EVALUATION PERFORMANCE OF MOLECULARLY IMPRINTED POLYMERS FOR CARISOPRODOL RECOGNITION IN FORENSIC MATRICES

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

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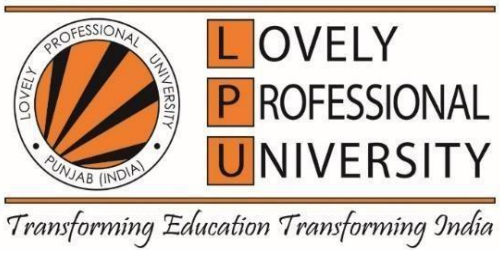

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Evaluation Performance of Molecularly Imprinted Polymers for Carisoprodol Recognition in Forensic Matrices**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision **Dr. Saurabh Shukla**, working as **Assistant Professor**, in the **Department of Forensic Science, School of Bio-engineering and Bio- Sciences** of **Lovely Professional University, Punjab**, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**Evaluation Performance of Molecularly Imprinted Polymers for Carisoprodol Recognition in Forensic Matrices**" submitted in fulfilment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the **Department of Forensic Science, School of Bio-engineering and Bio- Sciences**, is a research work carried out by **Sakshi Manhas**, (Registration No.) **11915235**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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This thesis is dedicated to my beloved parents, brother, family and friends for their endless love, support and encouragement.

ABSTRACT

In criminal investigations and legal procedures, forensic science plays a crucial role, particularly when it comes to the identification and analysis of drugs and poisons in biological samples for medico-legal reasons. This study explores the topic of forensic toxicology, giving light on cutting-edge analytical techniques that have the potential to have a substantial influence on forensics and broaden our knowledge of drug usage and its effects. There is an urgent need for comprehensive measures to address the expanding problems of substance dependency and addiction given the growing concern about drug usage, particularly among young people.

The current study focuses on muscle relaxants, particularly carisoprodol, which has been abused in the past and still continue to be abused because of its tranquil and euphoric effects. As a result of the widespread intoxication, dependence, and even mortality caused by carisoprodol usage, forensic toxicologists are now required to identify and examine drugs of abuse in biological samples. Carisoprodol (CSP) is one of the most common antispasmodic drugs used to reduce muscle spasms and is abused for its mind-altering effects. Its abuse has another major concern of increased risk of fatal road accidents due to impaired driving. Therefore, it is crucial and challenging for a forensic toxicologist to effectively extract and detect CSP from complex biological matrices. A notable aspect of this research is the synthesis of molecularly imprinted polymer (MIP), an innovative approach in forensic science. MIPs, with their high specificity and efficiency, have emerged as invaluable tools in forensic laboratories, enabling accurate and rapid detection and analysis of drugs. This method presents promising opportunities to revolutionize molecular recognition methods and advance various fields within forensic science.

In summary, by offering insightful knowledge about drug addiction and forensic analysis, this research significantly advances the field of forensic science. In the context of forensic science, the emphasis on effective and targeted detection techniques promotes a deeper knowledge of drug-related concerns, enables evidence-based drug misuse prevention efforts, and ensures safer use of prescribed drugs. The study also investigates the production and use of molecularly imprinted polymers made from various functional monomers, such as PVA and PEG. Despite initial difficulties, the researchers' flexibility and tenacity allowed them to construct a new MIP

employing methacrylic acid (MAA) with success. This adaptability in strategy highlights the promise of MIP-based technique for several applications outside of forensic science, including medication delivery, sensing, and separation research.

A novel analytical technique contingent on molecularly imprinted polymer (MIP) coupled with dispersive solid phase extraction (MIP-DSPE) has been developed for the simple, rapid, selective extraction and analysis of CSP by applying gas chromatography-mass spectrometry (GC-MS) from pharmaceutical and biological samples like urine and blood. MIP was successfully synthesized by bulk polymerisation utilizing CSP as the template, methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) as a crosslinker, azobisisobutyronitrile (AIBN) and acetonitrile as initiator and porogen, respectively. Further, characterization of the formed polymer was executed by Field emission-scanning electron microscopy (FE-SEM) and Fourier-transform infrared spectroscopy (FTIR). An equilibrium adsorption experiment was performed to determine the binding affinity of the polymer synthesized. Different parameters affecting the efficiency of MIP-DSPE were optimized for the selective estimation of CSP in urine, blood, and pharmaceutical samples. The developed method exhibits reasonable linearity for the calibration range of 0.1-10 µg mL^{-1} along with correlation coefficients (R^2) more than 0.9993, 0.9993, and 0.9996 for urine, blood, and pharmaceutical samples, respectively. Under the optimized situation, the percent recoveries of CSP from urine, blood, and pharmaceutical samples range from 83.9-113.2%. The limit of detection (LOD) and limit of quantification (LOQ) for urine, blood, and pharmaceutical samples were estimated to be 0.0052, 0.0076, 0.0050 µg mL^{-1}, and 0.0171, 0.0250, 0.0165 µg mL^{-1}, respectively. Moreover, the intra-day and inter-day precisions (n=5) were under 7% and 11%, respectively. In addition to this, the greenness of the proposed technique has been assessed with the help of the ComplexGAPI tool.

Using MIP-DSPE in conjunction with GC-MS, the proposed analytical approach for detecting carisoprodol in biological samples has successfully been validated, confirming its environmental friendliness, robustness, and sensitivity. The method's potential to extract trace amount of the analyte is highlighted by its precision, accuracy, and eco-friendliness, which make it a viable tool for regular analysis in clinical and forensic laboratories. The on-going development and improvement of

extraction techniques offer up new applications for MIP- based techniques, which have the potential to revolutionise drug analysis and detection in forensic science and other fields. These discoveries open the door for additional advancements and discoveries across a range of scientific fields, in addition to helping molecularly imprinted polymer method.

In conclusion, this study combines many forensic science disciplines, such as forensic toxicology, drug misuse, and molecularly imprinted polymer technologies. By combining these factors, this study deepens our understanding of drug-related problems and provides forensic science with cutting-edge tools and approaches to successfully combat drug addiction difficulties. Future criminal investigations might be revolutionised, with continued multidisciplinary cooperation and uncompromising dedication to scientific progress.

There are six chapters in this thesis. First chapter majorly consist of introduction, theory on muscle relaxant, carisoprodol, molecularly imprinted polymer, instrumentation. However, the review of literature is explained in chapter two. The experimental/research data is explained in chapter three, four and five. Chapter five contains the conclusion of the complete study.

Keywords: Carisoprodol, molecularly imprinted polymer, dispersive solid phase extraction, gas chromatography-mass spectrometry, green analytical procedure index

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List of Abbreviations

CNS	Central Nervous System
CSP	Carisoprodol
SMRs	Skeletal Muscle Relaxants
NDPS	Narcotic Drugs and Psychotropic Substances
EMs	Extensive Metabolizers
IMs	Intermediate Metabolizers
PMs	Poor Metabolizers
UV	Ultraviolet
GC-MS	Gas Chromatography/Mass spectrometry
MIP	Molecularly Imprinted Polymer
NIP	Non-Imprinted Polymer
MMA	Methylmethacrylate
MAA	Methacrylic acid
EI	Electron Ionisation
CI	Chemical Ionisation
EGDMA	Ethylene Glycol Dimethacrylate
THF	Tetrahydrofuran
DMF	N, N-dimethylformamide
WCOT	Wall-Coated Open Tubular
SCOT	Support Coated Open Tubular
SPE	Solid Phase Extraction
DSPE	Dispersive Solid Phase Extraction
MISPE	Molecularly Imprinted Solid Phase Extraction
MI-DSPE	Molecularly Imprinted Dispersive Solid Phase Extraction
FTIR	Fourier Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance

FE-SEM	Field Emission Scanning Electron Microscope
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS-MS	Liquid Chromatography tandem Mass Spectrometry
ACN	Acetonitrile
LOD	Limit of Detection
LOQ	Limit of Quantification
HEMA	Hydroxyethylmethacrylate
PVA	Polyvinyl alcohol
PEG	Polyethylene glycol
GAC	Green Analytical Chemistry
Mg	Miligram
µL	Microliter
ºC	Degree Celcius
MeOH	Methanol
Ng	Nanogram
Nm	Nanometer

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Chapter – 1
Introduction

1.1 Brief Description

Forensic science is generally the application of extensive ambit of sciences and scientific fields to criminal investigations and legal proceedings. Forensic toxicology is one of the important subfield of forensics in which detection and analysis of drugs and poisons is conducted in different biological samples in order to explicate questions that arise in medico- legal proceedings [Langman and Kapur, 2006; Dinis-Oliveira *et al.*, 2010]. Moreover, forensic toxicology further includes three specific subfields: 1) clinical toxicology; 2) forensic drug testing; and 3) analytical toxicology. Clinical toxicology is a kind of behavioral toxicology, depends on the toxicologists to check and measure the dose impact connection between drugs that evoke social changes. Forensic drug testing is generally done for liquor and drug testing in drivers and legal urine drug testing [Barry, 2020]. However, analytical toxicology, provides information about the presence (or absence) of chemicals in samples collected by forensic experts. One of the most serious situations is reporting a death to the coroner, where "toxicology" helps determine the cause and manner of death [Drummer *et al.*, 2013]. Understanding of pharmacology and pharmacokinetics are likewise imperative to comprehend the likely impact of any analysed results and their course of time [Drummer, 2010].

Nowadays drug abuse has become a huge medical and social issue. An associated concern is that smoking and drinking early could lead to more serious consequential involvement in the use of illegal drugs. Studies have shown that youngsters who use cigarettes and alcohol are more prone to use other harmful substances like depressants, stimulants, muscle relaxants or tranquilizers, sedatives, hallucinogens, opioids etc. [Griffin *et al.*, 2003]. Substance dependency or addiction is a consistently relapsing condition defined by a desire to try and take a drug and disruption of social and occupational operation. Reports have shown drug dependency due to chronic use which can lead to fatality sometimes [Koob *et al.*, 2004]. Biological specimens of an individual are generally analyzed to find the drug of abuse [Barry, 2020]. Through examining different substances and tissues collected during autopsy, the forensic toxicologist leads to the assessment of the cause and manner of death [Dinis-Oliveira *et al.*, 2010].

1.2 Muscle Relaxant

Muscle relaxants are among the most medications commonly used to relieve low back pain which is not specific. Approximately 35 % of the patients are prescribed for muscle relaxants. Signs and symptoms of strains and sprains may include muscle injuries causing stiffness, pain, and muscle spasms. Muscle relaxants can provide relief from such distress and prevent muscle tremors. Pain is a common feature in several medical conditions. For instance, in viral infections like Chikungunya, patients often experience severe muscle pain. Similarly, in cases of Arthritis, patients may initially feel discomfort and may become inactive in severe situations. Muscle relaxants are a diverse class of medications with a variety of modes of action. Antispasmodic and antispasticity drugs are the two types of muscle relaxant as shown in figure 1.1. Antispasmodic medication is mainly used to reduce muscle spasm and conditions like low back pain. Examples of antispasmodic drugs are cyclobenzaprine, carisoprodol, chlorzoxazone. However, antispasticity medication is specifically utilized to decrease spasticity that hinders treatment or function, like in cerebral palsy, multiple sclerosis and injuries of spinal cord for example dantrolene sodium, baclofen [Tulder *et al.*, 2008]. Evidences have shown that chronic use of muscle relaxant can increase the considerable chances of dependency, which can ultimately lead to fatality. While muscle relaxants are very rarely the main drug of abuse, they are generally used in combination with other drugs like central nervous system depressants (CNS), e.g. opioids or alcohol [Toth and Urtis, 2004].

