fructose from the complex and `dirty` sample matrix of '100%' fruit juices in order to improve their accurate analysis. The '100%' fruit juice samples were purchased from retail shops in Palapye, Botswana. Scanning electron microscopy (SEM) revealed G-F MIP particles that were somewhat spherical with an estimated particle size of > 20 μm. Both of which are excellent morphological characteristics for adsorption. The prepared G-F MIP powder demonstrated high selectivity, effective extraction and isolation for glucose and fructose from real samples of `100%` fruit juice samples as evidenced by the calculated high extraction efficiencies (EEs) of over 90%, with low percentage relative standard deviations (%RSD) of below 7% for n = 6, for both glucose and fructose when compared to the low EEs of below 28% by the non-imprinted polymer (NIP), regarded as the control. Furthermore, the G-F MIP showed lower selectivity towards the analogue molecules; maltose and lactose as supported by the low EEs of below 34%. All binding studies were performed at optimal conditions; 60 mg and 20 min for sorbent dose and reaction time respectively. With the high affinity for glucose and fructose, the selective sample preparation strategy proposed herein presented itself as a potential procedure to be employed to improve the accurate analysis of juices adulterated by artificial sugar sweeteners that are usually illegally added to the so-called `100%` fruit juices by producers to improve their taste.

Keywords: Molecularly imprinted polymers, selective isolation, selective pre-concentration, selective sample preparation, `100%` fruit juice, beverage adulteration, Palapye, Botswana

List of Abbreviations

AFM: Atomic force microscope

AIBN: Azobisisobutyronitrile

BET: Brunauer, Emmet and Teller

DFT: Density functional theory

EDTA: Ethylene diamine tetraacetic acid

EGDMA: Ethylene glycol dimethacylate

FE-SEM: Field emission scanning electron microscope

FI: Freundlich

FTIR: Fourier tranform infrared

GCB: Graphitized carbon black

GC-MS: Gas chromatography mass spectrometry

G-F MIP: Glucose-fructose molecularly imprinted polymers

GPC: Gel permeation chromatography

h: Hours

HPLC: High pressure liquid chromatography

IAEs: Immunoaffinity extraction sorbents

IASPEs: Immunoaffinity solid phase extraction sorbents

ISs: Immunosorbents

LC-MS/MS: Liquid chromatography tandem mass spectrometry

L-FI: Langmuir-Freundlich

LLE: Liquid liquid extraction

LME: Liquid membrane extraction

MAA: Methacrylic acid

MAX: Mixed-mode anion-exchange

MCX: Mixed-mode cation-exchange

mg: Milligrams

MIA: Molecularly imprinted adsorbents

min: Minutes

MIPs: Molecularly imprinted polymers

MIT: Molecular imprinting technology

mL: Milliliters

MMLLE: Microporous membrane liquid-liquid extraction

NIPs: Non-imprinted polymers

NMR: Nuclear magnetic resonance

1H-NMR: Proton nuclear magnetic resonance

PHWE: Pressurized hot water extraction

PS-DVB: Polystyrene divinylbenzene

RAMs: Restricted access materials

RI: Refractive index

SEM: Scanning electron microscopy

SFE: Supercritical fluid extraction

SLM: Supported liquid membrane

SPE: Solid phase extraction

TEM: Transmission electron microscope

TRIM: Trimethalolpropane trimethacrylate

UV-Vis: Ultra-violet Visible

μm: Micrometer

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Chapter 1: Introduction

1.0 Background

Premium quality 100% fruit juices are on high demand due to their many important health benefits [1], [2]. The continued high demand has made the juices command higher prices compared to other liquid refreshments. This reason has made the product become a target for adulteration. Unscrupulous 100% fruit juice producers have resorted to the addition of trace concentrations of sweeteners in the form of artificial glucose and fructose that would not be easily detectable or even be easy to differentiate from the natural glucose and fructose present as part of the fruit [3], [4]. This practice is gaining popularity in order to keep up with the high demand and also maintain excellent sweet taste enjoyed by the many consumers. The addition of the sugars without proper labelling is an illegal act and contributes to beverage adulteration [5]. This has given birth to a new crop of 100% fruit juices with a stinging sweet taste which are referred to as `100%` fruit juices in this study. In USA alone, over 56 million gallons of fruit juice is served in school districts [6]. Stake holders have expressed concern that the juice served may be adulterated with less expensive ingredients and may not be accurately labelled. The magnitude of the fraud costs governments billions of dollars annually [6].

Food and consumer service is tasked with the responsibilities of protecting consumers from this type of fraud. Different government agencies, for example, Botswana Bureau of Standards (BOBs) is responsible for inspecting and grading food products. The illegal addition of the artificial sugars has necessitated the monitoring of fruit juice quality and authenticity, with an aim of upholding consumer rights. Sensitive analytical instruments such as isotope ratio mass spectrometer [5], fourier transform infrared spectroscopy [7], and enzymatic methods [8] are some of the instruments usually employed to differentiate the adulterated artificial sugars from the natural sugars that form part of the natural fruit juices. The analytical instruments with very high sensitivities possess the capability to accurately monitor adulteration in juice matrices even at trace levels; however, they have proved to be challenged by the `dirty', complex matrix of the fruit juices which is often exacerbated by sample to sample variability of the fruits or fruit juices. The challenge is further compounded by the existence of analogous sugars in the matrix. Due to the complexity of juice matrices, samples often have to undergo targeted, selective and extensive sample preparation procedures such as selective sample isolation, pre-concentration and clean-up

prior to instrumental analysis. In the past, sample preparation strategies based on physical and chemical affinity of sugar molecules such as zeolite adsorption and activated carbon adsorption have been employed to extract sugar molecules from sample matrices [9], [10]. These techniques however have the limitation of poor selectivity [11] which has led to research in the development of selective sorbents, characterized by high porosity, larger surface area and specific binding sites. One example of such selective sorbents is the molecularly imprinted polymers (MIPs) [12], [13]. In many cases they have been combined with solid phase extraction [14]–[17] and led to the advent of solid phase extraction-molecularly imprinted polymers (SPE-MIPs) technique which combines the advantages of SPE; low cost, speed, flexibility and the selectivity of MIPs.

1.1 Main Objective

The overall main objective of this thesis was to develop a selective pre-analytical procedure that employed a molecularly imprinted polymer as an adsorbent for the simultaneous, selective extraction, isolation and pre-concentration of total glucose and fructose from the complex and `dirty` sample matrix of '100%' fruit juices in order to improve their accurate analysis.

1.2 Specific Objectives

- To synthesize a G-F MIP employing the simple method of free radical bulk polymerization.
- To characterize the prepared MIP particles using SEM.
- To optimize the binding parameters and characterize the prepared G-F MIP through optimized rebinding experiments.
- To apply the prepared MIP to '100%' fruit juice.

Chapter 2: Sample Handling Strategies for Beverage Samples

2.0 Beverage assaying

As part of beverage samples surveys, numerous beverage samples are tested daily to protect consumers from misleading product labelling [18]. Due to the complexity of beverage matrices, samples often have to undergo extensive sample handling procedures prior to instrumental analysis to achieve precision and accuracy in assaying.

2.1 Sample handling of beverage samples

Sample handling refers to all steps involved in preparing samples for accurate assaying before feeding the samples to analytical instruments [19]. Sample handling steps are sometimes referred to as pre-analytical steps and they include sampling and sample preparation procedures as depicted in Figure 2.1. Improper sample handling contributes to misleading results as well as compromising the proper functioning of analytical instruments [20]. B. Budowle *et al.,* reported that the condition of a sample being received for testing is of primary importance. They further explained the significance of reducing the time between sample handling and analysis [21].

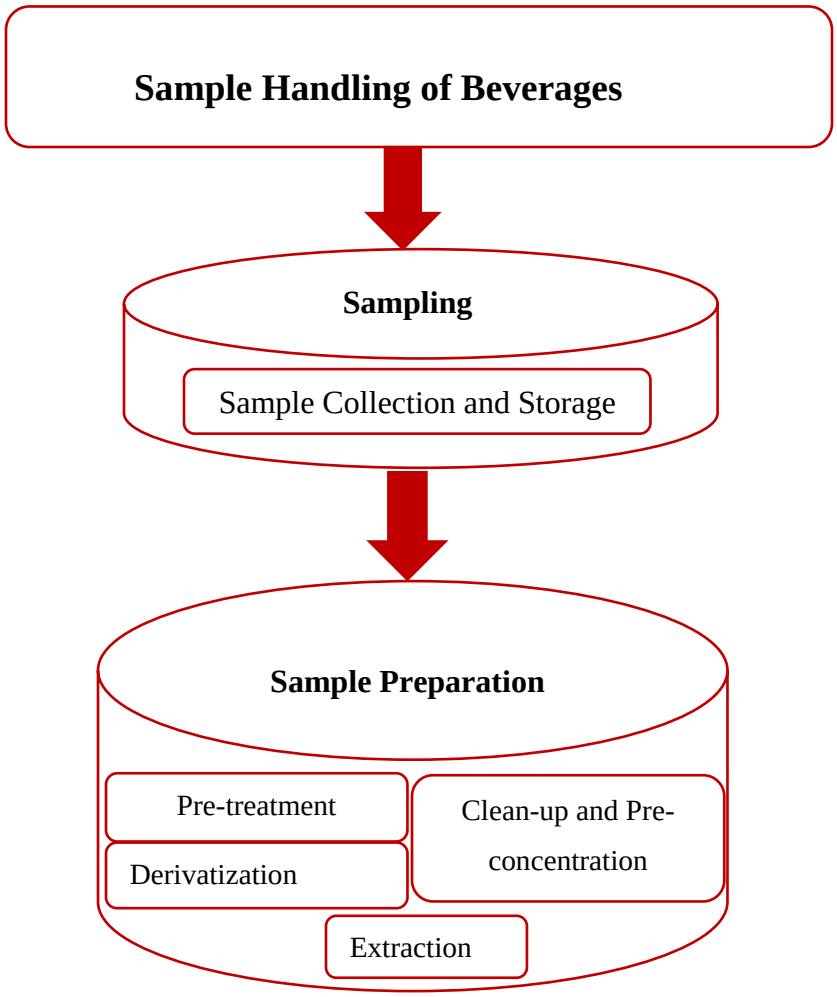


Figure 2. 1: A schematic diagram showing sample handling procedures for beverages

2.1.1 Sampling of beverage samples

Sampling is a systematic process of collecting a representative portion of a larger quantity of materials to be analyzed in the laboratory while the results obtained still maintain an accurate representation of the materials being sampled [20], [22]. Sampling assists in solving the challenge of analyzing large quantities of beverage in their entirety, which can be cumbersome and impossible [23]. Therefore, samples original conditions should be maintained as nearly as possible to the original materials [24]. There are different types of sampling methods which include; grab, composite, systematic and random sampling. The choice of sampling method to employ is dependent on the sample matrix and target analyte(s).

2.1.2 Sample preparation of beverage samples

Sample preparation refers to all steps performed on a sample prior to instrumental analysis [25]. Sample preparation is a key component in any analytical analysis, despite that, it is the most time consuming and tedious to perform and optimize [26], [27],. Extensive sample preparation is vital when handling complex matrices to eliminate interferences, pre-concentrate trace analytes and convert analytes into suitable detectable forms [28], [29] Additionally, sample preparation steps help to minimize the destruction of some sensitive instrumental components, for example, chromatographic columns, that are liable to column overload as well as injector ports that may become jammed [30], [31]. These steps also help reduce the expense of maintenance and increase the life of analytical instruments [26]. These steps include; pre-treatment, clean-up, pre-concentration, derivatization and extraction. In this thesis, these steps were applied for the selective extraction, isolation, purification and enrichment of glucose and fructose from fruit juice.

2.1.2.1 Pre-treatment

Pre-treatment of a sample involves procedures aimed at removing obvious dirt from a sample, pre-conditioning the sample and maintaining the integrity of the sample. Some examples of pre-treatment procedures include: filtration, homogenization and adjusting pH. Homogenization [26] and filtration [32] are the most employed pretreatment procedures in beverage samples. According to J. Medvě *et al.*, the choice of a pre-treatment procedure(s) is entirely dependent on the sample matrix as well as the target analytes [33], [34].

2.1.2.2 Clean-up and pre-concentration

Clean-up and pre-concentration are sample preparation procedures that simultaneously result in purification and enrichment of the analytes of interest in the sample for ease of detection. Clean-up involves eliminating undesired interfering matrix from a sample at molecular level, whilst pre-concentration refers to the process in which the concentration of target analyte(s) is increased by reducing the total volume of solution in which the analyte of interest is contained [35]. Often, analyzing a sample matrix with target analytes in trace concentrations poses a challenge for sample matrix characterized by ‘dirty’ samples that are usually very dilute relative to analytes of interest.

Therefore, it's very important to introduce these procedures to purify samples and enrich the trace components [36]. Food samples including beverages are usually characterized by 'dirty' matrices with analytes of interest at trace levels [37]. Directly injecting such samples into an analytical instrument usually leads to high down time of instruments resulting in chromatographic instruments that are liable to column overload and injector ports that may become jammed [30], [31]. For this reason, these sample preparation procedures are key as they introduce cleaner samples for analysis resulting in removal of sources of noise whilst also reducing clogging of chromatographic columns and other sensitive compartments of the analytical instruments [34], [36]. Additionally, these procedures lead to notable improvement in chromatographic separation along with instrumental detection of target analytes [38].

2.1.2.3 Derivatization

Derivatization is a sample preparation procedure that chemically transforms an otherwise non-responsive or less responsive analyte of interest into a modified compound that will be responsive to a particular analytical instrument [39], [40]. For example, in gas chromatography (GC) analysis, target analytes must have the following properties; be volatile and have good thermal stability. However, some analyte compounds, for instance, reducing sugars do not conform to these properties hence they are not amenable to GC analysis [41]. This implies that these simple sugars have to be derivatized before they are introduced into the GC. These reducing sugars also respond poorly to specific detectors coupled with HPLC due to the limited sensitivity and selectivity of the measurement. Reducing sugars lack chromophores which show electronic transition under visible light or ultraviolet. This implies that these compounds cannot be detected by diode-array detector (DAD) or ultraviolet (UV) detectors which rely on electronic transitions that can only respond to visible or UV light [42]. Therefore, these compounds need to be tagged with a functional group that will improve detection, for example, (DAD) is able to detect glucose-1-phenyl-3-methyl-5-pyrazolone derivatives but responds poorly to glucose thus, 1-phenyl-3-methyl-5-pyrazolone is usually employed to derivatize glucose [43]. Derivatization as a result, improves suitability, response and also improves resolution between co-eluting compounds and overlapping peaks in chromatography [44], [45].

2.1.2.4.1 Extraction and extraction techniques

Extraction refers to separation of desired analyte(s) from a sample matrix [46]. The procedure often results in simultaneous clean up and pre-concentration of analytes of interest. Conventional extraction methods that are usually non-selective, for example, filtration or mass exclusion have been observed to adequately work for simple sample matrices [47], [48]. However, when employing sensitive analytical instruments for analysis of complex matrices, it is imperative that the samples achieve a high standard of purity before introducing it into the instrument [49]. The non-selective extraction methods alone may not be able to achieve this high standard of purity in a sample. Therefore, for more complex sample matrices, novel extraction methods possessing high selectivity are needed to efficiently bring the analyte of interest out of the sample matrix to detectable levels. Extraction methods that are commonly employed in beverage samples are such as liquid-liquid extraction (LLE) [50], supercritical fluid extraction (SFE) [51], pressurized hot water extraction (PHWE) [52] and solid phase extraction(SPE) [53]. These methods prepare samples into formats compatible with analytical instruments such as HPLC/ RI , GC/MS and LC/MS/MS [54].

2.1.2.4.1.1 Liquid-liquid extraction (LLE)

LLE also known as solvent extraction employs the use of two immiscible solvents to separate compounds and relies on the different distribution of the compounds to be separated between the two liquid phases [50], [55]. The process is dependent on the mass transfer of the compounds to be extracted from the first liquid phase to the second one, meaning components are separated based on their relative solubility in the two immiscible liquids (see Figures 2.2 and 2.3). The selection of the solvents to be employed in LLE is important to achieve maximum transfer of solute from the carrier into the solvent [56]. The ideal solvent has the following properties; cheap, low viscosity, high boiling point, high resistance to thermal degradation, high solubility for solute and low solubility for the carrier liquid, density difference greater than 150 kg/m^3, nontoxic, not corrosive to process equipment and nonreactive with other chemicals taking part in the extraction process [57].

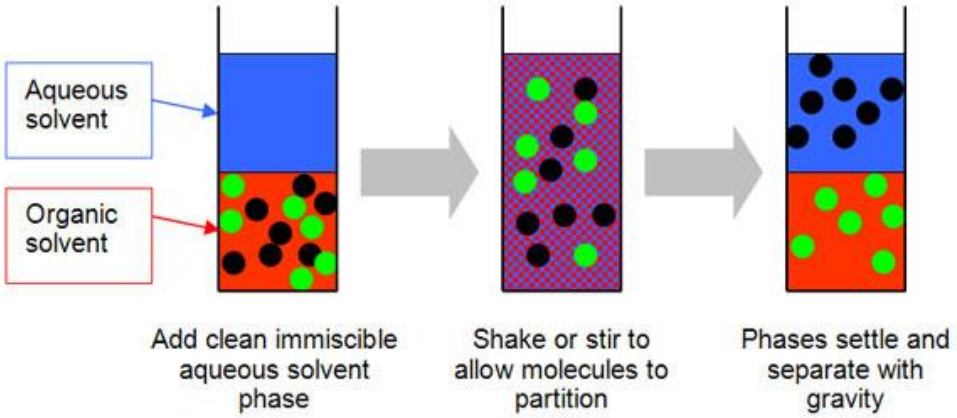


Figure 2. 2: A schematic representation of the principle of liquid-liquid extraction [55]

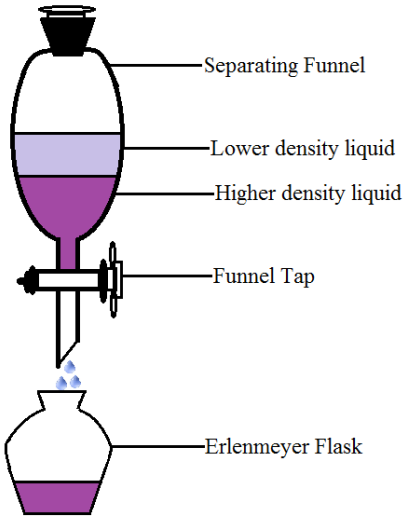


Figure 2. 3: A simple diagrammatic laboratory set up employing a separating funnel apparatus for LLE [56]

LLE is frequently employed for the extraction of toxicants due to its advantage of readily available solvents and the use of low cost apparatus. For instance, LLE has been previously employed for the extraction of low relative molecular mass compounds as reported by D Duarte *et al.*, in a study on quantification of sugars in tubers of *Solanum tuberosum*. They reported that 50% (v/v) aqueous methanol extraction solution gave the highest level of sugar analytes, among the four solvent extraction methods investigated. From their work, extraction recoveries ranging between 94.14% to 99.77% were obtained [58]. However, the method demonstrated may not have been ideal to selectively target one sugar molecule due to the similarity in the chemical type of the different

sugar analytes, therefore, D Duarte *et al.*, reported the extraction of all sugars simultaneously and to the same extent of at least 94% [58]. LLE is challenged by poor selectivity and it is also not easily automated [57].

2.1.2.4.1.2 Liquid membrane extraction (LME)

The liquid membrane extraction is a method which combines stripping and solvent extraction in one single step [59]. It employs facilitated diffusion transport mechanism to extract analytes of interest. In principle there are two variants of LME, supported liquid membrane (SLM) extraction which is suitable for the extraction of polar compounds and is compatible with reversed phase high pressure liquid chromatography (HPLC) and microporous membrane liquid-liquid extraction (MMLLE) which is suitable for the extraction of hydrophobic analytes and is compatible with gas chromatography (GC). Within the past few years a number of studies have evaluated interactions of saccharides with different carriers [8]. The investigations concluded that boronic acids as carriers are more efficient in transporting sugars through liquid membrane. SLM extraction has been reported to be highly selective to sugars due to the specificity of the carrier-substrate complex [8]. However, the studies have highlighted a major drawback associated with this extraction technique, which is associated with membrane instability arising from the partition of the organic carrier to the aqueous phase [59], [60].

2.1.2.4.1.3 Supercritical fluid extraction (SFE)

The extraction technique separates one component (extractant) from another (the matrix) using CO_2 at supercritical conditions as the extracting solvent [61]. Supercritical fluids are highly compressed gases at critical temperatures and critical pressure [62]. They possess the combined properties of gases and liquids making their reactions impossible to achieve using conventional solvents [51]. The extraction technique is fast and yields pure residue. A study has been carried out focusing on fractionation of complex carbohydrate mixture employing super critical carbon dioxide with different ethanol/water mixture. The study reported recoveries of 94% for mainly tri- and tetra- saccharides [63]. However, this technique has limitations of being very expensive plus consistency and reproducibility may vary in continuous production [62]. See Figures 2.4 and 2.5

showing a schematic diagram of the SFE setup and a phase diagram of a supercritical fluid respectively.

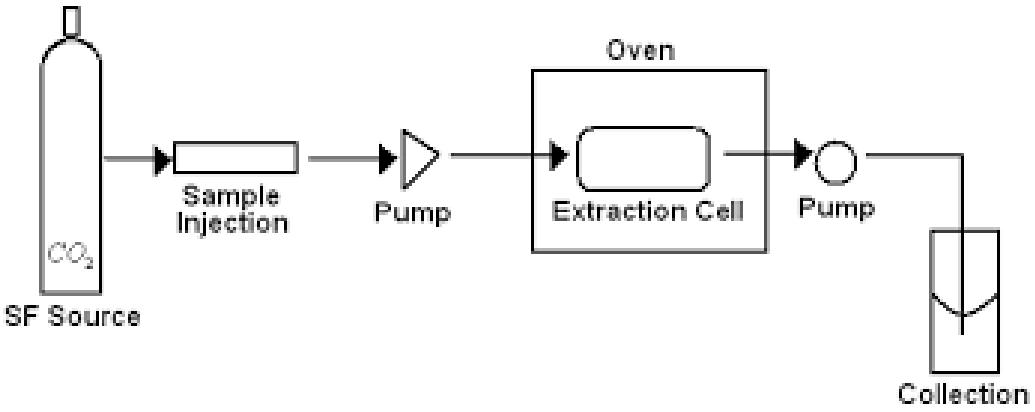


Figure 2. 4: A simple schematic representation of SFE set up [62]

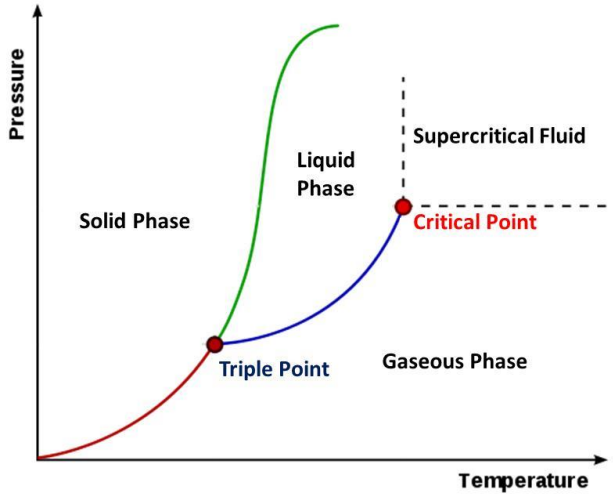


Figure 2. 5: A phase diagram showing the critical temperature and pressure of a particular super critical fluid [51]

2.1.2.4.1.4 Pressurized hot water extraction (PHWE)

PHWE is a green extraction method that employs water as a feasible solvent [64]. It is applicable in numerous kinds of matrices, such as, environmental, botanical and food samples. In sample preparation the process is mostly employed to extract organic contaminants from food stuff in food safety analysis [65]. PHWE utilizes pressurized water at controlled pressure and elevated temperatures, resulting in varied water polarity. Hence, the pressurized water can dissolve a wide range of analytes depending on their polarity [64]. The water density remains almost the same throughout to reduce the effect of pressure on the water properties. The temperature range for this process is between 100 °C to 374 °C, which is the boiling point and critical point of water respectively. To keep a condensed phase of water, minimal pressure is required such as 15 bar at 200 °C and 85 bar at 300 °C. Super-heated steam results if this pressure decreases below the boiling point [52], [66]. Benito-Roman *et al.,* found out that PHWE has been successfully employed to extract β-glucan from barley. Their obtained results have proven that under optimal conditions β-glucans were not degraded and the extraction time was significantly reduced [67]. Another major advantage associated with PHWE is the reduction in the consumption of organic solvents [64].

2.1.2.4.1.5 Solid phase extraction (SPE)

SPE is a form of solid adsorption extraction technique that employs principles akin to chromatographic separation such as, hydrophobic, hydrophilic, ion exchange and affinity [68]. Principally, it involves partitioning of the analyte(s) of interest between a liquid phase of the sample matrix and a solid phase known as the sorbent. Depending on the affinity of the analyte of interest for the liquid or solid phase, it may be retained by the solid phase or it may prefer to be in the liquid phase of the sample matrix [69]. If the analyte of interest is retained by the sorbent, then a small quantity of solvent with high elution strength will be employed to desorb (recover) the analyte. Thus, the analyte will be contained in a small quantity of the solvent resulting in pre-concentration. On the other hand, if the analyte remains in the liquid phase, then it would mean that the interfering species will be trapped by the sorbent. Therefore, the analyte of interest will be freed and thus clean-up will be achieved. The analyte of interest will easily be detected due to a cleaner sample matrix free of interference [70], [71]. Thus, SPE has been primarily employed for isolation, pre-concentration, clean-up and matrix removal purposes [72]. For instance, J Barnes *et*

al., has reported excellent isolation of sugar nucleotides employing SPE. The developed method was reported to be simple yet effective to purify sugar nucleotides [73]. Additionally, SPE has been successfully employed for the extraction of fructose, glucose and sucrose from sugarcane molasses as reported by W. Xu *et al.,*. The results showed superb linear ranges for fructose, glucose and sucrose as demonstrated by a wide range of concentrations from 1.80 to 29.70 g/L [74].

SPE may be performed either in column or batch format mode [75], [76]. In batch mode, which is the simplest and easiest mode, a known quantity of sorbent and sample matrix consisting of solvent, analyte and interference are mixed and shaken together to achieve equilibration. The solution mixture is filtered off or centrifuged. In the case that the analyte of interest is retained in the sorbent, it is desorbed and detected. If it is in the filtrate or supernatant, then is it determined directly or by subtraction as shall be discussed in the subsequent chapters. On the other hand, column or cartridge mode involves packing of a sorbent in a column format allowing the sample to pass via the column bed as depicted by the SPE protocol procedure in Figure 2.6. In the Figure, the analytes of interest are retained on the sorbent while the interference passes through (step c). The sorbent bed is further washed to remove any undesired interferences that may have been left behind. Finally, the purified analytes are eluted from the column using an appropriate solvent with a higher elution strength. Note that, if it is the interference trapped, then the elution step (step d) is left out.