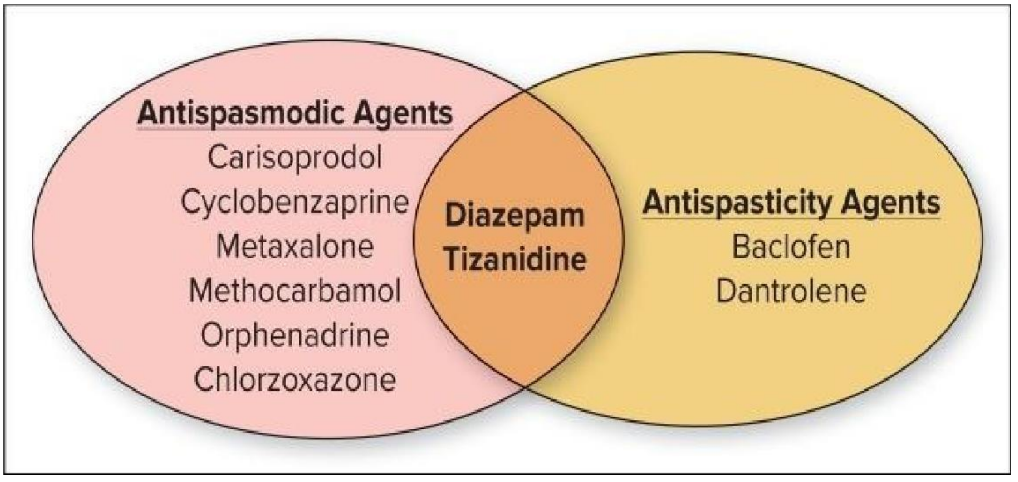


Figure 1.1: Showing classification of Skeletal Muscle Relaxants [Witenko *et al.*, 2014].

Antispasticity agents function by improving muscle hypertonicity and involuntary spasms either directly on the skeletal muscles or on the spinal cord. The term spasticity refers to the undesirable and uncontrolled movements that arise from increased muscle tone or stiffness [Chou *et al.*, 2007]. Spastic conditions including multiple sclerosis, cerebral palsy, and spinal cord injuries are all treated with baclofen. Dantrolene is another frequent antispasticity medication, although it is not suggested for treating musculoskeletal problems such as low back pain due to a lack of data supporting its efficacy. Furthermore, the use of dantrolene is accompanied with a boxed warning about the possibility of deadly hepatotoxicity [Chou, 2010; Witenko *et al.*, 2014].

Antispasmodics work by altering nerve conduction in the CNS. They are classified into two types: benzodiazepines, which inhibit GABA transmission on postsynaptic neurons, and non- benzodiazepine drugs, which act on the brain stem and spinal cord [Chou *et al.*, 2007]. Low back pain can be temporarily managed with the use of diazepam and other benzodiazepines, which are also used as sedatives, anxiolytics, and anticonvulsants. In addition to benzodiazepines, antispasmodics such as carisoprodol, cyclobenzaprine, metaxalone, and methocarbamol are routinely used to treat muscular spasms [Tulder *et al.*, 2003].

1.3 Carisoprodol

Carisoprodol (CSP) is a skeletal muscle relaxant with vaguely defined working mechanism and high potential of abuse [Boothby *et al.*, 2003]. It is a synthetic compound initially created in 1959, shares structural similarities with meprobamate, mebutamate, and tybamate [Robertson and Marinetti, 2003]. Although the specific mechanism of action is unknown, abnormal communication among neurons in the reticular formation and spinal cord has been related to both CNS depression and pain perception decrease. There are some indications that the primary mode of action is sedation, and that it does not have a direct impact on skeletal muscles [Gonzalez *et al.*, 2009]. It is lightly sedative and has decent antispasmodic effects. CSP is commercially available as a prescription medication prescribed as a muscle relaxant. It is marketed under several brand names, including Soma® tablets, Soma® compound, Soma® with codeine, and Sodol® Compound [Kumar, 2015].

CSP inhibits the activities of neurons in both descending reticular formation and spinal cord. Further after ingestion, CSP is metabolized into meprobamate. Duration of action of metabolite meprobamate is similar to barbiturates. Immoderate use has reported to have psychological and physical dependence [Tulder *et al.*, 2003]. This drug is prescribed in 250- mg and 350-mg tablets and should be taken thrice a day. It has been proven effective in treating acute low back pain, but only for a limited duration of two to three weeks. The medication takes around 30 minutes to take effect and typically lasts between 4-6 hours [Witenko *et al.*, 2014].

CSP is generally abused for mind altering effects that are sedation and euphoria. Addicted users also use this drug to prevent withdrawal and for improving sexual performance. Although, dependency, tolerance and abuse is the property of all the drugs coming under class of skeletal muscle relaxant (SMRs) but abuse of CSP have reported to increase more as compared to other available SMRs [Boothby *et al.*, 2003]. New findings validate that the misuse of CSP results in various negative consequences, including impaired psychomotor function and intense withdrawal symptoms, which could potentially increase the risk of experiencing hallucinations, seizures, and even fatality [Bramness *et al.*, 2004; Fass, 2010; Zacny and Gutierrez, 2011; Zacny *et al.*, 2012]. The misuse of CSP has witnessed a significant surge in recent times. Individuals who engage in recreational drug use exploit CSP for its muscle-relaxing properties, anxiolytic effects, and sedating qualities. It is common for them to combine it with other CNS depressants to amplify its sedative or euphoric impact [Hardon and Ihsan, 2014]. The Drug Enforcement Agency (DEA) has observed a rise in instances of CSP intoxication. The number of seizures triggered by the misuse of CSP escalated from 3,988 in 2008 to over 5,000 in 2010, surpassing those caused by other commonly abused substances such as lorazepam and methylphenidate [Witenko *et al.*, 2014]. The rising prevalence of CSP usage among high school students is an issue that is causing worry. According to the Monitoring the Future National Survey on Drug Use (2009), high school seniors used 1.3% and 1.4% of CSP annually for non-medical purposes in 2007 and 2008, respectively. The percentages of CSP misuse amongst senior and high school students are greater compared to other abused prescription drugs such as chlordiazepoxide (0.2%) and lorazepam (0.4%), and similar to clonazepam (1.3%) [Reeves *et al.*, 2012].

CSP's potential for abuse stems from its ability to modulate GABAA function, which can be intensified by the active metabolite meprobamate [Reeves and Burke, 2010]. CSP's physiological effects are frequently related to meprobamate's effects. [Reeves *et al.*, 1999], on GABA type A receptors (GABAARs), which are the primary receptors for the inhibitory neurotransmitter in the brain [Sigel and Steinmann, 2012]. Meprobamate, a Schedule IV prohibited chemical substance with a similar mechanism of action to barbiturates, was first marketed as an anxiety-relieving medication in the 1950s. Its half-life is usually around 10 hours, but with prolonged use, it can extend up to 48 hours [Reeves *et al.*, 2012]. People who regularly use CSP are more prone to experiencing withdrawal symptoms if they suddenly stop taking it. Withdrawal symptoms may manifest as anxiety, difficulty sleeping, irritability, tremors, muscle twitching, and lack of coordination [Reeves and Burke, 2010; Gatch *et al*., 2012].

Illicit diversion is increasingly common within legal medical practices and community pharmacies, involving various strategies such as engaging in "doctor shopping" or fabricating prescriptions. The occurrence of CSP misuse escalated as it gained recognition as a readily available sedative capable of enhancing or modifying the impact of other substances. Patients have reported using CSP to intensify the sedative properties of benzodiazepines or alcohol, alleviate restlessness when consuming cocaine, and promote relaxation following cocaine use. When combined with certain medications, CSP can generate synergistic effects that lead to profound relaxation and euphoria [Reeves *et al.*, 2012; Witenko *et al.*, 2014]. Another major concern of CSP abuse is the increased risk of road accidents due to impaired driving. This is leading road accidents and even death in some cases. Therefore, it is important for forensic toxicologists to the samples and detects the drug of abuse [Bramness *et al*., 2004]. In cases of death due to overdose, samples like urine, vitreous humor, heart and femoral blood is collected and sent for analysis to forensic expert. It has been reported that both CSP and its metabolite, meprobamate are found in all the above mentioned samples. It is also observed that the probability of both morbidity and mortality are low in cases of pure SMR ingestions; whereas mortality increases when muscle relaxant is ingested with multiple substances [Boothby *et al.*, 2003].

1.3.1 Clinical Use of Carisoprodol

The approval for clinical use of CSP as a skeletal muscle relaxant was granted by the Food and Drug Administration (FDA) in 1959 [Fass, 2010]. CSP exhibits a rapid onset of action, with effects lasting for approximately 4-6 hours [Littrell *et al.*, 1993]. It is available as tablets with strengths of 250 mg and 350 mg. Generally, it is administered 3-4 times daily for a maximum period of 2-3 weeks. In the year 2000, with 21% of all skeletal muscle relaxant prescriptions, CSP was the second-most often advised muscle relaxant in the United States. In the years 2003 and 2004, the trio of CSP, cyclobenzaprine, and metaxalone collectively constituted 45% of all SMR prescriptions distributed [Luo *et al.*, 2004; Toth and Urtis, 2004]. The primary adverse effects experienced medicinally with CSP usage include drowsiness, dizziness, and headaches [Bramness *et al.*, 2007]. Additional side effects encompass nausea, vomiting, low blood pressure, rapid heartbeat, impaired coordination, dizziness, tremors, and seizures. Typically, these adverse effects are noted when high doses are administered or when the drug is abruptly discontinued. In cases of toxic doses of CSP, individuals may exhibit agitation, myoclonus, and unusual movements [Roth *et al.*, 1998; Bramness *et al.*, 2005].

1.3.2 ADME and Chemistry of Carisoprodol

CSP exhibits the properties of a white crystalline powder, possessing a subtle distinct scent and a bitter flavor. It comprises a racemic blend of two stereoisomers known as (2 RS)-2- [(Carbamoyloxy) methyl]-2-methylpentyl (1-methylethyl) carbamate as shown in table 1.1 and figure 1.2. CSP has a molecular weight of 260.33 and an octanol-water partition coefficient of 2.36. The solubility of CSP remains unaffected by changes in pH. It readily dissolves in organic solvents such as DMSO, chloroform, acetone, and alcohol, and displays moderate solubility in water (30 mg/100 mL at 25°C) [Littrell *et al.*, 1993].

The pharmacokinetics of CSP, including the blood concentrations of CSP, is affected by the individual's CYP2C19 genotype. CSP is metabolized by extensive metabolizers (EMs) and intermediate metabolizers (IMs), resulting in a half-life of 1.5 hours [Olsen *et al.*, 1994; Dalen *et al.*, 1996]. Approximately 2-3% of individuals of Caucasian descent are classified as poor metabolizers (PMs). In PMs, the elimination of CSP

takes longer, with a half-life exceeding 6 hours, and there is limited production of meprobamate [Tamminga *et al.*, 2001]. The involvement of drug interactions is likely asignificant factor in fatalities associated with CSP [McIntyre *et al.*, 2012].

Factors such as age, gender, and the concurrent use of other medications that engage with the CYP2C19 enzyme can also influence the metabolism of CSP, consequently impacting its blood concentrations [Tse *et al.*, 2014]. The utilization of oral contraceptives affects the metabolism of CSP in a manner dependent on the individual's CYP2C19 genotype. Intermediate metabolizers (IMs) who take oral contraceptives tend to accumulate higher levels of CSP compared to extensive metabolizers (EMs) who are also using those [Bramness *et al.*, 2005]. Given that oral contraceptives are utilized by over 25% of women between the ages of 20 and 40 [Ronning, 2001], it is crucial for healthcare providers to be mindful of the potential impact on CSP concentration among individuals using these contraceptives.

Table 1.1 Showing IUPAC Name, Chemical Formula and Molecular Weight of CSP.

IUPAC Name	Chemical Formula	Molecular Weight
(2 RS)-2-[(Carbamoyloxy) methyl]-2-methylpentyl (1-methylethyl) carbamate	$C_{12}H_{24}N_2O_4$	260.33 g/mol

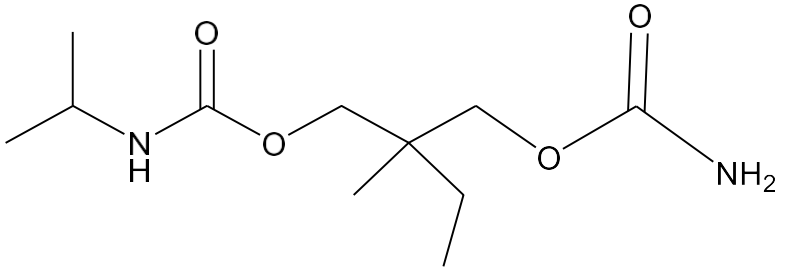


Figure 1.2: Showing chemical structure of CSP

1.3.3 Abuse and Withdrawal Symptoms

An initial study indicated that CSP did not serve as a replacement for morphine or barbiturates and did not induce intoxication or withdrawal symptoms resembling those of morphine or barbiturates. Consequently, it was deduced that CSP lacks the potential for abuse [Littrell *et al.*, 1993]. Nonetheless, recent findings have substantiated its propensity for abuse, as well as its ability to cause subjective and psychological impairment, along with acritical withdrawal syndrome that could increase the likelihood of seizures and potentially fatal outcomes [Ni *et al.*, 2007; Gonzalez *et al.*, 2009; Fass, 2010; Reeves and Burke, 2010; Zacny *et al.*, 2012; Reeves *et al.*, 2012; McIntyre *et al.*, 2012]. In 2008, the American Association of Poison Control Centers documented 2632 instances of deliberate CSP ingestion that necessitated medical intervention [Bronstein *et al.*, 2009].

One supra-therapeutic dosage of CSP at a level of 700 mg was found to cause both subjective and psychomotor deficits in studies on healthy adults. On the other hand, psychomotor impairments were predominantly caused by a single therapeutic dosage of 350 mg, although mild subjective impairments were also present [Zacny and Gutierrez, 2011]. When 10 mg of oxycodone was given within an hour of taking 350 mg of CSP, the psychomotor impairments were worsened [Zacny *et al.*, 2012]. Additionally, a research in northern region of India examined the effects of CSP that depended on dosage [Nebhinani *et al.*, 2013]. The use of 1-3 pills during that particular trial led to feelings of well-being and an increase in energy. 4-10 pills were used to produce psychomotor excitation, happiness, increased sociability, and increased self-assurance. However, taking 10 pills in one dose resulted in a condition that resembled acute organic brain syndrome, which was marked by confusion and partial forgetfulness of the events that had taken place while taking the drug. Ina second trial, Reeves and Burke found that those who used 12–30 pills daily, either on their own or in conjunction with alcohol, benzodiazepines, or tramadol, reported feeling euphoric and having hallucinations [Reeves and Burke, 2010]. CSP abuse has also been connected to suicidal thoughts [Bailey and Briggs, 2002].

There was a noticeable inclination to continue using CSP among individuals who were given prescriptions for long-term use. According to research by Owens *et al.*, patients continued to get CSP in almost 80% of reported cases even if their health coverage no longer covered the costs [Owens *et al.*, 2007]. A test of 40 people who

had been using CSP for more than three months found that 50% of the cases had a history of drug misuse. This group of 20 people included 40% who admitted to exceeding the recommended dosage, 30% who admitted to taking CSP for purposes other than treating low back pain, and 10% who admitted to using it to intensify the effects of other drugs they were abusing, and 5% who admitted to using it to reverse the effects of other drugs. When considered collectively, the data clearly suggests that CSP has addictive qualities and a substantial potential for misuse. It is concerning that doctors may not be sufficiently informed of the possible hazards connected to CSP [Reeves *et al.*, 1999]. Now that CSP is a scheduled drug, there ought to be greater awareness of the risks associated with its abuse.

Breastfeeding exposes nursing new-born to CSP. The concentrations of serum were examined before to and during a simple delivery as well as during the first month of breastfeeding to evaluate the amount of CSP and meprobamate in a nursing mother. The mother had been taking three times as much CSP as is recommended daily to treat her acute back spasms [Briggs *et al.*, 2008]. CSP and meprobamate in significant amounts were found in the infant's blood as well as the breast milk. Despite this, there was no sign of developmental harm. The infant had some mild drowsiness while being breastfed, but there were no withdrawal symptoms seen after nursing was stopped.

CSP misuse has significantly increased during the past few years. When compared to other illegal narcotics, it is more affordable and accessible, which makes it a desirable alternative for those who have a tendency towards substance misuse. CSP abusers commonly mix it with other psychoactive medications to boost or change their effects. For example, it can be used with alcohol or benzodiazepines to increase its sedative effects, with cocaine to lessen the usual anxiety brought on by using it, or with other drugs to produce a synergistic state of pleasure and relaxation [Reeves and Liberto, 2001; Reeves and Burke, 2010]. People may decide to take CSP as a substitute if they are unable to obtain opiates or benzodiazepines [Reeves *et al.*, 1999]. CSP was one of the 25 medicines recognized in 2008 as being often misused by the National Forensic Laboratory Information System. The FDA and the Drug Advisory Council rejected the Drug Enforcement Administration's (DEA) recommendation to schedule CSP in 1996 on the grounds that the data supporting the medication's risk for misuse was insufficient [Fass, 2010].

In 2010, decision was made to add CSP to schedule IV of the Controlled Substances Act of 1970 after holding more hearings discussing the drug's prospective scheduling. The revised timetable became effective on January 11, 2012. Following the first evaluation, there has been a significant rise in CSP misuse and a better understanding of the possible mechanism that underpins its effects. For instance, in 2009, CSP usage was directly blamed for around 30,000 of the roughly 50,000 emergency room visits linked to the misuse of musculoskeletal relaxants. In comparison to visits connected to CSP misuse that were documented five years earlier, this statistic shows a doubling [Reeves *et al.*, 2012]. CSP usage increases the risk of road accidents and accidents involving heavy machinery because of the subjective aspect of the impairments, psychomotor effects, and CNS depressive qualities associated with the drug [Hardon and Ihsan, 2014]. CSP and meprobamate are actually included as the seventh most common drug groups associated with drunk driving. If you behave in this way, you might be accused of operating a vehicle while inebriated [Bramness *et al.*, 2007].

Since there is evidence of CSP usage in many nations across the world, the issue goesacross the borders of the India and United States [Bramness *et al.*, 2007; Nebhinani *et al.*, 2013; Hardon and Ihsan, 2014]. In Norway, CSP was categorized as a class "A" medicine in 2007. This category includes drugs including opioids and hypnotics and is equivalent to the highest scheduling level in Norway. In a recent assessment, the Committee for Medicinal Products for Human Use found that CSP's risk for misuse outweighs its therapeutic advantages. The European Medicines Agency decided to take CSP off the market in 2008 as a result. It is currently only available to people who have no other effective therapy choices [Hoiseth *et al.*, 2009]. In South Sulawesi, Indonesia, sex workers put in a large amount of their salary on CSP [Hardon and Ihsan, 2014]. To meet the demands of their line of work, they take up to 10 tablets each day, sometimes in conjunction with alcohol and other psychotropic prescription medicines. CSP has also grown in importance internationally. It is feasible to buy thousands of tablets all at once in Mexico, which are then smuggled into the country and trade to youngsters [Davis, 2009]. According to the Los Angeles Police Department, CSP is one of the most often found prescription medicines at US-Mexico border crossings, which is consistent with this [Reeves and Burke, 2010].

Due to its potential for quick tolerance building, which causes increased pill intake, CSP presents a significant difficulty. Withdrawal syndrome may develop if CSP use is

abruptly stopped [Gatch *et al.*, 2018]. In humans, withdrawal symptoms such sleeplessness, cramping in the abdomen, headache, chills, and nausea can be brought on by abruptly stopping the administration of CSP at a dose of 100 mg/kg/day, five times the recommended daily dosage. CSP usage has been linked to cases of hallucinations, delusions, tremors, seizures, and deadly results [Ni *et al.*, 2007; Reeves and Burke, 2010]. Prisoners in Norway who had been using CSP at dosages between 700 and 2100 mg/day for at least 9 months had withdrawal signs when the medication was abruptly stopped. These signs were restlessness, worry, agitation, and soreness in the muscles [Wyller *et al.*, 1991]. Notably, withdrawal symptoms have been reported while stopping low to moderate dosages of CSP as well as when using 4 to 8 pills per day [Reeves *et al.*, 1997]. While the GABAergic system is predominantly linked to toxic and withdrawal symptoms, it's crucial to remember that other systems can also be affected. There have been many occasions when people ingested large amounts of CSP, up to 65 tablets, either by themselves or in conjunction with other sedative/hypnotic drugs [Bramness *et al.*, 2005]. Studies found that these people had specific symptoms that may be related to serotonergic activation, including tachycardia, hypertension, choreiform movements that resembled robots, tremors, and shivering [Roth *et al.*, 1998]. The serotonergic syndrome symptoms and indicators in the aforementioned investigation were non-specific. It is significant to highlight that the possibility of additional co-administered medicines contributing to the observed serotonergic effects cannot be ruled out due to the lack of a thorough drug screening. There is currently no particular treatment that is appropriate and effective for CSP withdrawal and intoxication. Barbiturates and benzodiazepines, however, are often used to treat withdrawal episodes in order to reduce agitation and anxiety [Reeves *et al.*, 2007]. Managing a CSP overdose is difficult because of the previously noted serotonergic-like symptoms. It's vital to understand that while a benzodiazepine antagonist is frequently given in situations of CSP intoxication, CSP does not work at the benzodiazepine site.