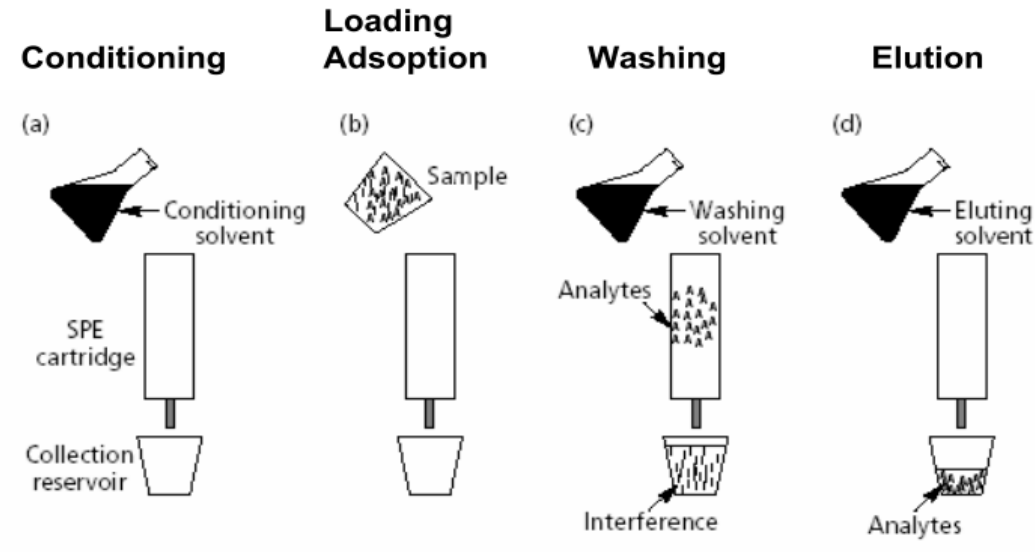


Figure 2. 6: A diagrammatic representation of column SPE illustrating the four steps involved [75]

SPE has many advantages that include; highly purified extracts, high recoveries, good reproducibility, applicability to a wide variety of sample matrices, ease of automation and reduction in solvent consumption [77], [78]. Despite these benefits, SPE has shown some limitations, most of which are associated with the type of sorbent employed. These limitations include; greater cost per sample, high time consumption and poor selectivity [79]–[81]. Conventionally, a wide range of non-selective sorbents that include, polymeric materials with C18/C8 skeletal backbones, silica based sorbents, weak anion-exchange sorbents, mixed-mode anion-exchange, mixed-mode cationic-exchange and hydrophilic-lipophilic balanced macro-porous copolymers have been employed [82]–[84]. Some of these conventional sorbents have proven to be effective in different extraction matrices for the purpose of isolation and pre-concentration of organic analytes [85]–[87]. However, these sorbents have proven to be non-selective as shown by their limitations which include; co-elution of matrix analytes and poor selectivity which consequently interfere with analyte(s) identification and quantification. For instance, co-extracted compounds may produce a signal suppression effect when mass spectrometry is employed as the detection technique [88]. Thus in recent times, there has been a growing interest to develop smart functional materials with superior selectivity among other attributes, to be employed as selective SPE sorbents.

2.1.2.4.1.5.1 Selective SPE sorbents

Selective SPE sorbents are based on molecular recognition mechanism and have attracted more research in the recent years due to their enhanced extraction selectivity [76]. These sorbents have the ability to be applied to virtually all matrices for the purpose of achieving cleaner extracts that could be directly injected into analytical instruments. Examples of these sorbents include; immunosorbents, restricted access materials (RAMs) and molecularly imprinted polymers (MIPs).

2.1.2.4.1.5.1.1 Immunosorbents

Immunosorbents are sorbents that have both reversible and selective antigen-antibody or enzyme-substrate interactions [89]. The sorbents are employed to trap analytes with similar structure and use the principle of an affinity ligand to trap the target analyte [90]. The development of immunosorbents involves covalently bonding an appropriate antibody or enzyme to an appropriate support. The process involves immobilizing the antibody or enzyme onto a solid support to form an antibody/enzyme support adduct which will then act an immunosorbent. Rigidity and porosity should be considered when choosing the ideal support for an immunoaffinity sorbent so as to enhance the flow of the sample. The support should also have sufficient functional groups to couple with either antibodies or enzymes to be immobilized [90]. Finally, the support should be hydrophilic in-order to avoid any non-specific interactions and be pressure-resistant for use in on-line techniques [89]. Immuno-affinity sorbents have the advantage of high selectivity resulting from the specificity of the antigen-antibody or enzyme-substrate spatial fitting and interactions. Immunosorbents achieve extraction, pre-concentration and clean-up of the sample matrix simultaneously [90]. However, theses sorbents are not cost effective and are easily denatured when exposed to harsh conditions thus making them suitable only for limited applications. Additionally, these sorbents need highly skilled manpower to develop them.

2.1.2.4.1.5.1.2 Restricted access materials (RAMs)

RAMs are ‘intelligent’ sorbent materials that are obtained by modifying the external surfaces of conventional sorbents with hydrophilic groups (chemical barriers) as well as by the presence of small pores (physical barriers) accessible only to small molecular size molecules Examples of some conventional sorbents include; polymers, active carbon, carbon nano-tubes, and silica-based materials [92]. Supramolecular solvents have also been employed as RAMs, due to their abilities to exclude proteins through precipitation with solvents or according to size [93],. RAMs are ideal for handling biological samples since they prevent access of large molecular size matrix components such as proteins, while smaller targeted molecules are selectively retained in the interior of the sorbent by partition, adsorption and/or ion exchange either via a chemical or physical diffusion barrier [93], [92]. These sorbents have a drawback of limited selectivity hence they may not be employed for a wide variety of analytes especially those with small molecular sizes [93],

[94]. The development of RAMs and Immunosorbents have proven to be time consuming and very expensive thus increasing the need to develop sorbents that are smart, inexpensive, stable, widely selective and robust. One such sorbent has been identified as Molecularly Imprinted Polymers (MIPs).

2.1.2.4.1.5.1.3 Molecularly imprinted polymers (MIPs)

MIPs are biomimetic polymeric materials with tailor-made binding sites that are complimentary in size, shape and functionality to the target analyte(s) [95]. MIPs are referred to as synthetic receptors since they mimic natural selection systems such as enzyme-substrate and antibody-antigen systems [96]–[98]. They have many advantages as SPE sorbents that include; ease of synthesis and handling, high selectivity, reusability, excellent reproducibility, chemical and physical stability [12]. According to Pei *et al.,* MIPs have a broad range of application and are known for their versatility in recognizing both biological and chemical molecules [99], [100].

In this thesis a novel custom-made glucose-fructose dual templated MIP was developed to simultaneously and selectively extract, isolate and pre-concentrate total glucose and fructose from complex and `dirty` sample matrix of 100% fruit juices prior to instrumental determination of artificial glucose and fructose, that are usually illegally added by fraudulent producers to improve the taste of '100%' fruit juice.

Chapter 3: Molecularly Imprinted Polymers

3.0 Molecularly imprinted polymers (MIPs)

MIPs are polymeric materials prepared by a process known as Molecular Imprinting Technology (MIT) where selective binding sites are introduced in the polymer matrix [101]. As depicted in Figure 1, the selective binding sites are produced by the incorporation of a template (target analyte) into a polymerization solution to establish binding interactions between the template and the polymerisable chemical functionalities of the functional monomers. Subsequently the resultant adducts are co-polymerized with a cross-linker to hold the template in place and form a rigid, highly crosslinked 3-dimensional polymer [102]. The template is then removed to free specific recognition sites that are complementary in size, shape and functionality to the imprinted template molecule(s) [103], [12], [104] during rebinding.

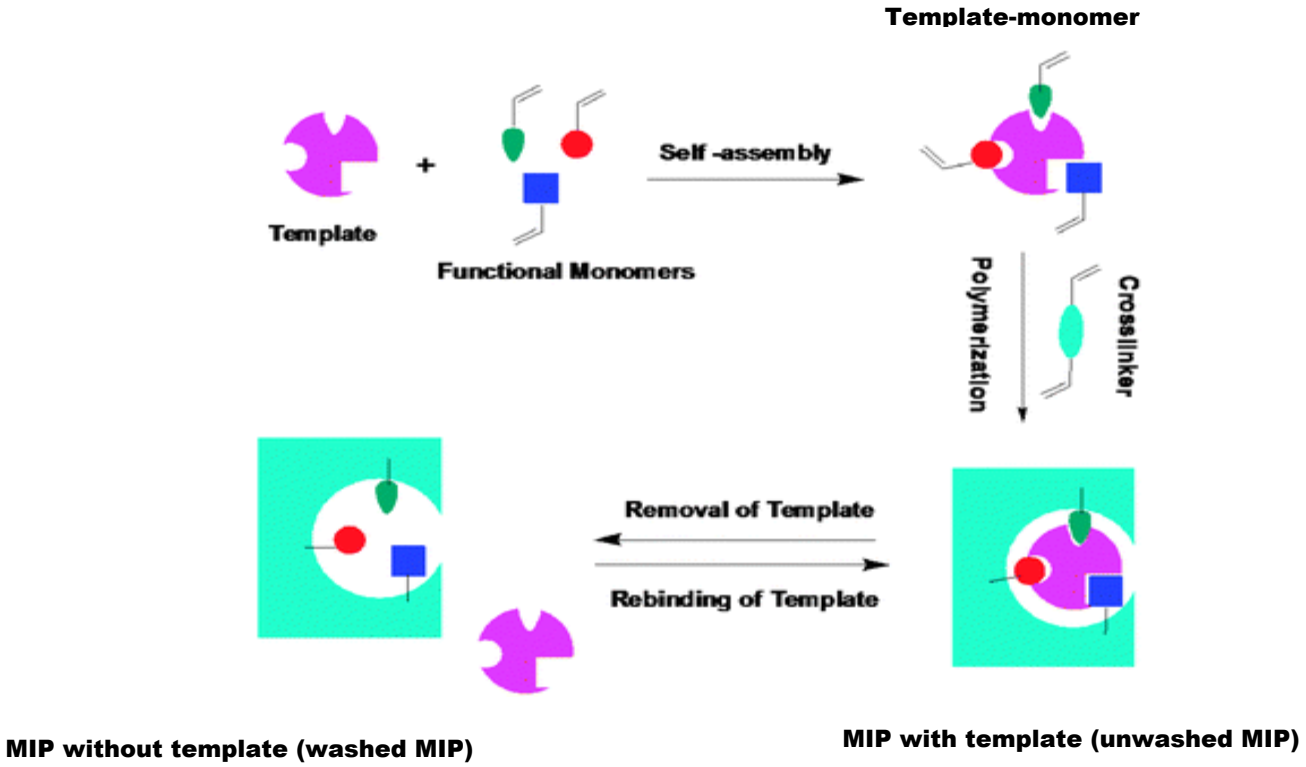


Figure 3. 1: Diagrammatic representation of the imprinting process showing all the general MIT reactants [105]

The ability to introduce selectivity in polymer matrices has made molecular imprinting a viable approach to design robust molecular recognition materials [106]. Molecular imprinting is often described as a technology that creates a specific molecular lock that matches a specific molecular key [97]. Since its inception, MIT has gained popularity due to its synthetic approach which mimics natural molecular recognition entities such as biological receptors and antibodies [105], [107]. MIT has sparked a lot of interest because of its three major unique features; recognition specificity, structure predictability and application universality in different fields [105]. Examples of areas where MIPs have been applied include; antibody simulation [108], biosensors [109], enzyme catalysis simulation [110], chiral resolution [111], and biochemical separation [112]–[114]. Additionally, MIPs have been successfully and extensively employed as selective solid-phase extraction sorbents in sample preparation resulting in a specialized technology called molecular imprinting solid-phase extraction MISPE [115].

3.1 Imprinting approaches

There are generally three imprinting approaches employed in MIT. The first is referred to as covalent imprinting approach and was developed by Wulff [116], [117]. It is also known as the pre-organized approach and it involves formation of covalent bonds between the template and functional monomers prior to polymerization. Hence, the template molecules require to be chemically modified with the functional monomers. The template molecule is subsequently removed after polymerization by cleavage of the covalent bonds. The covalent bonds are re-formed upon rebinding of the analyte of interest to the binding sites.

The second approach is the non-covalent imprinting or the self-assembly approach which was introduced by Mosbach [108]. Non-covalent imprinting basically relies on physical interactions between the functional monomer and template molecule [118], [119]. These relatively weak non-covalent intermolecular interactions are based on hydrogen bonding, electrostatic interaction, hydrophobic and/or van der waals forces [102], [100]. The resulting recognition is dependent on those non-covalent interactions [120], thus, the selection of functional monomers having favourable interactions with the template is very important to generate high affinity binding sites [15]. The non-covalent approach is advantageous as it has a wide range of polymerizable units having different functional groups that allow interaction with a template molecule. It also requires

limited organic synthesis knowledge. Due to these advantages and its simplicity, the non-covalent approach has advanced tremendously relative to the other [121]. Thus, even in this study it is the approach of choice. However, the non-covalent strategy suffers from limited imprinting of recognition sites, due to the non-stoichiometric mixing of monomer-template ratios during the initial stage of the imprinting process [103], [122], [123]. Recognition sites of heterogeneous nature are usually formed within the MIP structure. Furthermore, the MIPs designed with this strategy have been described to be 'group-specific' that is, selective not only to the template but also towards other structurally related compounds [103], [123].

With the aim of combining the advantages of both the covalent and the non-covalent approaches, Whitcombe introduced a third imprinting approach known as the semi-covalent or sacrificial approach [124]. This approach can be viewed as a fuse between non-covalent and covalent approaches due to the template being covalently bonded to the polymer, whereas non-covalent interactions are exploited during rebinding [118]. Semi-covalent approach is usually advocated for when employing templates with no functional groups pendant on them [125]. In this approach, a spacer group is introduced between the template and monomer in the imprinting process in order to achieve easy non-covalent rebinding. This approach can in principle yield MIPs with higher binding capacities due to the fact that the template molecule is covalently bound to the functional monomer at the onset, thus there is much better binding site integrity in the course of polymerization [126].

Generally, the quality of the binding sites is dependent on the interaction between template molecule and polymer matrix, which varies depending on the choice of polymerization method. Covalent imprinting produces well defined binding sites with the template being fixed at a particular position [126]. However, in comparison to the non-covalent approach, the covalent imprinting yields lower binding capacity, since the process of binding functional units with reversible covalent bonds is slow and cumbersome therefore limiting the covalent approach in imprinting [12]. On the other hand, the non-covalent approach does not require carbon-carbon bond formation but rather physical interactions between the template molecule and the monomer which differ depending on the template analytes [126]. When the template is organic the imprinted polymer is referred to as MIP whilst when the template is inorganic then the imprinted polymer is better known as ion imprinted polymers (IIPs) [13].