1.3.4 Pharmacology of Carisoprodol

Instead of directly inducing relaxation in skeletal muscles, CSP largely affects the CNS [Gonzalez *et al.*, 2009]. CSP's ability to relax muscles is thought to be due to the suppression of inter-neuronal communication in the spinal cord and descending

reticular development. Although the detailed mechanism of action is not completely known, it is thought that meprobamate, a metabolite of CSP, is responsible for the effects of the drug. At the federal level, meprobamate, a sedative-hypnotic with a history of use in the treatment of anxiety, is now categorized as a schedule IV prohibited drug. Its potential for abuse is comparable to that of benzodiazepines [Roache and Griffiths, 1987; Mounier *et al.*, 2012]. Miltown and Equnil were the first brand names for meprobamate when it was first made available in 1955. However, it was soon supplanted by benzodiazepines within a few years because of its substantial misuse potential [Reeves *et al.,* 2012]. CSP shows unique pharmacological and physiological effects; even though meprobamate is probably involved in both the drug's beneficial and negative effects. This shows that CSP has special properties all its own [Gonzalez *et al.*, 2009]. Toxicological symptoms appear within 30 minutes of a CSP overdose (half-life: 1.5 hours), before the drug is metabolized into meprobamate (half-life: 11 hours) [Littrell *et al.,* 1993].

The researchers used complete cell patch electrophysiology in HEK cells to show that CSP directly activates GABAARs (GABA type A receptors) and performs allosteric regulation on them. CSP blocks the receptor at high doses, which causes rebound currents to occur after the medication is stopped, a similar effect to that seen with barbiturates. The highest inhibition of motor activity is shown within 10 minutes of CSP intake, according to in vivo investigations, suggesting that these effects are not primarily attributable to the drug's conversion to meprobamate [Gonzalez *et al.*, 2009].

1.4 Narcotic Drugs and Psychotropic Substances (NDPS) Act, 1985

In the beginning, the main reason of drug addiction was the use of opium and morphine in prescription medicines throughout the time of the Civil War. Veterans were dependent on these chemicals as a result of their exposure to them during the war, which is why the disorder is known as "soldier's disease" [Grey, 2012]. Though hemp (marijuana) cultivation had been successfully outlawed in 1937, during World War II governments came to realize the surprising importance of hemp, especially for basic requirements like rope and other cordage applications. In the Indian context, there is historical evidence of cannabis use going all the way back to the Vedic era. The Atharvaveda documents the use of cannabis, which occurred many hundred years

ago [Sharma *et al.*, 2017]. In India, cannabis and its derivatives—bhang, charas, and ganja—were extensively consumed for recreational purposes up until 1985 when they were made lawfully accessible for purchase. India made a position against the US-proposed 1961 Single Convention on Narcotics, which sought to control all narcotics. As a result, the convention came to a consensus and gave India a 25-year "Grace period" during which it would be allowed to use cannabis only for research and medicinal purposes, with no exceptions.

In light of India's international responsibilities and the political sensitivity of the subject, the government felt obliged to confront the deeply rooted cultural use of cannabis. As a result, on November 14, 1985, the narcotic drugs and psychotropic substances (NDPS) Act was passed, essentially outlawing all narcotic substances with low levels of resistance in India. As stated by Britto in 1989, the 1985 Act did contain a clause permitting the non-medical cultural usage of beverages prepared from cannabis leaves. Apart from specific situations for medical and scientific purposes, the NDPS Act prohibits the following acts linked to these drugs and substances: production, possession, sale, transit, and cultivation [Kethineni *et al.*, 1995]. This law covers all narcotic substances, including cannabis, coca, and opium. The primary objectives of this law's execution were to control the production and distribution of pharmaceuticals, guarantee their quality, require the disclosure of medicinal components, and stop substance addiction in society [Sharma *et al.*, 2017].

1.5 Molecularly Imprinted Polymers

Synthetic biology has experienced a dramatic revolution, utilizing multiple multidisciplinary methodologies to develop a plethora of creative technology-based applications that have a substantial impact on the advancement of human evolution. Numerous research focused on the reconfiguration of technology as they explored its processes, which included biological components such as purines, pyrimidines, proteins, genes, and metabolic pathways, throughout the transformational process [Benner and Sismour, 2005]. The redesigning of biomolecules has permitted the development of more specific and specialised applications, such as Molecular Imprinting Polymers (MIPs), biomimetics, biomarkers, and biosensors, by exploiting data libraries [Checa *et al.*, 2012]. To grasp the underlying processes involved in biomolecule redesign, it is critical to first understand the many interactions within

systems that lead to the production of dynamic and complex structures. Despite their weakness, non- covalent bond interactions serve an important function in sustaining certain dynamics within these complexes throughout the affiliation or dissociation of micro or macro molecules. These features of association and dissociation constitute the basis for recognising the chemical transition of complex molecules inside biological systems. In biomolecules, weak covalent interactions such as Vander Waal, hydrogen, and hydrophobic bonds outnumber strong covalent connections [Hishiya *et al.*, 2002; Hao *et al.*, 2016].

Numerous synthetic strategies for achieving molecular recognition by imitating biomolecules have been developed. One intriguing advanced strategy in this domain is molecular imprinting, which involves creating specialized cavities suited to a template inside a polymer network. The process of molecular imprinting has gotten a lot of attention in a variety of domains. It uses polymer network methods as the imprinting process's base. In the absence of the template molecule, recognition is created by the use of functional groups with precise sizes and shapes that match to the template molecule. Molecular imprinting methods are used to build synthetic compounds that imitate bio-active receptors, assuring effective recognition capabilities that stay stable under varied physio-chemical circumstances, both high and low [Nicholls *et al.*, 2015].

Non-covalent molecular imprinting is the clear winner among the three techniques for polymer network construction. This approach is distinguished by two critical elements: the effective removal of the template and the attainment of efficient recognition. These elements add to the method's favorability. However, limitations in terms of low binding affinities and
the use of solvents in rebinding investigations have been found. Furthermore, the occurrence of heterogeneous binding sites has been discovered to be restricted or limited [Schneider and Yatsimirsky, 2008; Matsui et al., 2009; Kempe et al., 2015; Line et al., 2020].

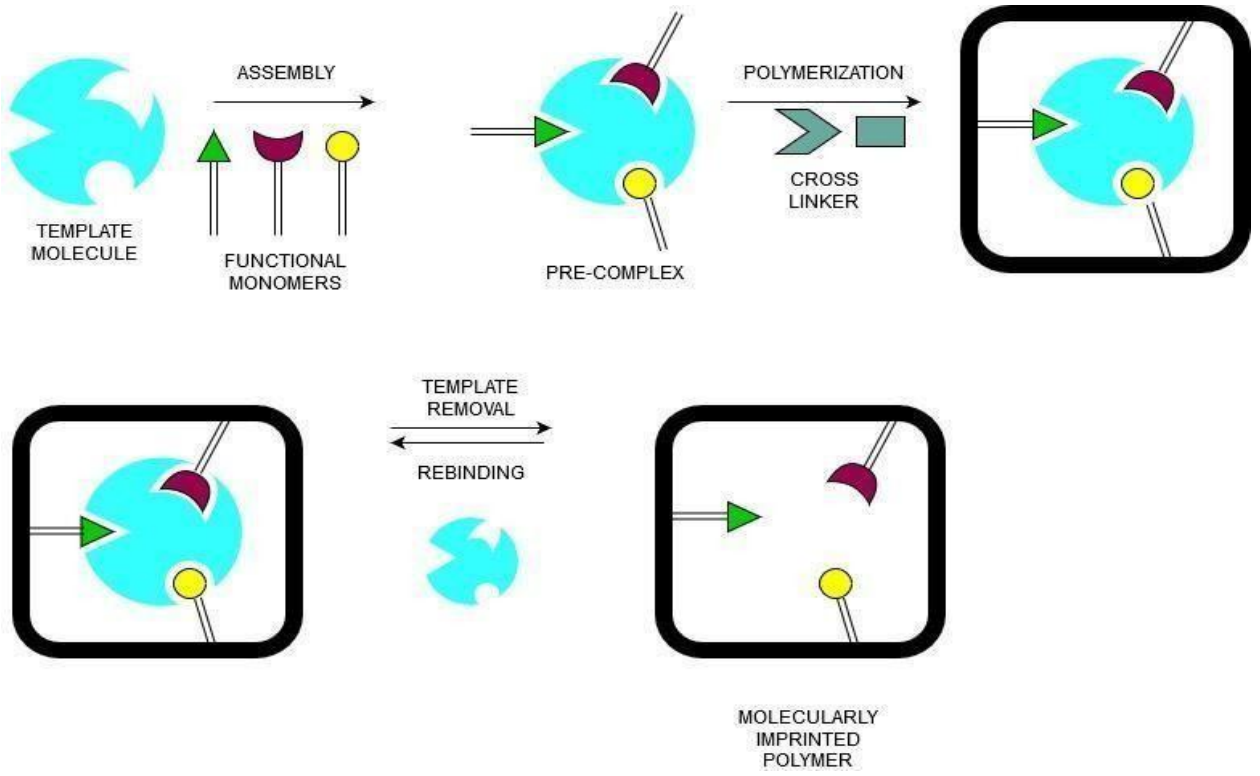


Figure 1.3: Diagrammatic representation of molecularly imprinted polymer synthesis

The covalent approach is efficient with a wide variety of solvents and pre-polymerization, but it has drawbacks such as the inability to remove the template molecule due to strong bond contacts and the difficulty in maintaining its bonds. To overcome these restrictions, a hybrid imprinting method that combines covalent and non-covalent procedures was devised. Some of the limitations connected with the separate covalent and non-covalent approaches have been successfully solved by this hybrid method [Kamplain and Bielawski, 2006; Wulff and Liu, 2012; Whitcombe *et al.*, 2014; Nagase *et al.*, 2015; Takeuchi and Sunayama, 2018;].

MIPs display preferential binding to the molecules with which they were templated during their manufacture. They bring specificity and selectivity to biological systems and have the added benefit of being usable throughout a wide temperature range. While Polyakov laid the framework [Polyakov, 1931], the notion of molecular imprinting gained acceptance in the early 1970s [Wulff *et al.*, 1973]. The capacity of

MIPs to template a wide range of things, including atoms, ions, molecules, ionic assemblies, macromolecular assemblies, and even microbes, is a significant property. MIPs' distinct qualities, such as their simplicity, predictable structure, ease of manufacture, cost- effectiveness, high selectivity, and sensitivity, have piqued the interest of researchers in a variety of domains [BelBruno, 2018]. Diagrammatic representation of MIP synthesis is given in figure 1.3. The template molecule, functional monomer, cross- linker, polymerization initiator, and porogenic solvents are important components in the production of MIPs. The interaction amongst the monomers and the template molecule is the first and most important step in building traditional MIPs, followed by polymerization using cross-linkers to generate a cross-linked polymeric network that integrates the template molecule. Following that, the imprinted molecules are removed from the polymer matrix, leaving precise binding sites that may successfully rebind the template with great specificity.

The target compounds in analytical techniques are the template molecules. A template molecule must fulfill the following requirements to be called ideal:

1.It should not include any groups that might obstruct polymerization.
2.Throughout the polymerization procedure, it should be chemically stable.
3.It should include functional groups capable of forming compounds with functional monomers [Chen *et al.*, 2011].

A functional monomer is typically made up of two parts: a recognition unit and a polymerizable unit. The affinity of the MIP for the template is checked by the strength of the interactions between the template and the monomer. As a result, choosing a good functional monomer is critical throughout the MIP creation process [Karim *et al.*, 2005]. Acrylamide, methylmethacrylate (MMA), methacrylic acid (MAA), aniline, thiophene and its derivatives, and pyrrole are some of the most often utilised functional monomers. Because of its excellent properties as both a hydrogen bond donor and acceptor, MAA is well recognized as a versatile functional monomer [Golker *et al.*, 2013].

Cross-linkers are essential in the immobilization of functional monomers around template molecules. Their purpose is to maintain an extremely cross-linked and stiff polymer structure even after the templates are removed. The quantity and kind of cross-linkers used have a direct influence on the sensitivity and binding capability

of the MIP towards the template molecules [Yan and Ho Row, 2006]. Cross-linkers used in traditional MIP production include ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM), N, N- methylenebisacrylamide (MBAA), and divinylbenzene (DVB). These cross-linkers are responsible for creating and maintaining the polymer's highly cross-linked structure long after the templates have been removed. Porogens, also known as porogenic solvents, operate as dispersion media and contribute to the creation of pores during the polymerization process. They are extremely important in determining the porosity nature of the polymeric network. 2- methoxyethanol, methanol, tetrahydrofuran (THF), acetonitrile, dichloroethane, chloroform, N, N-dimethylformamide (DMF), and toluene are common solvents used in MIP synthesis [Esfandyari-Manesh *et al.*, 2011].

1.5.1 Approaches to Molecular Imprinting

Based on the interactions between functional monomers and templates, three separate molecular imprinting procedures have been developed: covalent imprinting, non-covalent imprinting, and semi-covalent imprinting. A template-monomer complex is produced by flexible non-covalent or metal ion coordination contacts in the non-covalent or self-assembly technique. Prior to chemical synthesis, a covalent contact between the template and monomer occurs in the covalent or pre-organized method. Andersson and his colleagues pioneering work was largely focused on using non-covalent interactions between the host and target [Andersson, 1984]. Wulff and his colleagues, on the other hand, conducted substantial research into the usage of covalently bound imprinting polymers. Methanol, THF, acetonitrile, dichloromethane, chloroform, DMF, and toluene are all examples of organic solvents [Wulff *et al.*, 1985].

The imprinted molecule is covalently bonded to a polymerisable molecule in the covalent method. Covalent bonds that are reversible are created during the polymerization process. The imprinted molecule is chemically removed from the highly cross-linked polymer after copolymerization with a cross-linker occurs. As a result, the MIP produced has precise binding sites with a high affinity for the template molecule. Covalent imprinting guarantees that functional monomer residues are only found in the imprinted cavities. The removal of the template molecule, however, becomes difficult in this approach, making it a less versatile method. The strong

covalent contacts make achieving thermodynamic equilibrium challenging, resulting in delayed binding and dissociation rates [Alexander *et al.*, 2006].

Ionic interactions, hydrogen bonding, van der Waals forces, and Π-Π stacking interactions are examples of non-covalent interactions. Non-covalent imprinting has recently acquired prominence as a generally applicable synthesis technique. The key advantages of non- covalent imprinting are quick and efficient template removal, simple preparation procedures, the flexibility to use a wide range of templates, and faster binding and rebinding kinetics [Herrera-Chacón *et al.*, 2021]. Semi-covalent imprinting is a new approach that combines the stability of covalent imprinting with the fast target uptake capabilities of non-covalent imprinting. This method is an intermediate option in which the template is covalently attached to the functional monomer and subsequent rebinding is based on non-covalent interactions [Chen *et al.*, 2011].

1.5.2 Scheme of Molecularly Imprinted Polymer Synthesis

In the production of MIPs, two separate techniques, bulk and surface imprinting, are used. MIP materials with dimensions ranging from "bulk" to micron-sized particles are commonly produced by conventional processes such as bulk polymerization, precipitation polymerization, and emulsion polymerization. The resultant MIPs are acquired after the imprinting process and pulverized into powder form. Bulk imprinting is well-known for its speed, low cost, and ease of use. However, bulk imprinted particles have a comparatively small number of binding sites positioned on or near the surface [Pietrzyk-Le *et al.*, 2010]. As a result, binding kinetics is slow. Significant limitations of bulk imprinting include difficulties in eliminating target molecules from internal binding sites, insufficient rebinding capacity, a limited number of binding sites on or near the surface, and limited accessibility to deeply embedded binding sites within the particles [Ding and Heiden, 2014].

Surface imprinting overcomes the constraints of bulk imprinting by forming voids on or near the surface of the polymer. This favourable arrangement makes template removal and rebinding easier. Mosbach and his research team were the first to reveal groundbreaking work on surface imprinting technology for the manufacture of imprinted polymers [Ramstroem *et al.*, 2002]. Surface imprinting provides a strategy

for the full removal of template molecules while also facilitating increased access to target molecules. Surface imprinting can be done directly by grafting a thin polymer film onto a support, which can result in a reduction in binding capability. This problem, however, can be minimized by using nanomaterials with enormous surface areas for molecular imprinting. Surface imprinting on nanostructured materials have received a lot of interest in the scientific community as a good way to improve binding kinetics. Nanomaterials with larger surface area-to-volume ratios provide for easier access to target species, resulting in better binding kinetics and binding capacity [Li *et al.*, 2005].

Nanomaterials are defined as materials with dimensions less than 100 nm and possessing distinct and outstanding electrical, thermal, catalytic, and mechanical capabilities. These materials are distinguished by their inherent stability, exceptional mechanical strength, and large surface-to-volume ratios [Zhang *et al.*, 2013]. Various particles, such as silica nanoparticles, magnetic nanoparticles, gold nanoparticles (AuNPs) [Ahmad *et al.*, 2015], and carbon nanomaterials [Ding and Heiden, 2014], have been used as supports in the surface imprinting process. Carbon nanomaterials include fullerenes, carbon nanotubes (CNTs) [Choong *et al.*, 2009], graphene [Xu *et al.*, 2017], and nanofibers, among others.

1.5.3 Recent Application Advances

Significant research efforts have been spent over the last several decades towards enhanced techniques that enable the examination of selective recognition with high affinity for molecules or complexes inside complicated biological systems. MIPs have been produced as artificial analogues for a variety of targets including aptamers [Yao *et al.*, 2013], antigens, antibodies [Haupt, 2010], and enzymes [Moreira *et al.*, 2016]. Synthetic MIPs have revitalized the notion of natural selection by overcoming some of the restrictions observed in natural systems, resulting in increased selectivity, specificity, durability, resistance, cost reduction, and speedier manufacture for human applications [Haupt, 2010]. MIPs have unique properties that make them biocompatible, extremely stable and cost- effective for biosensor development [Moreira *et al.*, 2016]. Extensive research has been conducted on a variety of applications [Moczko *et al.*, 2019], including separation techniques, analytical validation studies, drug screening utilizing synthetic receptors produced by MIPs, and

the creation of biosensors for early and efficient disease detection [Jenkins *et al.*, 2012; Kempe *et al.*, 2015].

MIPs have been used as biocatalytic agents as well as in immunological tests and drug delivery systems, where they provide improved selectivity, recognition, and efficiency. Furthermore, MIPs have shown considerable promise in commercial applications, notably in the separation of enantiomers and racemers in amino acids, proteins [Janiak and Kofinas, 2007; Moczko *et al.*, 2019], and bioactive compounds [Zhang *et al.*, 2006]. Their application in chromatographic purification methods such as High- Performance Liquid Chromatography (HPLC) [Bhatia *et al.*, 2019] and other electrophoresis systems such as capillary and membrane systems has piqued the interest of pharmaceutical companies. MIPs have been used successfully in chromatography as stationary phase columns [Cacho *et al.*, 2008] and as Solid Phase Extraction (SPE) devices for a wide range of chemical substances [Moczko *et al.*, 2019]. Different applications of MIP are shown in Figure 1.4.

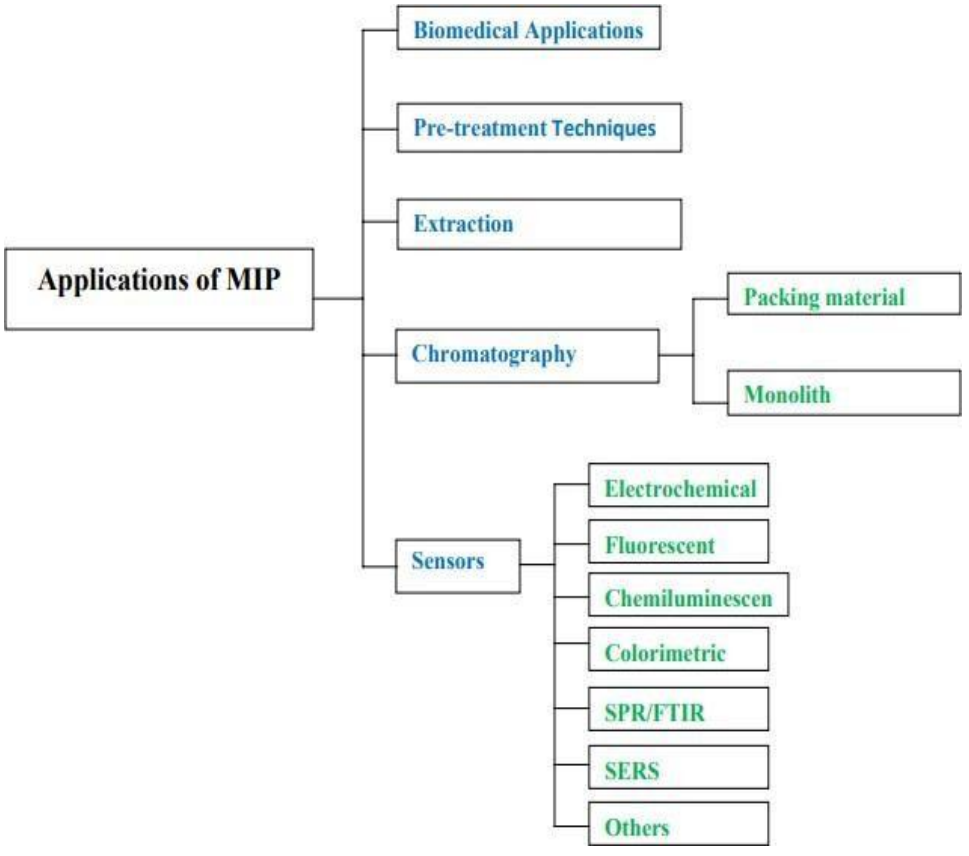


Figure 1.4: Different applications of MIP

1.6 Solid Phase Extraction

The measurement and identification of constituents in various compounds are getting more and harder every day. Many chemicals require some sort of initial sample preparation before they may be directly determined. Analyte and their associated compounds are present in real samples in considerable volumes but at low concentrations [Kantipuly and Westland, 1988].