3.2 General reactants for MIPs synthesis

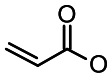
The synthesis of MIPs involves incorporation of a template molecule in a polymer matrix during polymerization. In which the template interacts with functional monomers and cross-linkers, all dissolved in a suitable solvent in the presence of an initiator. The polymerization process is usually activated by heat (thermal polymerization) or activated by ultraviolet light (photochemical polymerization). Primary interactions between matter and light are always non-thermal. Thermal polymerization on the other hand is always associated with thermally labile templates whilst the photochemical polymerization is associated with optically active templates [127] that are sensitive to heat.

3.2.1 Templates, functional monomers and their interactions

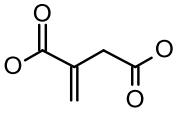
Template molecules in imprinting are usually the target analytes. The structure and the chemical moieties of the template molecules define the subsequent properties of the binding sites. On the other hand, functional monomers are the reactant molecules that provide functional groups that interact with the template to form a template-functional monomer abduct (complex) see Figure 3.1. The choice of functional monomers is dependent on matching the groups on the template with those on the monomers. The ideal condition in preparation of MIPs is that there must be some level of interaction between the functional monomer and the template; either via non-covalent or covalent bonding [115]. The MIPs performance is a factor of these interactions as well as selectivity and binding capacity with the analyte of interest [106]. Favorable interactions together with favorable template-monomer ratio produce better molecular recognition and selectivity of the prepared MIPs. Polymers with both excessive binding sites and steric mismatching binding sites commonly arise from an excess of functional monomers in template-monomer equilibria. These binding sites result in highly non-specific adsorption [128]. In the same way, insufficient monomer leads to a polymer sorbent with a deficient degree of self-assembly, thereby resulting in low selectivity [128]. Therefore, it is very important to have an ideal balance in the template-monomer equilibria for the success of MIPs in molecular recognition [129]. In order to select the best functional monomer(s), computer simulation method known as MIP dialing is commonly

employed [102]. Density functional theory (DFT) is one example of computer simulation methods, which uses Gaussian 03 software [130] to calculate binding energies of both the monomer and the template making available the best template-monomer interaction choice. One other approach that is usually employed for the selection of an ideal functional monomer is the trial and error method. In the trial and error method, deep knowledge of synthesis chemistry is employed in matching the template to the functional monomers. Consequently, the method has major drawbacks of high time consumption in matching the reactants as well as waste of reactant materials. Thus, computer simulation is preferred since the reactants can be matched up-to a certain degree of certainty, reproducibility and the method is relatively faster thus reducing the challenges experienced using the trial and error method [118]. Once the template and monomer have been matched, their interactions are optimized and characterized employing several techniques that include; fourier-transform infrared spectroscopy (FTIR), ultraviolet–visible spectroscopy (UV/VIS) and nuclear magnetic resonance spectroscopy (NMR). Generally, the template-monomer mole ratio that yield good recognition sites have been found to be 1: ≥4 [131]. Some examples of common functional monomers employed in molecular imprinting are shown in Figure 3.2.

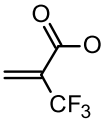
Acidic functional monomers



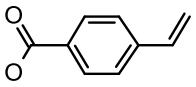
acrylic acid



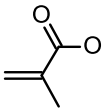
itaconic acid



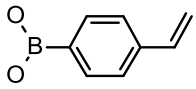
2-(trifluoromethyl)acrylic acid



p-vinylbenzoic acid

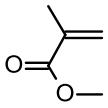


methacrylic acid

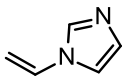


4-vinylbenzene boronic acid

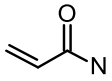
Neutral functional monomers



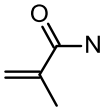
methyl methacrylate



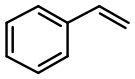
1-vinylimidazole



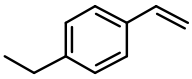
acrylamide



methacrylamide



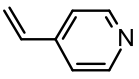
styrene



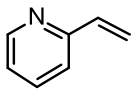
1-ethyl-4-vinylbenzene

acrylonitrile

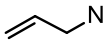
Basic functional monomers



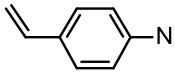
4-vinylpyridine



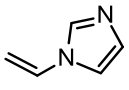
2-vinylpyridine



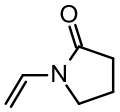
prop-2-en-1-amine



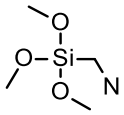
4-vinylaniline



1-vinylimidazole



N-vinyl-2-pyrrolidone



(trimethoxysilyl)methanamine

Figure 3. 2: Examples of functional monomers for molecular imprinting.

3.2.2 Cross-linkers

Cross-linkers are the second monomers that complement the functional monomers by successfully fixing the functional monomers around the template. Therefore, the choice of an appropriate cross-linker is as equally crucial as that of choosing the functional monomer. Cross-linkers are responsible for mechanical stability, control of morphology build-up as well as the general highly cross-linked three dimensional geometry of MIPs [102]. Nonetheless, when in excess, the cross-linker impedes the binding sites due to the difficulty in completely removing the template thereby resulting in MIPs with low binding capacity and poor selectivity [12]. The most commonly used cross-linker have been (EGDMA) ethylene glycol dimethacrylate and (TRIM) trimethalolpropane trimethacrylate due to their ease of availability and compatibility with a wide variety of functional monomers. However TRIM gives more rigid binding sites that are more effective compared to EGDMA [132]. In bulk polymerization EGDMA is commonly applied, while TRIM is employed in precipitation polymerization [97], [133]. From literature template-crosslinker ratio must be 1: ≥10 [131]. Below are examples of the most common cross-linkers.

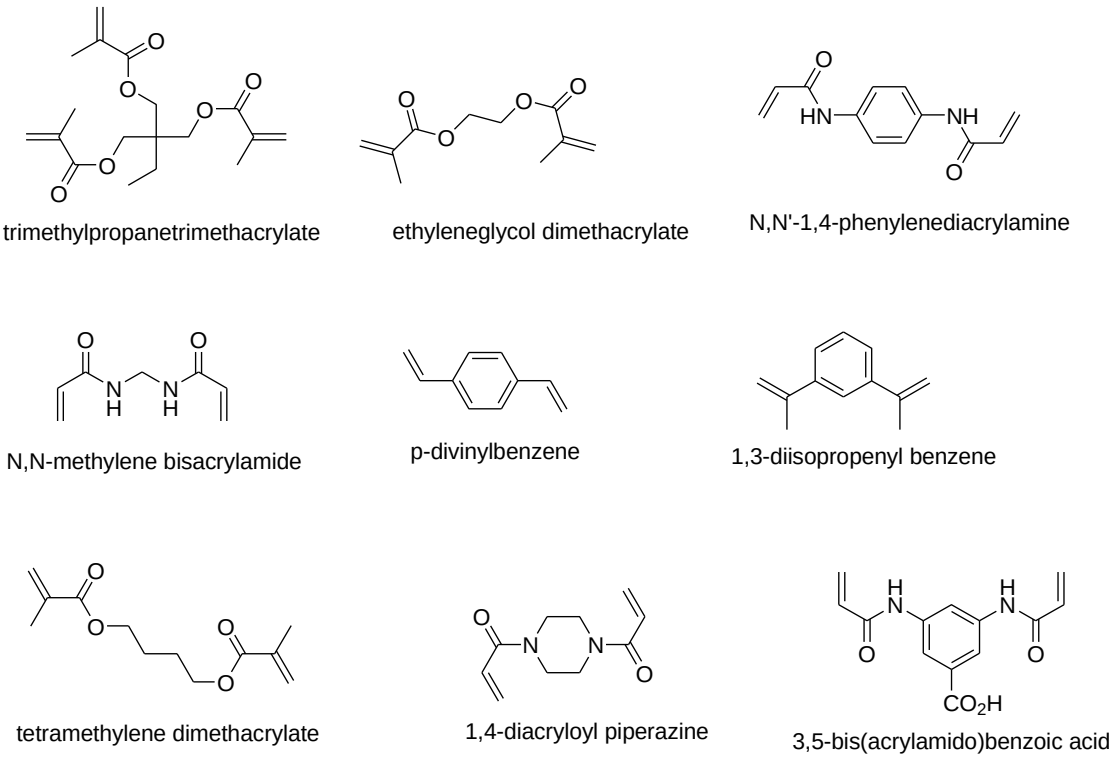


Figure 3. 3: Examples of cross-linking monomers for molecular imprinting.

3.2.3 Porogen (solvent)

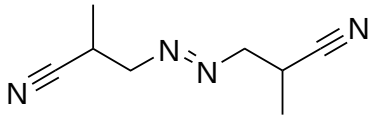
Porogens are referred to as all solvents and/or mixtures of solvents that are used for dissolution of all reactants employed during the imprinting process. The role of a porogen in these polymeric interactions is as equally important since it plays a part in defining the MIP porous structure, morphology and the surface structure. It is important that the monomers, template and initiator be soluble in the porogen. However, the porogen should have minimal interactions with template and monomers to ensure that the strength of pre-polymerization complex is not weakened [97]. Hence, the use of aprotic solvents is encouraged for non-covalent imprinting approach having monomer-template interactions that involve hydrogen bonding [125]. Table 3.1 lists some common solvents employed in MIP synthesis.

Table 3. 1: Common porogens [134].

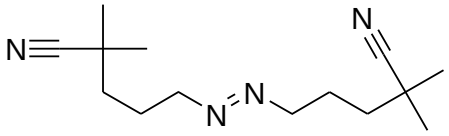
Solvent	**Boiling point (°C)**	**Density (g mL^{-1})**	**Polarity index**	**Dielectric constant***	**H-bond strength**	**Mw (g mol^{-1})**
Acetone	56.2	0.786	5.1	20.7(25)	7.0	58.08
Acetonitrile	81.6	0.786	5.8	37.5	6.1	41.05
Carbon tetrachloride	76.7	1.587	1.6	-	-	153.82
Chloroform	61.7	0.795	4.1	4.81	5.7	119.38
Dichloromethane	39.8	1.326	3.1	9.08	7.1	84.93
Dimethylformamide	189.0	0.944	6.4	36.7	11.3	73.09
DMSO- acetonitrile	-	-	-	-	-	-
Ethanol	78.5	0.789	-	24.6	19.4	46.07
Ethanol-water	-	-	-	-	-	-
Methanol	64.6	0.791	5.1	32.6(25)	22.3	32.04
Methanol-water	-	-	-	-	-	-
2-methoxy ethanol	125.0	0.965	-	16.9	-	76.09
Tetrahydrofuran	66.0	0.886	4.0	7.60	8.0	72.11
Toluene	110.6	0.867	2.4	2.38	2.0	92.14

3.2.4 Initiators

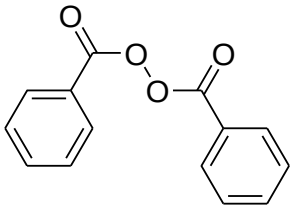
An initiator is defined as any chemical species that reacts with a monomer to form an intermediate compound capable of linking successively with a large number of other monomers to form a polymer. Aliphatic azo-compounds from literature are the most commonly used to polymerize vinyl chloride, methacrylate and other monomers employed in MIP preparation [102], [135], [136]. Thermal and photochemical activation of initiators is employed based on the type of template used as discussed in Section 3.1. Choice of initiator also plays a key role in the morphology of the polymer matrix and the binding capacity of MIPs. Hence, the choice of an initiator should be performed accordingly and the stability of the templates and monomers under such initiation needs to be handled carefully. Below are a few examples of initiators;



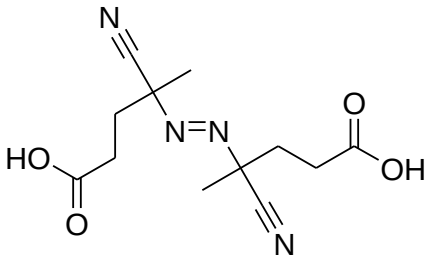
azobisisobutyronitrile (AIBN)



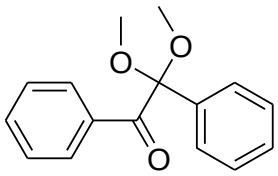
azobisdimethylvaleronitrile



benzoylperoxide



4,4-azo(4-cyanovaleric acid)



2,2-dimethoxy-2-phenylacetophenone (DMPA)

Figure 3. 4: Examples of initiators for molecular imprinting

3.3 Polymerization and fabrication methods for the preparation of MIPs

Various MIPs applications require different shapes (geometry), sizes (magnitude) and texture (morphology) of the polymer material for the best performance. Therefore, it is imperative to produce MIPs with functional and befitting geometry, magnitude and morphology for the intended application. Shape, size and texture of MIPs are dependent on the type of polymerization method employed to prepare them [105], [137], [138]. In some cases, size, shape and texture of MIPs can be designed through different molds with various artifacts such as grooves or indents to suit a particular application. Molding or achieving different shapes, sizes and texture other than through polymerization is referred to as fabrication. This section discusses the various polymerization methods that have been employed in the preparation of MIPs as well as one of the current fabrication methods that is currently gaining popularity since it presents an opportunity for producing nano-fiber based MIPs with superior sensitivity and selectivity [139].

3.3.1 Bulk polymerization

Bulk polymerization is the most popular method in MIP preparation owing to its simplicity [140]. The polymerization process is simply carried out in one reaction vessel in which all reactants; template, functional monomer, initiator and cross-linker are dissolved in an appropriate solvent. The solution mixture in the reaction vessel is cooled and purged with nitrogen gas to remove reactive dissolved air and provide an inert environment. Bulk polymerization usually follows free radical polymerization and is thermally or photochemically activated [141]. The resultant product of this polymerization is a monolith that precipitates out of the bulk reactants. The monolith is ground to provide particles of smaller sizes and sieved for homogeneity. Bulk polymerization has drawbacks such as; irregular size particles that arise from grinding and are irreproducible, grinding may also destroy some high affinity binding sites which results in formation of low affinity binding sites, low yield of polymer sorbent due to wastage during sieving and difficulty in accessing binding sites which results from the bulkiness of the particle materials produced [142], [143].