SPE method is utilized for sample pre-concentration, extraction and clean-up before most of the above mentioned analytical techniques [Coulter *et al.*, 2012]. Although SPE's first experiment-based applications date back fifty years, it wasn't until the middle of the 1970s that this technique was applied to analytical tasks [Liška, 2000]. However, SPE is a traditional technique with concerning demerits. It is a multi-step and time consuming method as the sample containing target analyte goes through different stages; like clean-up, pre- concentration and fractioning which may correspond to significant drop of target analyte throughout these stages [Šafařı´ková and Šafařı´k, 1999; Wierucka and Biziuk, 2014; Andrade- Eiroa *et al.*, 2016]. Additionally, lack of selectivity results due to co- extraction of unwanted analyte during sample preparation from complex samples. Usage of extensive volumes of toxic organic solvents for elution and washing of SPE adsorbents also poses negative impact on environment as well as analyst. This also warrants an added pre-concentration step of last extract before further evaluation.

In intend to minimize the adverse outcome of analytical processes upon nature, the idea of green analytical chemistry (GAC) has emerged which emphasizes the usage of least or zero amount of toxic organic solvents and minimal generation of organic waste. Therefore, a future analytical procedure has to be established considering 12 principles of GAC [Baggiani *et al.*, 2007; Andrade-Eiroa *et al.*, 2016]. These concerns have paved the way to evolve new methods, such as dispersive solid phase extraction (DSPE) which is a miniaturized configuration of SPE.

1.6.1 Dispersive Phase Extraction

DSPE, in this context, is one of the forms of the technology that streamlines the process and shortens the amount of time needed for extraction and clean-up. DSPE does not need packing the sorbent into a column, in contrast to traditional SPE. There

is no need for a conditioning step because it interacts immediately with the liquid sample containing the analyte. In order to ensure good interaction between the packed sorbent and the target molecules (and to avoid channeling), maintaining a steady and regulated flow velocity of the sample is essential in traditional SPE. However, DSPE permits quicker and more effective contact between the two phases. The direct analysis of materials containing microparticles or microorganisms is particularly well suited for DSPE, it should be noted. By adding the liquid sample or extract, which contains the analyte and possible matrix interferences, to the primary sorbent material, DSPE is frequently used as a purification technique [Wilkowska and Biziuk, 2011; Socas- Rodríguez *et al.*, 2015].

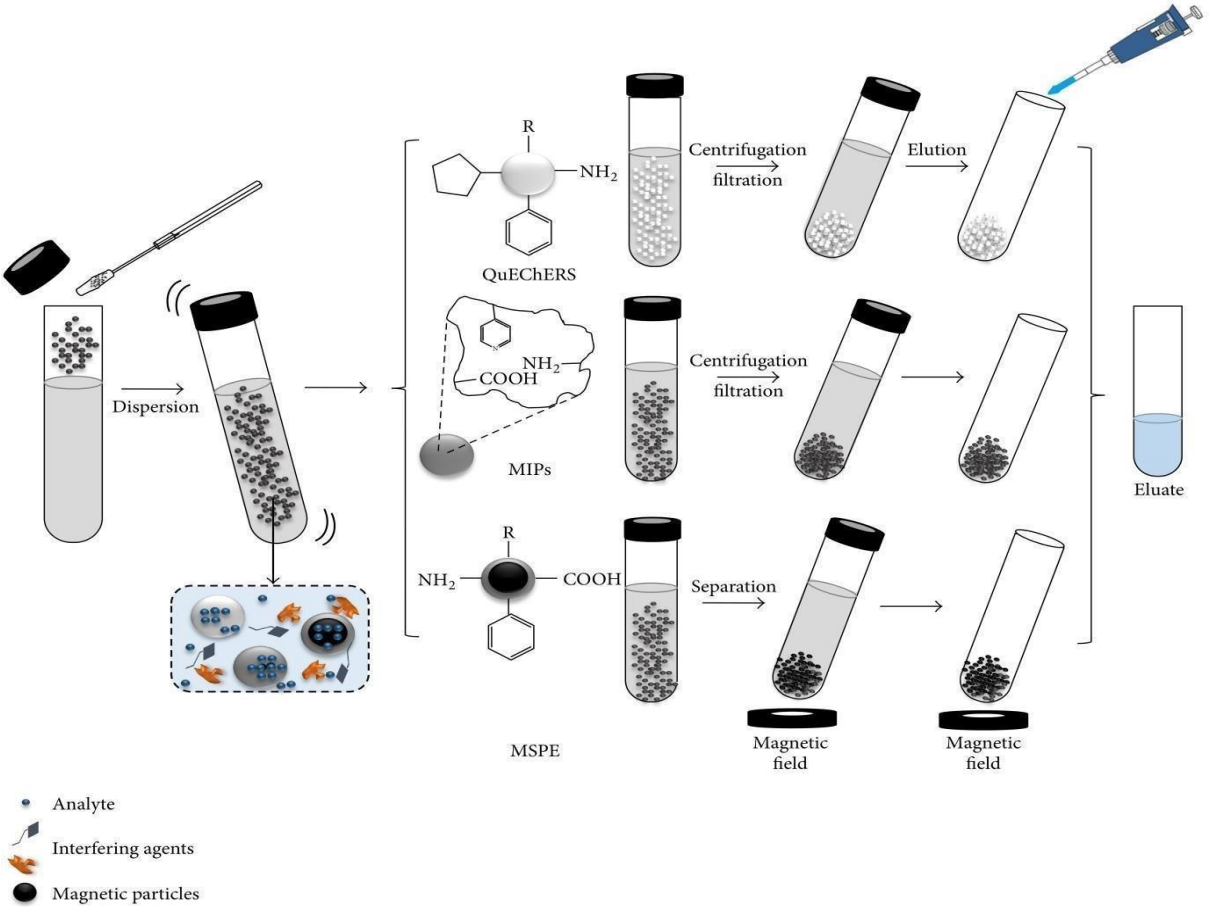


Figure 1.5: Diagrammatic representation of dispersive solid phase extraction (DSPE) applied in by various methods [Islas *et al.*, 2017]

Both the bulk of the loaded sample as well as the solvent selection are important factors in DSPE techniques. The effectiveness of the extraction process is directly influenced by the sample mass, and the solvent used to transport the sample is crucial

for keeping the chemicals on the adsorbent material. Additionally, depending on the DSPE method being utilized, different desorption solvents or solvent mixes are used during the washing and desorption processes in DSPE. It's crucial to choose a desorption solvent for the target chemicals that closely matches their polarity when it comes to desorbing them, keeping to the adage "like dissolves like" [Montes *et al.*, 2003; Büyüktiryaki *et al.*, 2020]. It functions by direct inclusion of a sorbent within the sample solution continued by dispersion facilitated by vortex agitation or ultra-sonication to facilitate sorbent-analyte contact. After finishing the dispersion process, the sample is subjected for centrifugation and the sorbent is collected in a separated vial where adsorbed analyte on sorbent are eluted or back extracted in little amount of organic solvent. In contrast to conventional SPE techniques, DSPE is rapid; offer high sample throughput, cost-effective and greener [Han *et al.*, 2014; Islas *et al.*, 2017], figure 1.5 shows different methods applied with DSPE.

1.7 Blood and Urine as Important Forensic Matrices

Examining biological samples like blood and urine for the purpose of identifying and measuring drugs and other compounds, such as toxins, falls under the category of forensic toxicology [Levine and Kerrigan, 2020]. To prepare samples for analysis, a broad variety of methods are available, ranging from easy methods to complex ones. These methods include phospholipid depletion, liquid-liquid extraction, supported liquid extraction, SPE, and dilutions (such as the "dilute and shoot" approach). Each extraction technique varies in terms of its level of complexity, the quantity of steps it requires, and its capability for automation. Additionally, these methods may be modified and scaled to meet the demands of the particular matrix type and sample volume. Blood and urine are two biological fluids that are frequently studied in forensic toxicological investigation as toxicokinetics and level of drug is generally higher in them as compared to other samples. The choice of alternative biological matrices, on the other hand, relies on a number of variables, including the availability of specimens, the interpretation criteria, the target analyte, sample acquisition (antemortem or postmortem), sample appropriateness, and stability. Additionally, the degree of decomposition might limit the number of appropriate specimens that are available for examination in postmortem forensic toxicological investigation [Mella *et al.*, 2018].

1.7.1 Blood and its Components

Blood, sometimes referred to as "whole blood," is a popular liquid media in the area of forensic toxicology; red and white blood cells, platelets, cellular fragments, and other molecules are all suspended inside the plasma, along with other components as shown in figure 1.6. About 8% of the weight of the human body is made up of blood [Dean, 2005; Burkhart *et al.*, 2012; Basu and Kulkarni, 2014]. The usual blood volume of an adult is five litres, or 1.3 gallons. Approximately 45% of blood is made up of whole blood, 54% is made up of plasma, and 1% or so is made up of white blood cells or platelets. Blood's red colour is caused by a substantial amount of haemoglobin and can vary in intensity depending on the interaction of heme groups with other substances, the degree of oxygenation, and other factors. Blood that has lost oxygen has a dark crimson colour. Notably, harmful drug levels may be determined by the colour of the blood [Hirsch *et al.*, 1977; Wirthwein and Pless, 1996]. The therapeutic half- life of the substances, the method of administration, previous exposure to the chemical (such as acute or chronic usage), pharmacogenomics, and other variables all play a role in the identification and quantification of medicines and their metabolites in blood.

Based on the type of collecting tube utilized, complete blood or certain blood components like plasma or serum may be obtained while taking samples [Basu and Kulkarni, 2014; Mathew and Varacallo, 2020]. In terms of interpretation, the choice of collecting site—such as arterial, venous, femoral, or cardiac blood—is important [Dinis-Oliveira, 2016]. Plasma is the name for the light-yellow liquid component of blood that, unless intentionally eliminated, retains suspended blood cells. In contrast, serum is extracted from blood using centrifugation, where the liquid component (serum) is then placed in a different tube. The most often examined blood components in forensic toxicological analysis are whole blood, plasma, and serum, but additional separated blood components may rarely be found. When preparing the sample and interpreting the data, it is crucial to take into account different sample preservation techniques and anticoagulants that may be added to the collecting tube, such as potassium oxalate, sodium fluoride, or ethylenediaminetetraacetic acid (EDTA) [Mathew and Varacallo, 2020; Jones *et al.*, 2021].