These drawbacks related to bulk polymerization have led to the birth of better alternative polymerization methods some of which result in the elimination of crushing, grinding and sieving thereby achieving polymer materials of smaller and uniform sizes. Furthermore, the alternative

methods achieve better yield since there is no sieving as well as improved accessibility to reach binding sites during rebinding experiments since the resultant are of smaller sizes. These methods include: suspension polymerization [144], emulsion polymerization [145], multi-step swelling polymerization [146], precipitation polymerization [147] and core-shell surface imprinting [148].

3.3.2 Suspension polymerization

Suspension polymerization is a type of heterogeneous polymerization that employs two immiscible phases having the same kinetic conditions as in bulk polymerization. These two phases include an organic phase with dispersion abilities and a continuous aqueous phase. Water is commonly used as the dispersing medium, however perfluorocarbon liquid and liquid paraffin can also be employed [149]. During synthesis, the organic phase containing all the reactants is dispersed into the aqueous phase as liquid droplets, each of which behaves in the same manner as a chemical reactor in which an independent polymerization reaction is kick started in the presence of a soluble initiator resulting in solid polymer particles in the form of beads. The size of the polymer beads is controlled by using stabilizers such as poly vinyl-alcohol or salts such as calcium and magnesium carbonates which are responsible for improving the dispersion viscosity in the aqueous phase. Water as a dispersing solvent has a continuous phase which suspends a droplet of pre-polymerization mixtures in the presence of a stabilizer or surfactants [144], [150]. The use of water in this technique as a dispersing solvent has been reported to serve as a drawback, since water interrupts template-monomer bonding especially if the non-covalent approach is employed [138], [151]. Therefore, the use of perfluorocarbon liquid as a dispersing solvent yields MIPs with good recognition and chromatographic properties. Perfluorocarbon liquids have an advantage of immiscibility with most organic solvents and also they are chemically inert hence, no interference with non-covalent interactions during imprinting, which results in specific recognition sites [145]. However, the use of perfluorocarbon also requires per-fluoro polymeric surfactant which interferes with monomer-template interactions in a similar way stabilizers cause interference within conventional suspension polymerization [152]. The use of mineral oil as investigated by Kempe and Kempe is also an effective alternative for water interference. However, the downfall of this method is that some porogenic solvents are immiscible with the mineral oil [153], [154]. In an interesting study, performed by Mayes A. *et al.*, it was reported that MIPs prepared using

suspension polymerization demonstrated lower retention capacity and selectivity compared to MIPs prepared using bulk polymerization [152].

3.3.3 Precipitation polymerization

Precipitation and bulk polymerization utilizes similar pre-polymerization mixtures, however, precipitation employs a higher quantity of solvent, approximately 10 times the volume of solvent used in bulk polymerization, resulting in a diluted system [147]. The distinguishing feature between the two polymerization methods is that when the growing polymeric chains reach a critical mass, they precipitate forming microspheres that are characteristically 10 µm diameter for precipitation polymerization while in bulk polymerization a monolith is formed. The resultant microspheres immediately go through template removal since they do not need to go through grinding and sieving as in the case of bulk polymerization. Hence, this process is made simpler since no sieving and grinding procedure is required prior to template removal [155], [156]. Precipitation polymerization does not involve the use of surfactants and/or stabilizers, which is another advantage of this polymerization process as particles of controlled size and size distribution are produced [157], [124], [131]. One notable drawback is that precipitation polymerization is not as robust as bulk polymerization, reason being that the 'imprintability' factor which also means the possibility to imprint a template remains a major concern. This is because the presence of template may either increase particle size or reduce the polymer yield [158], [156]. Nonetheless, precipitation polymerization is considered to be a simple and better method as it uses no stabilizers and surfactants which may contaminate the final product.

3.3.4 Emulsion polymerization

This method requires an oil-soluble functional host, emulsion stabilizer, water-soluble template and a polymer matrix. Self-assembly of molecules occurs at oil-water interface resulting in recognition sites on the polymer surface. In this case, emulsion polymerization using oil-water medium produces microsphere particles. Similar to precipitation and suspension, emulsion polymerization produces particles with monodispersed particle size range [103]. However, it also suffers from interference resulting from the added surfactants [159]. Emulsion polymerization

method is more suitable for imprinting higher molecular weight compounds, although it can also be employed to imprint small analytes such as sugars and peptides [159]. Although not often, emulsion polymerization may be employed especially when surface imprinting proteins [116]. In general, two approaches are employed for templating proteins, the first approach is based on correct placement of functional groups that can form strong interactions with the template [159]. In this approach metal-chelating and electrostatic interactions help in template recognition. The second approach mainly deals with the MIP ability to recognize a template using both the shape complementarity and multipoint weak interactions from the monomers able to form hydrogen bonds or hydrophobic interactions [159]. The challenge in imprinting of large templates stems from the restriction in movement of the molecules within highly crosslinked polymer networks and the poor efficiency and reversibility in binding. Nonetheless, the method has been employed to imprint different target analytes successfully [138], [145], [151], [159].

3.3.5 Multi-step swelling polymerization

Multi-step, also known as two-step swelling polymerization [160] is a method that was developed to control the size distribution and the geometry of the MIP particles more efficiently. The method involves two steps; the initial synthesis of uniform polystyrene beads (seeds or 'shape templates') by emulsion procedure in the presence of an emulsion activating solvent (for example, dibutyl phthalate), usually containing an appropriate stabilizer (for example, polyvinyl alcohol) and an initiator. The resultant beads are employed for the subsequent swelling steps. For the second emulsion polymerization step, the polymer beads will be equilibrated with a solution containing polymeric reactants until the droplets of polymerization mixture are adsorbed on the swollen particles [146], [161]. The polymer beads are then washed with a range of organic solvents. The size of the original seeds increases considerably although the shape is maintained. To control the final bead size, the amount of activating solvent and the volume of the dispersion phases are varied. Masci *et al.,* prepared and compared two imprinted polymers, MAA and acrylamide-based clenbuterol-imprinted microbeads employing two-step swelling and polymerization method and successfully used them for the chromatographic separation of clenbuterol from other β-adrenergic molecules [162], [163]. Though the baseline separation can be achieved, the clenbuterol peak is observed to be very dispersed in the MAA based MIP. On the other hand, for the acrylamide based

MIP, the peak becomes less broadened at optimum eluent conditions. The results implied that the chromatographic performance was dependent on the recipe of cross-linker and monomer, and the eluent composition but not the MIP format [162]. Multi-swell polymerization is more advantageous as the size and the number of polymer particles can be controlled, however, the method is elaborate and time consuming since it involves multi-swelling and polymerization steps [121]. Additionally, the presence of water which is employed as a continuous phase inhibits non-covalent interactions between the functional monomer and the template [164].

3.3.6 Core-shell surface imprinting

The core-shell surface imprinting method represents a rather new trend that produces polymer materials with imprinting sites located on the surface of the shells or cores of nanoparticles [148], [165]. The preparation of core-shell MIPs is a complex polymerization reaction which employs other polymerization methods such as precipitation or emulsion methods [166]. The involvement of nano/micro-structures as functional core for surface imprinting has appeared as an advantageous protocol for fabricating MIPs with diverse functionalities. The core materials are supports of MIPs and are normally synthesized according to the characteristics of the functional monomers and template. There are few main categories of cores employed namely; magnetic nano- particles, silica, nanoclusters, quantum dots and up-conventional materials [166], [167]. The core-shell MIPs have been successfully employed as SPE sorbents as well as sensors [166], [167].

3.3.7 Electrospinning

Electrospinning is a technique employed to fabricate continuous fibres with diameters from between 10 nm to some micrometers. This is achieved by subjecting a spinnable polymer solution or melt to a high voltage [168], [169]. Usually, the polymer solution or melt is allowed to flow through a capillary tube with a conducting tip (the spinneret) until the surface tension of an about to be ejected drop of the solution or melt at the tip of the capillary is overcome and stretched into fibres by an induced surface electrical charge that pulls and stretches a generated taylor cone polymer jet formed at the spinneret to the second oppositely charged terminal referred to as the collector.

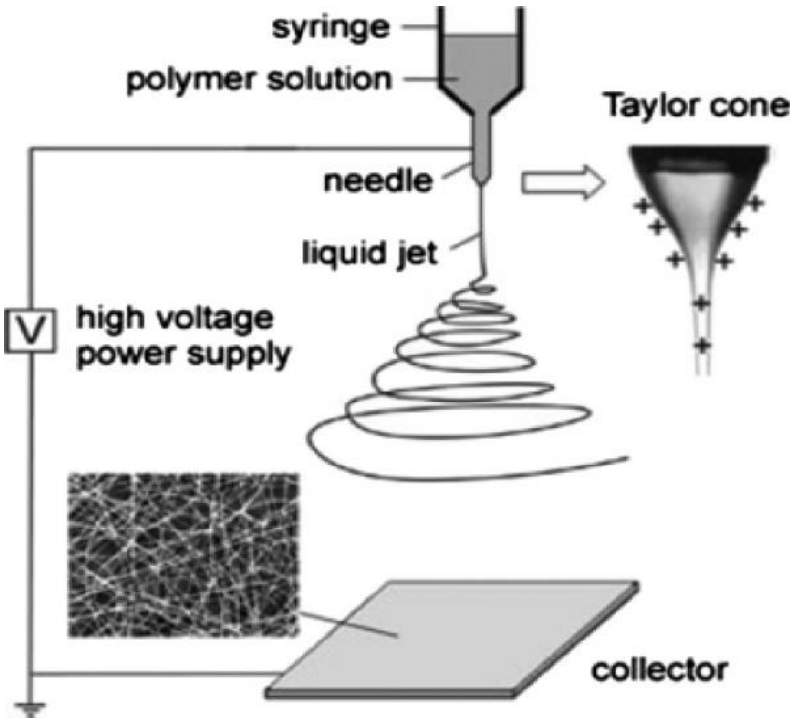


Figure 3. 5: Typical electrospinning setup [168]

Optimizing solution parameters (concentration, molecular weight and viscosity), ambient parameters (temperature, humidity) and processing parameters (voltage, collectors and the distance between the tip and the collector), result in fibers of different morphology and sizes including nanofibers [169]. Nanofibers are sensitive materials owing to their one dimension with high surface area to volume ratio. It is these traits and their ability to be fabricated into fiber mats that have led to their popularity and application in various fields of science such as conducting nanofiber mats (optical sensor, electrical circuit, displays and lighting device) developments [139], tissue engineering [170], wound dressing [171] and sample preparation especially as fiber based SPE sorbents [172]. Even though fiber based SPE sorbents boast unparalleled sensitivity [172], they are less selective. To deal with this challenge, molecular imprinting technology with its high selectivity has been combined with electrospinning technology with its high sensitivity to produce smart, functional nanofiber materials known as molecularly imprinted electrospun nanofibers [173].

3.4 Characterization of molecularly imprinted polymers

Characterization of the polymeric particles is usually carried out to ensure a good match between the applicability and the features of the synthesized polymer particles. Characterization is also important for monitoring the progress of the imprinting process mainly the template monomer interactions.

3.4.1 Fourier transform infrared spectroscopy (FTIR)

FTIR technique characterizes the absorption of infrared radiation by a sample against a wavelength. This analytical technique is usually used for identification of organic compounds by using infrared absorption bands to help identify molecular components and structures [174]. In MIP synthesis, FTIR is commonly employed to assess the nature of binding sites. Ideally, the presence and/or absence of peaks (functional groups) within the starting materials and synthesized polymer materials is usually revealed by the IR-spectra obtained, giving rise to specific IR-peaks or lack of them due to the existence or inexistence of some functional groups respectively [175], [176]. Secondly, FTIR can be used to assess the nature of recognition sites in molecularly imprinted polymer particles and to examine the extent of complexation between the functional monomer and the template molecule during the imprinting process [177]. Thus, FTIR can be employed to identify unknown samples and determine the consistency or quality of a sample [178]. Zhu *et al.,* demonstrated the use of FTIR as a characterization technique for methanol gas sensor-based molecularly imprinted polymer and they obtained spectra for the synthesized polymer materials which were compared to those of the starting reagents. The spectra confirmed successful MIP formation, and also differentiated between the MIP with and without the template (methanol) as was supported by the presence and absence of some peaks in the spectra of the MIP with and without the methanol template, respectively [179]. Recently vibrational spectroscopy (especially FTIR) on pre-polymerization products has provided additional complementary information to nuclear magnetic resonance (NMR) studies by probing the vibrational signatures of the involved molecules and complexes [180].

3.4.2 Nuclear magnetic resonance (NMR)

As far as the preparation of MIPs is concerned, proton/1H NMR titration experiments facilitate observation of the hydrogen bonding between the carboxylic acid and bases. In molecular imprinting, 1H NMR technique has been introduced to investigate the extent of complex formation in pre-polymerization solutions. Additionally, the technique has been employed as a means of identifying the specific sites in interacting structures that take part in complexation. Hence, evaluating the shift of a proton signal due to participation in hydrogen bond is employed as the selection criterion for complex formation.

Lachlan J. Schwarz *et al.,* employed NMR technique for the selective recognition of the bioactive polyphone, (E)-resveratrol MIPs [181]. This is a representative of the general approach that has been deployed for these 1H NMR spectroscopy titration experiments. 0.1 mmol of (E)-Resveratrol dissolved in trideuteroacetonitrile was titrated with increasing molar equivalents of 4-vinylpyridine. The 1H NMR spectrum was observed after each addition and the change in aromatic –OH shifts followed until the presence of H bonding interactions was demonstrated by the consistent downfield shift of this aromatic–OH signal with increased additions. This process was continued until the aromatic –OH signal was no longer detectable due to peak broadening.

3.4.3 Scanning electron microscopy (SEM)

SEM is a technique commonly employed to examine the texture, size, structure and surface morphology of the MIP particles [182], [183], [184]. In addition, some other microscopy techniques such as atomic force microscopy (AFM) and transmission electron microscopy (TEM) may be used in tandem with SEM to provide information on surface topography, crystalline structure, chemical composition and electrical behaviour of the top 1 μm of the specimen respectively [150], [185], [129], [186].