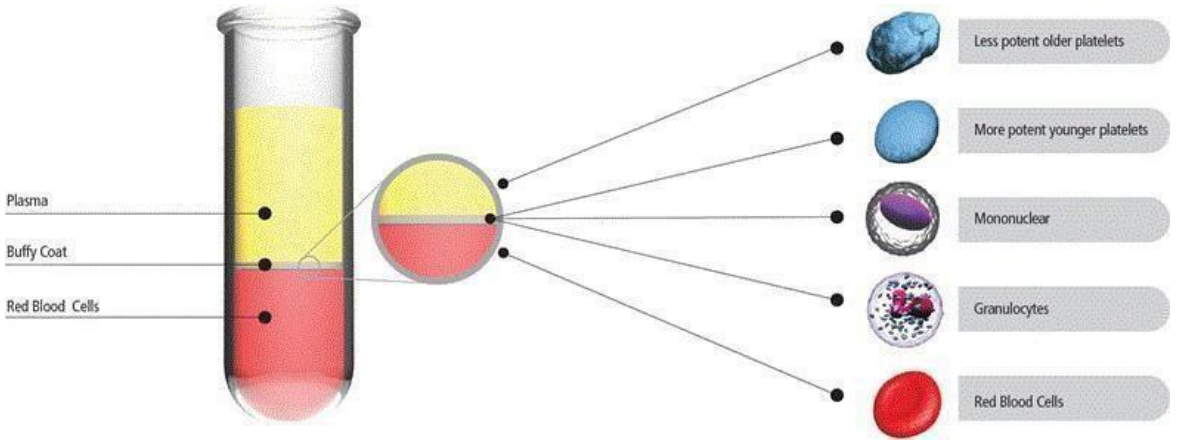


Figure 1.6: Showing composition of blood [Parrish and Roides, 2017]

1.7.2 Urine

The kidneys' liquid waste product, urine, is kept in the bladder [Fogazzi *et al.*, 2008]. It usually has a golden colour and is clear and translucent. A healthy adult typically excretes between 40 and 60 ounces of urine per 24 hours. Water, urea, uric acid, and salts make up urine, which has a ratio of 960 parts water to 40 parts solid particles [Verhamme *et al.*, 2008; Rohrig and Kunia, 2019]. Urine may, however, include sugars, albumin, bile pigments, or aberrant concentrations of its usual ingredients in people with health concerns. Urine from living humans is collected in a specialised container either by seen methods (such as workplace drug testing) or witnessed collection [Caplan and Goldberger, 2001]. The bladder in healthy humans has the ability to store up to 16 ounces of pee for 2 to 5 hours or longer [Moeller *et al.*, 2008]. Urine can be obtained postmortem by puncturing the bladder, collecting it using a syringe, and then transferring it to a suitable collection receptacle [Fogazzi *et al.*, 2008].

1.8 Gas Chromatography

The material is vaporized and then injected onto a column made particularly for gas chromatography (GC) as part of the GC procedure. A flow of inert gas carries the sample down the column. The column is made up of a support material, often fused

silica that is either packed with sorbent or covered with a thin liquid layer. In this method, the sample passes through the column while it is still a vapour thanks to a combination of adsorption and partition events. Due to these events, individual components in the sample mixture can be separated based on their particular affinities for adsorption and desorption processes. Figure 1.7 shows the diagrammatic representation of GC.

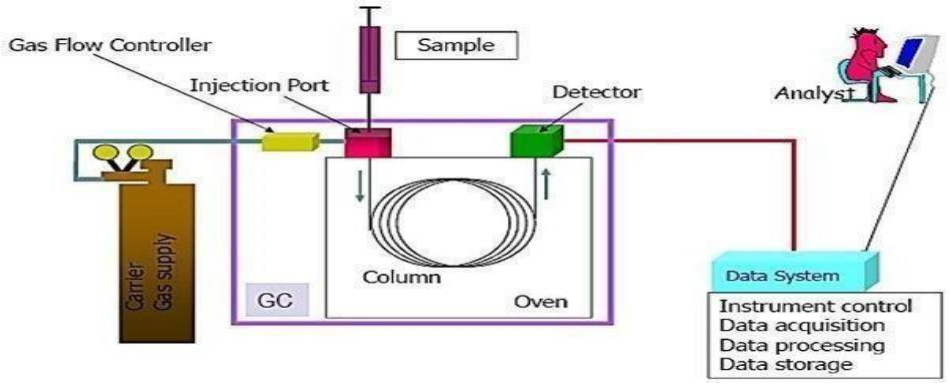


Figure 1.7: Shows the diagrammatic representation of gas chromatography [Obeidat, 2021].

1.8 Components

1.8.1.1 Carrier Gas

GC requires a carrier gas that is chemically inert. For this purpose, nitrogen, helium, argon, and carbon dioxide are often used gases. The type of detector being used is often taken into account while choosing the carrier gas. To remove water and other impurities from the carrier gas system, a molecular sieve is also included.

1.8.1.2 Sample Injection Port

It is crucial to insert a sample of the correct size onto the column as a vapour "plug" in order to attain the column's maximum efficiency. Large samples injected slowly risk band widening and resolution loss. The most often used injection method involves injecting the sample using a micro syringe inserted via a rubber septum into a flash vaporizer port located at the top of the column. The sample port's temperature is normally set to be around 50°C higher than the sample's least volatile component's boiling point. The sample size for packed columns might be anywhere between tenths of a microliter and twenty microliters. In contrast, capillary columns often require

much lower sample volumes, about 10-3 L. Injection with or without splitting are frequently used in capillary GC. Please see the illustration of a split/splitless injector supplied.

1.8.1.3 Columns

The two primary column types used in GC are packed columns and capillary columns, often known as open tubular columns. Packed columns are made of a finely split, inert solid support material—often diatomaceous earth—and a liquid stationary phase. The length and internal diameter of packed columns typically range from 1.5 to 10 metres and 2 to 4 millimetres, respectively. However, capillary columns sometimes have an internal diameter that is much smaller—just a few tenths of a millimeter. Wall-coated open tubular (WCOT) and support- coated open tubular (SCOT) are the two main varieties of capillary columns seen in GC. A capillary tube with walls coated in a liquid stationary phase is a characteristic of WCOT columns. On the other hand, SCOT columns consist of a capillary with the stationary phase adsorbed onto a thin layer of diatomaceous earth or another type of support material inside the capillary. It is important to remember that, generally speaking, WCOT columns tend to be more effective than SCOT columns. When compared to packed columns, both WCOT and SCOT capillary columns perform more efficiently. Capillary columns are strengthened by a polyimide coating, which adds strength, and have substantially less thicker walls as compared to glass capillary columns. These columns are adaptable and simple to coil. Capillary columns' physical toughness, adaptability, and low reactivity are benefits [Servcik, 1975].

1.8.1.4 Column Temperature

The boiling point of the material being examined determines the optimal temperature for the column in GC. The average elution time ranges from 2 to 30 minutes when the column temperature is chosen to be only somewhat more than the sample's average boiling point. Longer elution periods but often improved resolutions are the results of lower temperatures. The benefit of temperature programming may be seen when the sample has a wide range of boiling points. With this method, the column temperature is gradually raised as the separation advances, either continuously or in increments.

1.8.1.5 Detectors

There are several detector alternatives available in GC, each of which gives a distinct amount of selectivity. While selective detectors react to a certain spectrum of compounds possessing a similar physical or chemical feature, non-selective detectors only detect substances other than the carrier gas. Conversely, certain detectors are made to react to a single chemical molecule. Other types of detectors include those that depend on mass flow or concentration (table 1.1). The integrity of the sample is often unaffected by concentration- dependent detectors, which provide a signal proportional to the solute concentration. However, when makeup gas is used, sample dilution can have an impact on how these detectors respond. The sample is often altered by mass flow-dependent detectors, and their signals are dependent on how quickly solute molecules arrive at the detector. The presence of makeup gas has no impact on how mass flow-dependent detectors react. The separated components in the column are removed from it and put through the proper detector before being recognized.

Table 1.2 Showing types of detectors in GC [Lakshmi, 2015]

Detector	Type	Support Gases	Selectivity	Detectability	Dynamic Range
Flame Ionization (FID)	Mass flow	Hydrogen and air	Most organic compounds	100 pg	10^7
Thermal Conductivity (TCD)	Concentration	Reference	Universal	1 ng	10^7
Electron Capture (ECD)	Concentration	Make-up	Halides, nitrates, nitriles, peroxide, anhydrides, organometallics	50 fg	10^5
Nitrogen-Phosphorus	Mass flow	Hydrogen and air	Nitrogen, Phosphorus	10 pg	10^6
Flame Photometric (FPD)	Mass flow	Hydrogen and air possibly oxygen	Sulphur, Phosphorus, Tin, Boron, Arsenic, Germanium, Selenium, Chromium	100 pg	10^3

Photo Ionization (PID)	Concentration	Make-up	Aliphatics, Aromatics, Ketones, Esters, Aldehydes, Amines, Heterocyclics, Organosulphurs, some organometallics	2 pg	10^{7}

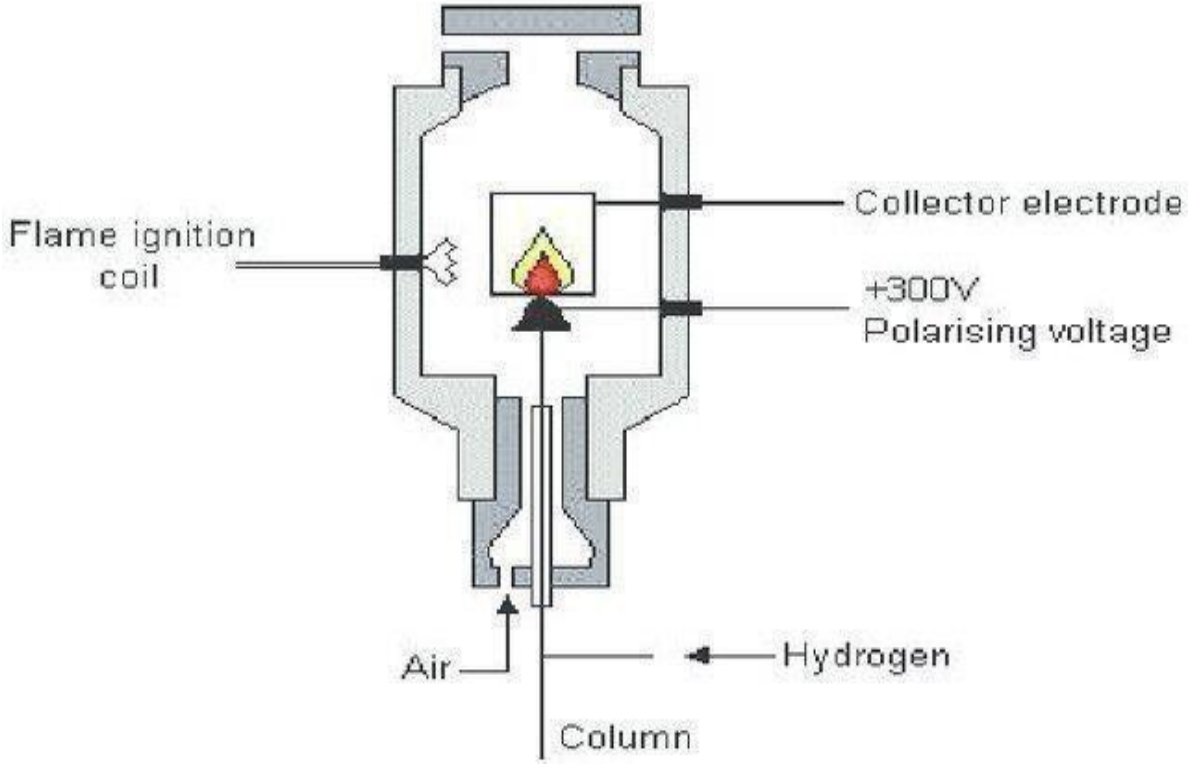


Figure 1.8: Diagram of flame ionization detector [Prasad, 2014]

As the effluent leaves the column, it is mixed with air and hydrogen before being burned. When organic materials burn, ions and electrons are released into the flame that have the ability to conduct electricity. A large electrical potential is created at the burner tip, and a collecting electrode is positioned above the flame. Any organic substances that are pyrolyzed produce a current that is measured. FIDs (flame ionisation detectors) (figure 1.8) are more sensitive to a compound's mass than to its concentration. This trait offers a benefit in that variations in the mobile phase's flow rate have no effect on the detector's response. FIDs are regarded as a good general detector for analysing organic chemicals since they are very sensitive, have a broad linear response range, display low noise, and do so. It should be emphasized,

nevertheless, that FIDs eliminate the sample. The literature offers an in-depth analysis of the operating concepts of GC detectors [Servcik, 1975].