3.4.4 Brunauer, Emmett and Teller (BET)

BET method is employed on a solid sample to determine the specific surface area and the pore size distribution. The obtained measurement data is used to predict the dissolution rate, as this rate is

proportional to the specific surface area [187]. The specific surface area of a powder is determined by physical adsorption of a gas at a given pressure on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface area of the test powder. The determination is usually carried out at the temperature of liquid nitrogen although any other gas may be employed, provided it is physically adsorbed by weak bonds on the solid surface and can be desorbed by a pressure decrease at the same temperature. The number of active sites are dependent on the surface area. Additionally, the obtained surface area is useful in evaluation of product performance and product development [187].

3.4.5 Chemical Characterization

Chemical characterization is key for assessing the binding and selective capability of the recognition sites within the prepared polymers. This is achieved through rebinding experiments, either by batch or column SPE [104], [113].

3.4.5.1 Characterization through binding experiments

The first insight into binding properties of the synthesized MIPs is provided by the rebinding studies. In particular, it has been instructive to compare the binding properties of imprinted and non-imprinted polymers (the control polymers) [188], [189].

In a typical re-binding procedure a known volume and concentration of the target molecule is introduced to a vial with a given quantity of MIP and shaken for a known period of time until an equilibrium is reached. Once the system has come to equilibrium, the mixture is centrifuged /filtered to separate the two phase concentration of the target molecules in the supernatant/filtrate and target molecules bound to the MIP are determined by an analytical instrument. Thereafter, the percentage of the target molecules adsorbed (bound) by the MIP sorbent usually referred to as percentage extraction efficiency (% EEs) is then calculated employing equation 3.1:

$$\%\ \text{Extraction efficiency (EEs)} = \frac{[\text{Ci} - \text{Cf}]\times 100}{Ci} \qquad [3.1]$$

Where $\mathbf{C_i}$ is the initial concentration of the targeted analyte before employing MIP/NIP, $\mathbf{C_f}$ is the final concentration of the targeted analyte found in the supernatant/filtrate after employing MIP/NIP.

The same experiments are usually performed using the non-imprinted polymer (NIP) for comparison and control purposes. The NIPs possess no recognition sites as no template molecules are included during their synthesis. Consequently, MIPs are always expected to have higher percentage extraction efficiencies for targeted analytes than NIPs since MIPs possess highly selective recognition sites while NIPs do not.

3.4.5.2 Selectivity studies

The selectivity of a MIP results from the presence of recognition sites designed for a template. It is studied by comparing the extraction performance of the MIP/NIP sorbent material in adsorbing the template molecules relative to adsorbing ability of competitive species. During selectivity studies an optimal quantity of MIP is added to a solution of known volume and concentration containing the target analyte and the competing species (analogue). The solution mixture is then equilibrated for an optimal period of time. Once the system has equilibrated, the quantity of target analyte and analogue remaining in solution (after adsorption by the MIP/NIP sorbent) is determined by a suitable analytical instrument and the EEs for both the target and the competing molecules calculated employing equation 3.1.

The selectivity towards the target molecules versus the analogue molecules is then evaluated by calculating a parameter known as the selectivity factor. Selectivity factor is a measure of affinity of the imprinted polymer towards a particular target analyte relative to the affinity of any competing species calculated via equation 3.2:

$$\text{Selectivity factor} = \frac{EE\ of\ target\ analyte}{EE\ of\ competing\ species} \qquad [3.2]$$

When applied to MIPs, the selectivity factor indicates how many times better the MIPs binds the target molecules relative to binding ability towards the analogue molecules. A positive outcome

(>1) of the selectivity factor indicates imprinted polymers exhibiting good selectivity towards the template/target analytes.

Other than re-binding and selectivity experiments, other analytical tools based on graphical methods have been employed to provide accurate description on binding behaviour of MIPs, especially those of non-covalent character. Generally, the methods enable MIP researchers to understand the distribution of the binding sites within the polymer matrix. The polymer matrix are then classified as homogenous or heterogeneous. Generally, MIPs have been characterized by a high degree of varying binding sites (heterogeneous) when compared to the homogeneous ones found in natural systems [151]. Graphical methods such as scatchard plots have been employed to fit binding isotherms to models such as that of Langmuir [190], Freundlich (FI) and Langmuir-Freundlich (L-FI) combined [191] to assist in describing the binding sites of any prepared MIP polymer as homogenous or heterogeneous [97], [194], [195].

3.5 Challenges of molecularly imprinted polymers

MIPs have had some very interesting advantages which have led to increased research in recent years, however they have some challenges.

3.5.1 Template bleeding

This still remains the biggest challenge in MIP synthesis [137]. Template bleeding simply means incomplete template removal. It mainly arises from MIP synthesis where some template molecules might be fused deeper in the polymer matrix which makes it very cumbersome for the desorbing solvent to gain access to them [137]. Hence, the resulting imprinted polymer has some template remains, which might lead to unavoidable template leaking that may influence the accuracy of quantification and identification of target analytes [194]. Researchers have proposed a strategy to avoid template bleeding by use of ‘dummy’ template, structural analogue of target analyte itself, during MIPs preparation [195]. In the event that the ‘dummy’ template bleeds out the analysis will still be effective since there will be no interference in the rebinding of target analyte(s) since it was not added during the polymer fabrication. Other post polymerization treatment methods, for

example, supercritical fluid extraction, microwave assisted extraction, thermal annealing and parallel extraction using blank samples have been employ to reduce template bleeding [137]. However these techniques have less effect compared to 'dummy' template method [195]. The 'dummy' template approach is more appropriate in instances such as; an analyte is too expensive, toxic analyte that involves handling safety consideration or an analyte that is susceptible to degradation [14], [107], [196]. The search for molecules to be used as dummy templates is not a straight forward one. Therefore more work has to be done to ameliorate template leaking. Moreover, surface imprinting has also received a lot of interest as a technique employed to ease the subsequent template removal due to the imprinting sites positions which are believed to be on the surface or approximately near the surface of the prepared polymer [13], [102]. On the other hand, semi-covalent imprinting is another method of interest believed to limit template leakage. The reason being that the template is covalently bound to the polymer and the conditions employed for template removal are less harsh than those employed for polymer formation. This means that during rebinding, the small amounts of template left in the sorbent will remain there and not interfere with the quantity of target analyte adsorbed [124], [125].

3.5.2 Incompatibility with aqueous conditions

The use of polar protic solvents for example water in MIP preparation has remained a challenge especially when using the non-covalent imprinting method. The physical interaction of template and monomer in non-covalent imprinting may be interrupted by water especially if the interactions are based on hydrogen bonding [99]. The property of water acting as both hydrogen donor and acceptor disrupts the recognition sites that are based on hydrogen bonding. Additionally, water favors hydrophobic bonding which results in non-specific binding sites. However, water has less influence on electrostatic interactions [99]. MIPs having high recognition for organic analytes pose a challenge during preparation in aqueous solutions, however, ionic interactions do not experience this limitation [123], [137], [196]. This led to the conclusion that incompatibility with aqueous conditions it is a template specific limitation. Therefore templates that possess ionic interactions are compatible with aqueous environment and the resultant binding sites have high recognition [97].

3.5.3 Heterogeneous binding sites

The formation of heterogeneous binding sites may arise from non-covalent imprinting since reactions/ interactions are usually not well controlled in pre-polymerization step [115]. A monomer and template form a number of interactions having different attachment ratios in this step. To ensure the template is in a fixed position, the ideal situation would be to form as many template-monomer interactions to limit unspecific rebinding. An excess of either template or monomer may result in more heterogeneous binding sites [191]. In this case, semi-covalent imprinting approach may be employed to solve the challenge of heterogeneous binding sites. The imprinting approach allows for the template to covalently bond to the monomer, hence well-defined binding cavities are formed. Moreover, there is lower kinetic restriction during rebinding of template due to the non-covalent bonding which occurs after template removal [115]. Stoichiometric non-covalent imprinting and selective modification of low affinity sites are also additional methods employed to solve the challenge of heterogeneous binding sites [137].

Chapter 4: Methodology

4.0 Materials and chemicals employed

Tetrahydrofuran (THF), dimethyl sulfoxide (D_6-DMSO) were purchased from AppliChem (Darmstadt, Germany), methanol (99%) purchased from Skylabs (Johannesburg, South Africa), acrylamide (99%), 4,4'-azobis(4-cyano pentanoyl chloride) (ABCC), (98%), ethylene glycol dimethacrylate EGDMA (99%), D-fructose, D-glucose, maltose, lactose monohydrate and 0.45 µm pore sized ashless whatman filter papers were purchased from Sigma-Aldrich (Johannesburg South Africa). Ultra-pure water, was prepared by a Millipore-Q purification system from Merck, (Darmstadt, Germany).

4.1 Instruments

High performance liquid chromatography – refractive index detector (HPLC-RID), Agilent 1200 infinite series (LA, California, USA) was employed to determine the concentrations of the sugars. A sacanning electron microscope (SEM) JSM-7100F purchased from JEOL (UK) Ltd (Welwyn Garden City, Hertfordshire) was used to obtain high resolved images of the synthesized polymers and assess their structural morphology. Thermo Scientific laboratory oven (TTM-J4) was purchased from Thermo Fisher Scientific Inc. (New York, USA), Benchmark hot plate was purchased from Benchmark Scientific (New Jersey, USA), Boeco GP Series micro-pipettes was purchased from BOECO (Berlin, Germany), a Tyler analytical balance from Mettler Toledo, A W.S Tyler^{TM} (Johannesburg, South Africa), and a Laval stainless steel sieve (45-200 µm) was purchased from Laval LAB (Minnesota, USA). Centrifuge *VWR* (24/16) was purchased from VWR Catalyst (Philadelphia, PA, USA).

4.2 HPLC-RID operating conditions

The concentrations of fructose, glucose, lactose and maltose were determined using HPLC-RID. Agilent Hi-plex Ca, 7.7 × 300 mm, 8 µm column was employed for isocratic separation using 100% DI water as the mobile phase, and a flow rate of 0.5 mL/min. Column oven temperature of 80 ºC was maintained. The injection volume was 20 µL.

4.3 Preparation of fructose and glucose imprinted polymer

In a nitrogen purged reaction vessel, 0.736 mmol of fructose and glucose, 10 mmol of acrylammide, 41.2 mmol of EGDMA and 0.53 mmol of ABCC, were added to a mixture of THF (1.68 mL) and DMSO (21.02 mL). The mixture was stirred at 900 rpm for 45 min. The reaction vessel was heated at 50 ºC and polymerization proceeded for 48 h under continuous stirring. The resulting polymer monolith was finely ground and dried in an oven at 50 ºC for 4 h. The resultant polymer powder was then washed twice employing ultra-pure water to remove unreacted materials and dried at 50 ºC. The powder was sieved to obtain homogeneous particles of <50 µm. The templates (fructose and glucose) were exhaustively removed from the imprinted polymer by washing severally in 99% methanol. After every 5 h washing cycle, the MIP powder was separated from the used methanol by filtration and fresh methanol was then added to further remove the templates. After every cycle, the concentrations of fructose and glucose in the filtrates were determined employing HPLC-RID until a point where the concentrations remained the same, thus marking optimal template removal. The resultant MIP powder particles were then recovered through filtration and then dried at 50 ºC. The voltage values from the HPLC-RID that correlated to concentrations were then plotted against the number of washing cycles. A control polymer, non-imprinted polymer (NIPs) was also prepared employing a similar procedure except that the template molecules were absent during preparation. The resultant NIP powder was then subjected to all procedures that were performed on the MIP powder.

4.4 Characterization of G-F MIP and NIP powders

4.4.1 SEM Characterization

The size homogeneity of the polymer particles was first achieved through the use of standard sieves. SEM was employed to further characterize size, geometry and surface morphology of the MIPs and NIPs. The powders were carbon-coated under a polaron range high vacuum pressure sputter coater and placed on a 1 cm tall sample holder. The operation was performed under high vacuum and beam acceleration voltage of 20 kV.

4.5 Batch rebinding experiments

To evaluate the binding capability of the prepared MIP, batch rebinding experiments were performed, after which the HPLC-RID was employed to determine the concentrations of glucose and fructose before and after application of the prepared MIPs. The percentage extraction efficiencies of the MIPs were calculated following equation 3.1.

For the pH, the real juice sample pH was obtained as pH 3.5, this pH value was employed in all the analysis. All experiments were performed in triplicates (n = 3). The temperature of the natural fruit juice was employed in the study (24˚C).

4.5.1 Optimization of quantity of MIP powder needed for maximum glucose and fructose extraction

Increasing quantities of MIP powder from 0 mg at intervals of 10 mg were added to 30 mL of 20 mg/L equimolar standard solutions of glucose and fructose. The solution mixtures with different quantities of MIPs were left overnight for equilibration and maximum extraction. The MIP powders were filtered off and the concentrations of the glucose and fructose in the filtrate before and after employing the MIP powders were determined using the HPLC-RID and calculated via equation 3.1. The experiment was repeated with increasing quantities of the MIP powder until a point where the calculated extraction efficiency remained constant with increasing quantity , thus marking the optimal quantity needed for maximum extraction. A plot of % extraction efficiency versus quantity of G-F MIP powder added was constructed.

4.5.2 Optimization of Time needed for maximum fructose and glucose extraction

Using the optimized MIP quantity in (4.5.1), optimal time needed for the MIP to remove maximum glucose and fructose from standard glucose-fructose solutions was investigated. The optimized quantity of the G-F MIP powder was added to various 30 mL of 20 mg/L equimolar concentrations of glucose and fructose standard solutions for an increasing duration at 10 min intervals starting from 0 min. Each mixture was left to equilibrate at room temperature. The experiment was repeated

with increasing time until a point where the calculated % extraction efficiency for the different times remained constant with increased time, thus marking the optimal time needed for maximum extraction. A plot of % extraction efficiency against time needed for maximum extraction was constructed.

4.5.3 Selectivity studies

To evaluate the ability of the prepared MIP powder to favorably extract the target analytes (glucose and fructose), analogue molecules; lactose and maltose were chosen to compete with the target analytes via rebinding experiments described in section 3.4.5.2. An optimal quantity of MIP powder was added to 30 mL of 20 mg/mL equimolar concentrations of glucose, fructose, lactose and maltose and allowed to equilibrate for optimal time. The MIP powder was filtered off and the concentrations of glucose, fructose, maltose and lactose in the filtrate obtained after equilibration under optimal conditions were analysed employing HPLC-RID and thereafter the extraction efficiencies by the prepared MIP for each analyte were calculated following equation 3.1.

Similarly, the same procedure was followed employing the NIP powder that was employed as the control and the extraction efficiencies for each analyte were calculated following equation 3.1.

4.5.4 Method Validation

4.5.4.1 Linearity

Linearity of the method was investigated through triplicate injections of spiked '100%' fruit juice at different glucose and fructose equimolar concentrations ranging from 0 to 50 mg/L. Calibration curves of concentration verses peak areas for glucose and fructose were plotted and the correlation coefficient (R^2) which is a measure of linearity was then obtained from the linear plots of each of the sugars respectively.

4.5.4.2 Detection Limits

The Limits of detection (LODs) and Limits of quantification (LOQs) were determined by employing a method based on the analytical curve parameters according to equations 4.1 and 4.2.

$$LOD = \frac{(\mathrm{SD} \times 3.3)}{\mathrm{m}} \quad [4.1]$$

$$LOQ = \frac{(\mathrm{SD} \times 10)}{\mathrm{m}} \quad [4.2]$$

Where m is the slope and SD is the standard deviation.

4.5.4.3 Application of method to real '100%' apple juice samples

`100%` apple fruit juice samples were diluted with distilled water (dilution factor 500). The samples were then filtered using 0.45 µm pore sized filter paper and the concentrations of glucose and fructose before and after application of G-F MIP in the filtrates were obtained employing HPLC-RID. Chromatograms of glucose and fructose concentration of the filtrates before and after employing the G-F MIP were obtained and the enrichment factor (EF) calculated using equation 4.3:

$$EF = \frac{\mathrm{a}}{b} \quad [4.3]$$

Where:

EF is enrichment factor, *a* is glucose or fructose concentration obtained after filtering and application of G-F MIP and *b* is glucose or fructose concentration obtained after filtering.

Precision was also expressed as a percentage relative standard deviation (% RSD) for n = 6 and was calculated following equation 4.4. Precision may be expressed as repeatability and it is a measure of the degree of conformity between independent measurement results acquired under set conditions.

$$\%\ \mathrm{RSD} = \frac{standard\ deviation}{\mathrm{mean}} \times 100\% \quad [4.4]$$

Chapter 5: Results and Discussion

5.0 Synthesis of Fructose-glucose MIP

It is imperative that the template molecules be removed successfully for optimum performance of MIPs as adsorbents [129]. For this, the MIP was extensively washed until there was no further observable changes in the voltage values (equivalence of the concentrations of the templates) obtained despite further washing with fresh solvent as marked by a plateau from the 5^{th} – 10^{th} wash in Figure 5.1. It was observed that after 5 subsequent interval washes, satisfactory amounts of glucose and fructose molecules were removed from the MIP. The plateau confirmed optimal template removal by the method that was employed. Optimal template removal is necessary as it is the one that frees reaction sites (cavities) for the subsequent rebinding of the target analytes. Furthermore, optimal template removal is vital for the elimination of template bleeding which leads to erroneous results (false positives).

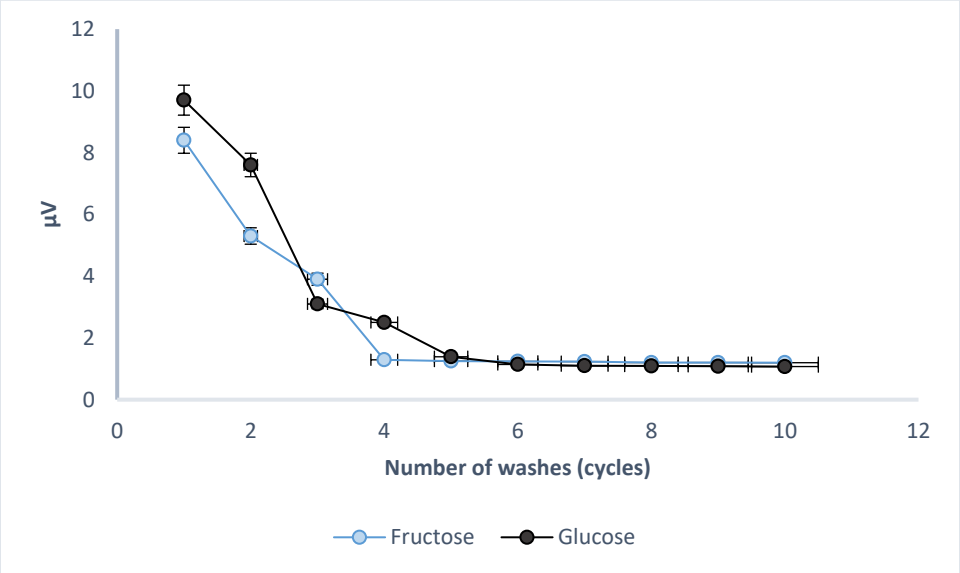


Figure 5. 1: A voltage (concentration) versus number of washes (cycles) plot confirming the removal of glucose and fructose templates from MIPs.

5.1 Morphology of the synthesized polymer particles

SEM micrographs of the MIPs and NIPs obtained did not show notable variations, hence, only the SEM micrograph of the MIPs was reported (see Figure 5.2). Preliminary particle size homogeneity was achieved with the help of standard sieves, allowing only 45 µm MIP particles to pass through the standard sieves for further processing and application. The size of the MIP particles were further estimated to be < 20 µm using SEM, see micrograph in Figure 5.2. This size is small enough to be associated with increased surface area resulting in enhanced sorbent capacity. The smaller the particle size the higher the surface area and sorbent capacity [197]. The image revealed that the MIP particles were somewhat spherical. Mayes *et al.,* and Mosbach *et al.,* reported spherical shape to be a good geometry for sorbent materials [152], [198]. The surface of the particles seemed to be rough and spongy which is an excellent characteristic for adsorption [199].

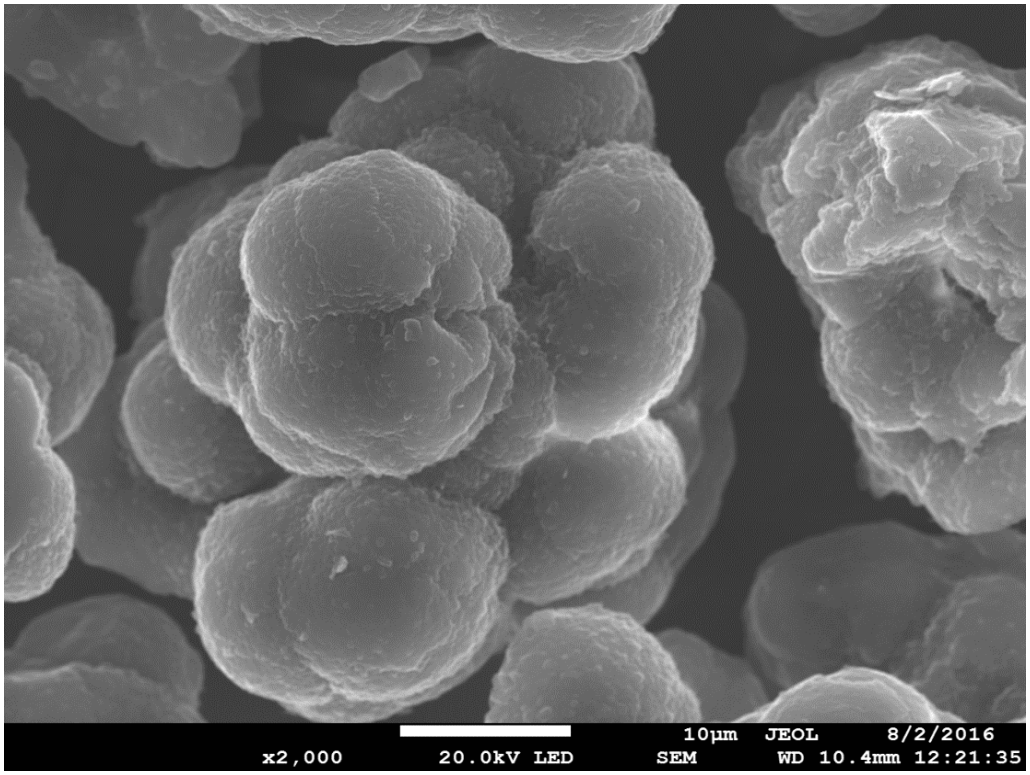


Figure 5. 2: SEM image of MIP particles.

5.2 Batch Rebinding Experiments

5.2.1 Optimization of MIP powder needed for maximum glucose and fructose extraction

Optimization of sorbent mass is important to avoid wasting of sorbent by adding excess unnecessarily or adding low sorbent mass thus compromising the results [14]. For this work, the optimum MIP powder needed for maximum extraction was found to be 60 mg, marked by a point at which the plateau starts to form on the plot of percentage extraction efficiency versus mass of sorbent (see Figure 5.3 below). Increase in sorbent mass from 10 to 60 mg resulted in significant adsorption, which was attributed to the parallel increase in binding sites. However, it was observed that after 60 mg there was no further change in percentage extraction which marked the saturation point of the binding sites. Therefore 60 mg was employed in this study.

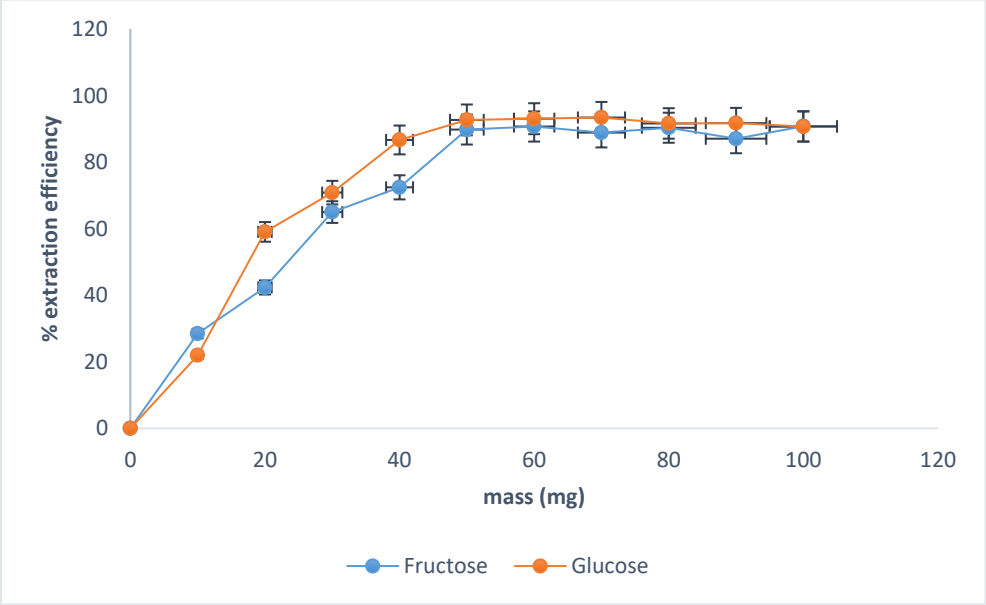


Figure 5. 3: Optimization of the maximum quantity of MIP required for optimal removal efficiency for fructose and glucose

The optimized mass was used to determine adsorption capacity following equation 5.1 below:

$$q = \frac{[\mathrm{Ci}-\mathrm{Cf}]\times V}{\mathrm{W}} \qquad [5.1]$$

Where, q is the adsorption capacity, *Ci* and Cf are the initial and final concentrations respectively, V is the volume of sample employed and W is the mass of the sorbent.

Adsorption capacity for the prepared MIP was obtained as 9.30 mg/g and 9.07 mg/g for glucose and fructose respectively. Parmpi *et a.,l* reported binding capacities of 7.15 mg/g for fructose and glucose. In their study, the authors findings have shown that the proposed MIP gels selectively bound glucose and fructose analytes in a water swollen state [200]. Rajagpal *et al.,* also reported a binding capacity of about 40 nmol/mg of fructose which signified an effective binding pocket [201].

5.2.2 Optimization of time needed for maximum fructose and glucose extraction

Sufficient equilibration time is required for target analytes to rebind to MIP recognition sites. In this thesis, the optimal time required for rebinding was found to be 20 min for both target analytes, fructose and glucose, marked by a point at which the graph plateaued (see Figure 5.4 below). Percentage extraction of the glucose and fructose analytes increased with increase in contact time from 0 min up to 20 min, after which the rate of adsorption was relatively uniform. The linear increase observed from time 0 min to 20 min, resulted from the increase in interaction time between the MIP and the analyte molecules which increased the chances of adsorption [95]. In a study carried out by Rajkumar *et al.,* on fructose recognition by imprinted polymers an optimum time of 2 hours was reported [201]. Indicating that the prepared G-F MIP in this thesis exhibited excellent equilibration time.

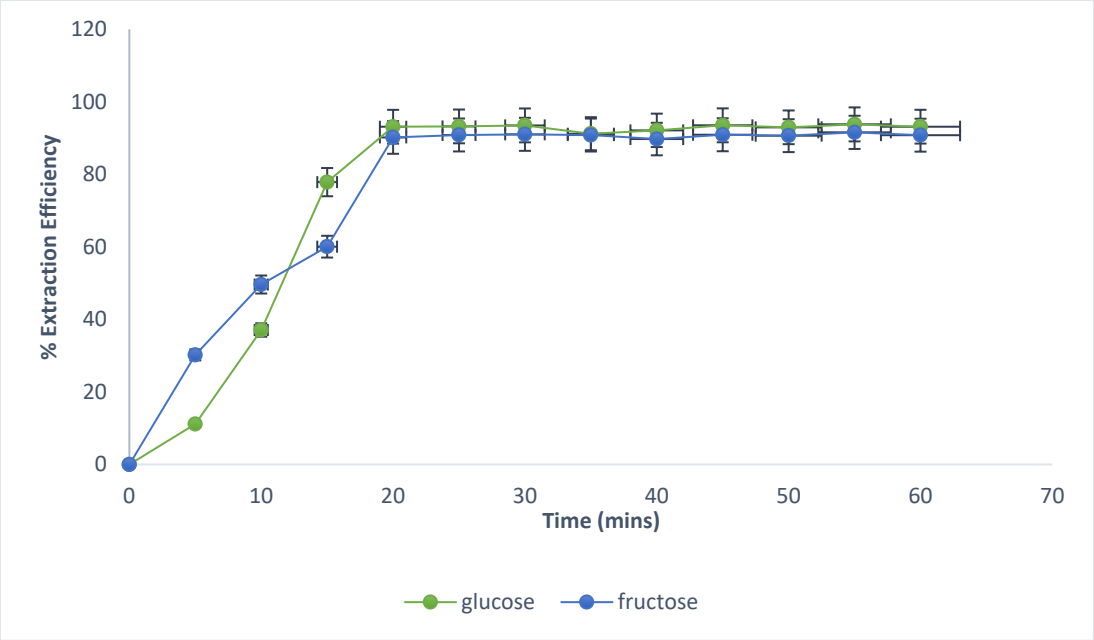


Figure 5. 4: Optimization of time required for maximum removal of fructose and glucose

5.3 Selectivity studies

MIP efficiency cannot solely be evaluated on the ability of the polymer to rebind the analyte(s) [136], but also on its discriminative ability towards target analytes in the presence of analogue molecules [136], [202]. Following Figure 5.5, the percentage extraction efficiencies by the MIP powder for glucose and fructose were observed to be much higher than those of lactose and maltose. These observations were an indication that the binding sites in the MIP had a higher affinity and selectivity for glucose and fructose. In addition, the extraction efficiency of the G-F MIP was observed to be superior in extracting glucose and fructose when compared to the NIP in the same environment. The MIP bound a higher percentage of 92.45% and 93.12% for glucose and fructose respectively compared to the much lower percentages of 23.6% and 31.4% for maltose and lactose respectively. This was attributed to the binding sites that were freed during template removal and left a memory for the target analytes to rebind. The NIP on the other hand showed non selectivity and lower performance than the prepared G-F MIP as demonstrated by statistically the same percentage extraction efficiencies that had a low range of (22.7 - 33.4 %) for both the target and the analogue molecules (see Figure 5.5). The non-selectivity of the NIP is attributed to the lack of inclusion of the templates during the synthesis process.

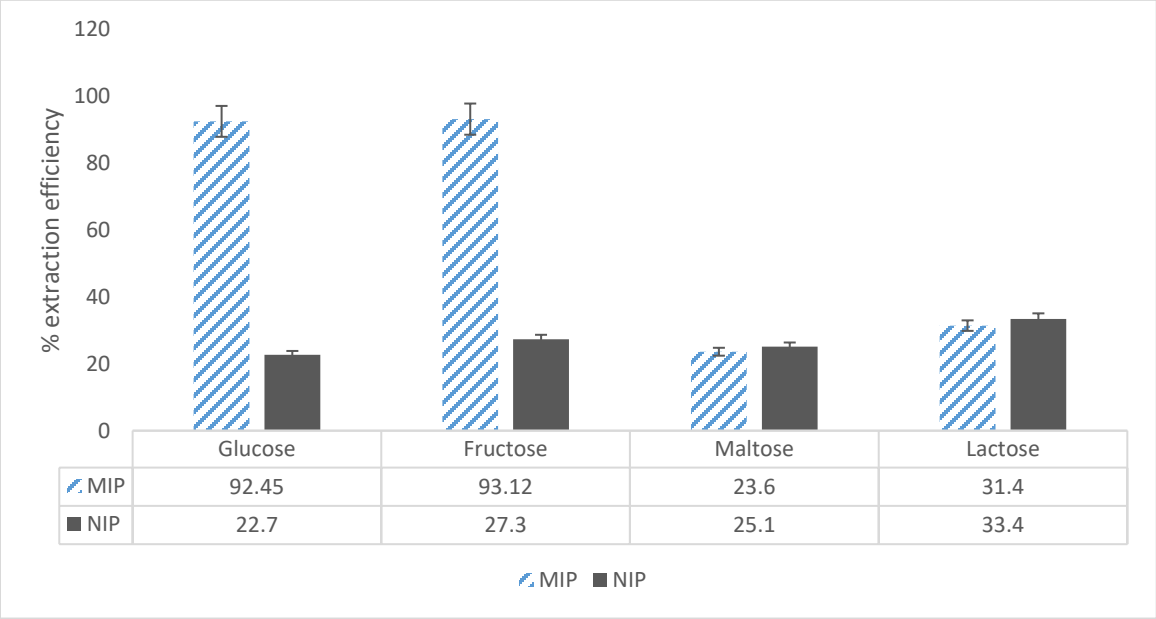


Figure 5. 5: Percentage extraction efficiencies of glucose, fructose, maltose and lactose by the MIP and NIP adsorbents.

Furthermore, the selectivity and affinity towards fructose and glucose was marked by selectivity factors of ≥ 2.9 in all cases;

Glucose/maltose (3.9), glucose/ lactose (2.9), fructose/maltose (3.9), fructose/lactose (3.0)

A positive outcome (>1) of the selectivity factor indicates imprinted polymers exhibiting good selectivity towards the template/target analytes. Thus, the greater the ratio the higher the selectivity of the prepared MIP [203].

5.4 Method validation

5.4.1 Linearity

Table 5.1 summarizes the linearity of the calibration curves for glucose and fructose in the form of correlation coefficients of 0.9959 and 0.9945 for glucose and fructose respectively within the concentration range of 0-50 mg/L. R^2 values > 0.995 are considered to show a good linearity of the data obtained, therefore the obtained correlation coefficient in this study were found to be

statistically acceptable [204]. Thus, the results proved that there was a fairly strong linear correlation between the sample concentrations of glucose and fructose and the corresponding determined chromatographic peak areas or response.

Table 5. 1: Linear regression parameters obtained from standard calibration curve for a 100% apple juice sample spiked with various concentrations of glucose and fructose within 0-50 mg/L

Sugar	Regression Equation	Regression coefficient (r^2)
D-Fructose	y = 6079.4 x - 439.8	0.9945
D-glucose	y = 2233 x -153.8	0.9959

5.4.2 Limits of detection (LODs) and limits of quantification (LOQs)

The calculated LODs and LOQs were obtained and found to have a low range of 3.975 - 7.185 mg/L and 12.04 - 21.77 mg/ L respectively (see Table 5.2). These LODs and LOQs indicated that the method was more sensitive compared to the relatively high LOD and LOQ values of major sugars in apple juice reported by Zielinski *et a.,l.* which ranged from 7.56 - 56.86 mg/L and 25.21-192.88 mg/L respectively for all sugars including glucose and fructose [205]. Other studies on LODs were also reported by other workers employing HPLC. Wanxia *et al.*, analysed monosaccharides and disaccharides by HPLC following SPE, and the LODs of glucose and fructose were 0.04 g/L and 0.16 g/L respectively [74]. Wei and Ding reported LODs between 0.2 and 1.2 µg for different carbohydrates in drinks using HPLC with evaporative light scattering detection (ELSD) [206].

Table 5. 2: Limits of detection and quantification for glucose and fructose

Sugar	LOD mg/L	LOQ mg/L
D-Glucose	7.185 ±0.007	21.77 ±0.007
D-Fructose	3.975 ±0.005	12.04 ±0.004

5.4.3 Application of method to real '100%' apple juice samples

A close study and evaluation of the peak intensities obtained in Figure 5.6, of before and after G-F MIP application to '100%' apple juice samples showed an increase from 8.5 to 15.8 mV for glucose and 15.9 to 31.9 mV for fructose. This marked a 2-fold enrichment factors (EFs) or pre-concentration factors for glucose and fructose. The EFs were calculated following equation 4.3. Relatively high extraction efficiencies of more than 90% and low RSDs of less than 7% for n=6 for both glucose and fructose were recorded and demonstrated fairly good efficiency of the G-F MIP within the concentration range of 5 to 25 mg/L(see Table 5.3)

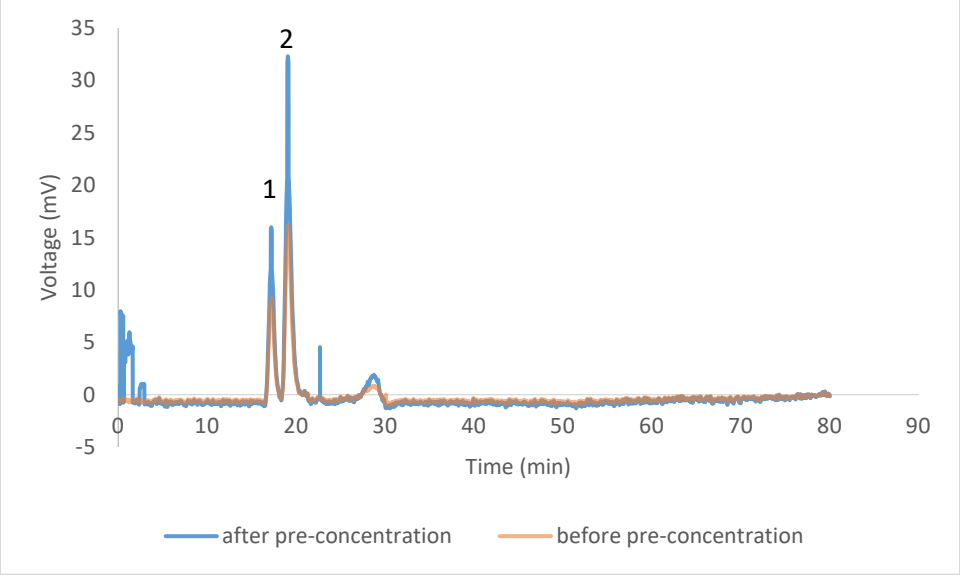


Figure 5.6: Chromatograms of apple juice sample, with (1) glucose and (2) fructose before and after MIP application

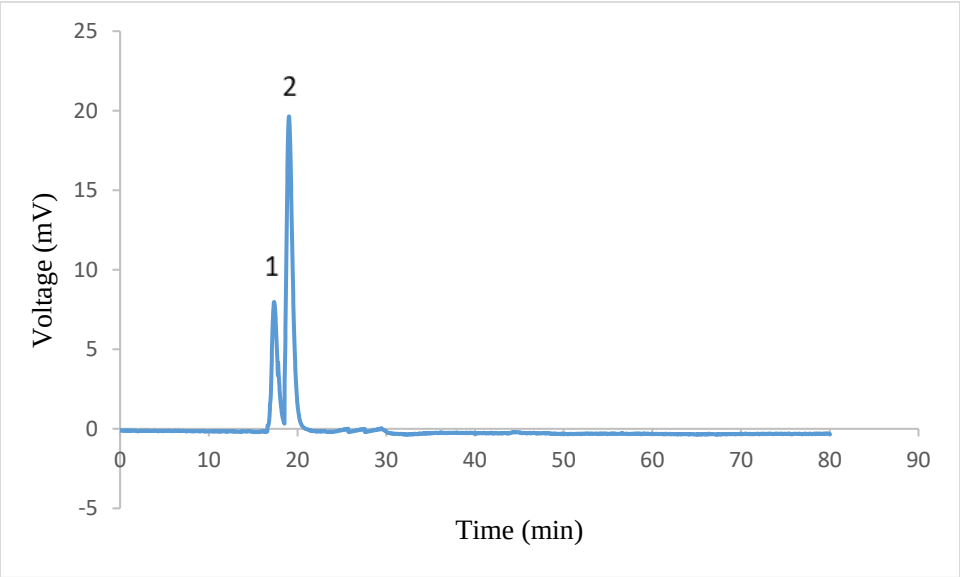


Figure 5.7: Chromatogram of equimolar standard of 5 mg/L glucose (1) and fructose (2) showing their retention times

Table 5. 3: Extraction efficiencies of glucose and fructose with the G-F MIP and associated % RSD calculated at three equimolar concentrations of glucose and fructose

Concentration mg/L	% Extraction Efficiencies (EEs)		% RSD	
	Glucose	Fructose	Glucose	Fructose
5	93.5	91.7	6.13	4.89
15	90.8	92.2	4.78	5.94
25	92.1	92.6	5.97	5.13

Chapter 6: Conclusion and perspective

This thesis presented a successful synthesis of a G-F MIP that exhibited excellent affinity and specificity towards fructose and glucose. The prepared G-F MIP demonstrated success in selectively binding a higher percentage (> 90% EEs) for the analyte molecules (glucose and fructose), than the analogue molecules, maltose and lactose (< 34% EEs) in competitive binding environments. The study presented herein demonstrated that extraction efficiencies of glucose and fructose (> 90% EEs) employing G-F MIP were significantly higher, with low %RSD of > 7%, than those obtained after employing the NIP; the control polymer (< 28% EEs). All binding studies were performed at optimal conditions; 60 mg and 20 min for sorbent dose and reaction time respectively. The developed method was relatively sensitive as indicated by LODs and LOQs found to have a low range of 3.975 – 7.185 mg/L and 12.04 – 21.77 mg/L respectively. Approximately 2-fold enrichment factors were achieved when the method was applied to selectively extract and pre-concentrate glucose and fructose from 100% apple juice samples simultaneously. Thus, the G-F MIP prepared in this thesis, presented itself as a potential effective sorbent that can be employed for the selective extraction, isolation and pre-concentration of glucose and fructose from 'dirty' complex matrices prior to their accurate analysis by sensitive analytical instruments.

To improve this work, I recommend that MIP dialing be employed to select the best MIP reagents for the preparation of the MIP polymers instead of the trial and error that was employed in this thesis. In addition to this, the use of a dummy template is also recommended to rule out completely the challenge of template bleeding which may result in positive error. Finally, the improved MIP must be applied in conjunction with a sensitive analytical instrument, especially stable isotope

ratio mass spectrometer, to accurately determine the concentration and differentiate the natural sugars from the illegally added sugars in the so call ‘100%’ fruit juices. This will greatly improve the process of authenticating 100 % fruit juices as well as protecting consumers from unscrupulous producers.

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Chapter 7: Appendices

7.0 Publications

H.W. Mukami and B.S. Batlokwa, “Application of a custom-synthesized molecularly imprinted polymer for the selective isolation of total glucose and fructose from `100%` fruit juice samples prior to instrumental analysis,” *Molecular Imprinting Journal.*, vol 5, no.1, pp. 16-24, 2018.