Quantifying volatile and thermally stable chemicals may be done very well with GC. Various stationary phases' excellent selectivity makes it possible to achieve the best column separations. Investigating the impurity profile of left over solvents in medicinal compounds benefits greatly from this method [Poole, 2012].

1.9 Gas Chromatography Mass Spectrometry

Gas chromatography mass spectrometry (GC-MS) is an analytical technique that utilizes both a mass spectrometer and a gas chromatograph to effectively separate, identify, and quantify complex chemical mixtures. It is especially well suited for examining a variety of low molecular weight molecules frequently encountered in environmental contaminants [Gómez *et al.*, 2009]. A chemical must have adequate volatility and thermal stability to be suitable for GC- MS analysis. Additionally, functionalized compounds would need to undergo chemical modification (derivatization) before analysis in order to get rid of unwanted adsorption effects that can impair the quality of the findings. As samples are frequently analysed as organic solutions, relevant materials such as soils, sediments, and tissues must first be extracted using a solvent before being subjected to different "wet chemical" procedures. Only then can a GC-MS analysis be performed [Lavagnini and Magno, 2006].

A carrier gas, often helium, transfers the sample solution from the GC's intake onto a chromatographic column once it has been converted into vapor. The components of the mixture are separated based on how the sample interacts with the stationary phase (column coating) and mobile phase (carrier gas) as it passes through the column. The generation of ions can be accomplished in one of two ways. The most often utilised technique is electron ionisation (EI), however chemical ionisation (CI), an alternate technique, is also rarely used. EI involves the ionisation of the sample molecules by an electron stream, which results in the loss of one electron. The molecular ion, or M^+ (a radical cation), is the name given to a molecule that is missing one electron. The molecular weight of the chemical may be determined from the existence of this ion's peak in a mass spectrum [Mottier *et al.*, 2002; Colombini *et al.*, 2005].

Due to the substantial energy (70eV) that the molecular ion receives, it often fragments, giving rise to smaller ions with differing relative abundances. The molecular structure may be identified by these distinctive abundances, which operate as a "fingerprint". This knowledge is useful for locating compounds of interest and supporting the identification of ambiguous components in mixes. When it comes to CI, the procedure begins with the ionisation of methane (or another appropriate gas), which results in the production of a radical. The sample molecule is subsequently ionised by this radical, producing [M+H] + molecular ions. The ionisation process used in CI is less energetic than that used in EI, which leads to less fragmentation. As a result, CI yields the molecular ion but offers less comprehensive information about the molecule's structure. The two techniques are complimentary in that the molecular ion may not always be detected via EI. After being ionised, the ions are repelled from the ionisation chamber using a modest positive charge [Chauhan, 2014; Fischer and Scholz-Böttcher, 2019].

The mass analyzer, also known as a filter, is the next element in the setup which works by dividing the positively charged ions into different groups according to their distinctive mass- related characteristics, which change depending on the particular type of analyzer used. Quadrupoles, ion traps, magnetic sectors, time-of-flight analyzers, radio frequency analyzers, cyclotron resonance analyzers, and focusing analyzers are just a few examples of the various types of analyzers that are available. The next component of the arrangement is the mass analyzer, also referred to as a filter. Depending on the specific type of analyzer being used, it divides the positively charged ions into several groups based on their distinctive mass- related properties. The many kinds of analyzers that are accessible include quadrupoles, ion traps, magnetic sectors, time-of-flight analyzers, radio frequency analyzers, cyclotron resonance analyzers, and focusing analyzers, to name a few [Maštovská and Lehotay, 2003].

1.10 Infrared Spectroscopy

The study of infrared radiation falls under the umbrella of infrared spectroscopy. Between the visible and microwave bands, infrared radiation is distinguished by longer wavelengths and lower frequencies than visible light. Infrared spectroscopy is used to identify and analyse chemical substances and is based on absorption

spectroscopy methods [Tan *et al.*, 2021]. The infrared (IR) spectrometer is a typical laboratory tool used for sample examination utilising infrared light. Based on its distance from the visible section of the electromagnetic spectrum, the infrared component of the spectrum is split into three parts. The near, mid, and far infrared regions are the names of these divisions. The near infrared zone, which ranges in wavenumber from 14000 to 4000 cm^{-1}, has the highest energy of these areas. Mid-infrared light, which has a wavelength between 4000 and 400 cm^{-1}, is used to study the basic vibrations of molecules. Last but not least, the far infrared ranges from 400 to 10 cm^{-1} and is located near to the microwave range [Kondepati *et al.*, 2007].

1.11 Scanning Electron Microscopy

For microanalysis and analysis of solid inorganic materials, scanning electron microscopy (SEM) analysis or SEM microscopy, is very efficient. Usually, a certain region of the sample's surface is used to collect data in order to produce a two-dimensional image that illustrates how attributes vary spatially. It is feasible to examine regions using traditional SEM methods that range in width from 1 cm to 5 microns in a scanning mode, with magnifications of 20X to 30,000X and a spatial resolution of 50–100 nm. The capacity of SEM to analyze individual point locations on a sample is another characteristic that comes in handy for qualitative or semi-quantitative investigations into the chemical compositions, crystalline structures, and crystal orientations. The SEM's design and operation have significant similarities to other instruments' in terms of their capabilities [Tomohiro *et al.*, 2017]. Figure 1.9 shows diagrammatic representation of scanning electron microscopy.

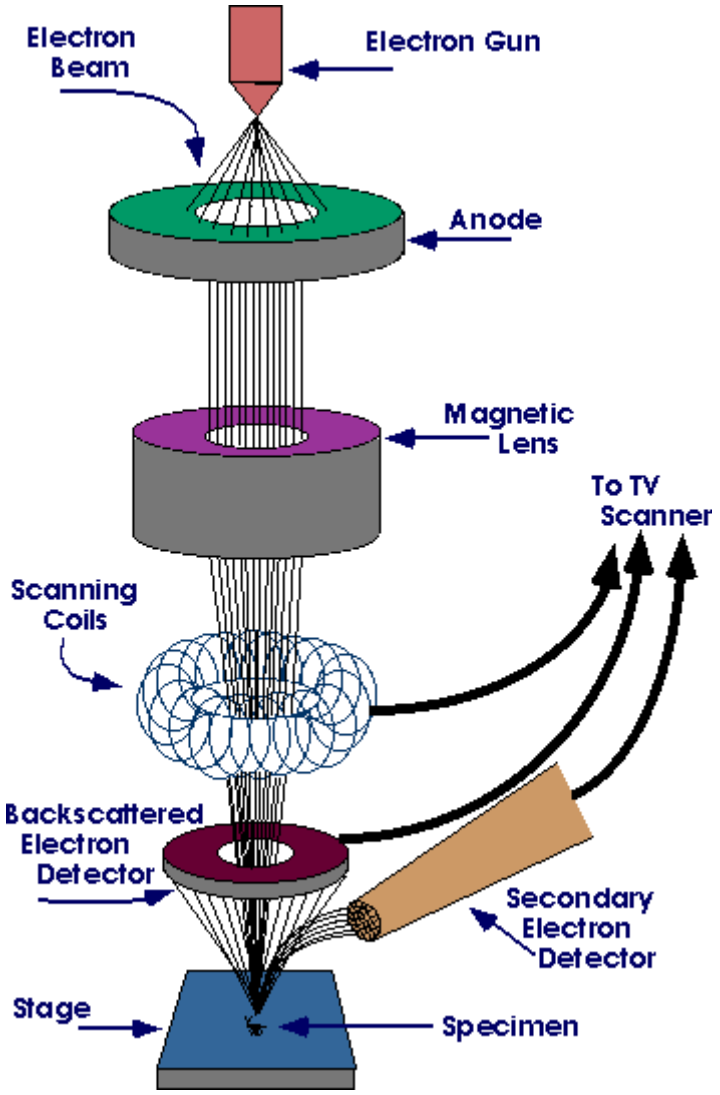


Figure 1.9: Diagrammatic representation of Scanning electron microscopy [Shawky, *et al.*, 2016]

1.11.1 SEM Image Development

Focused streams of powerful electrons are used by the SEM to create a variety of signals on the surface of solid objects. These signals are the consequence of electron association with the sample and provide details on its crystalline structure, material orientation, chemical composition, and external morphology (texture).

A SEM's high-speed electrons have a lot of kinetic energy, which they release through interactions with the sample as a variety of signals. When the incident electrons slow down inside the solid sample, these interactions take place. Secondary electrons, backscattered electrons, diffracted backscattered electrons, photons (specifically X-

rays used for elemental analysis), continuum X-rays, visible light, heat, and backscattered electrons are some of the signals that are produced; these secondary electrons are responsible for the formation of SEM images. Secondary electrons and backscattered electrons are frequently used for sample imaging. Backscattered electrons excel in revealing composition differences in multiphase samples, enabling quick phase classification, whereas secondary electrons are very good for displaying the morphology and topography of materials. Inelastic collisions between incoming electrons and electrons occupying particular orbitals (shells) of atoms inside the sample produce X-rays. The wavelength of the X-rays that excited electrons release when they transition to lower energy states corresponds with the energy difference between electrons in various shells of a particular element [Newbury and Ritchie, 2013; Abd Mutalib *et al.*, 2017].

1.12 Need and Scope of the Study

CSP is a centrally acting muscle relaxant which is commonly used by people suffering with musculoskeletal problems. Its abuse potential is similar to benzodiazepines and it has been reported to be used for its calming and soothing effects or to enhance the effects by combining with other drugs. CSP abuse has reportedly increased in past few years [Reeves and Burke, 2010; Reeves *et al.*, 2012]. A survey was conducted of 40 patients who used CSP for a longer period and it revealed that 40% of the patients accepted of consuming the drug in excess amount than prescribed, 30% patients were reported of consuming this drug for its effect other than its main function, 10% of the patients reported to use this drug to enhance the effect of other drugs, some were reported to obtain extra CSP with the help of prescription [Reeves and Burke, 2010]. A person‘s body builds resistance after chronic use of the same drug which ultimately leads its abuse and overdose of the drug can lead to fatalities. In a case report, three overdose cases of CSP were detected and it revealed that they were dealing with depression and took overdoses of CSP [Backer *et al.*, 1990]. Moreover, studies on CSP have shown traffic accidents due to psychomotor impairment after the intake of CSP [Bramness *et al.*, 2008]. Hence, several drug abuse and death related to overdose and impaired driving cases come in forensic labs. Therefore, it is important to make easy, highly specific and less time consuming drug extraction and detection method for better and fast detection. Pie chart below is showing the increased use of pain

relievers and muscle relaxants (figure 1.10). Therefore, CSP was considered for the current research.

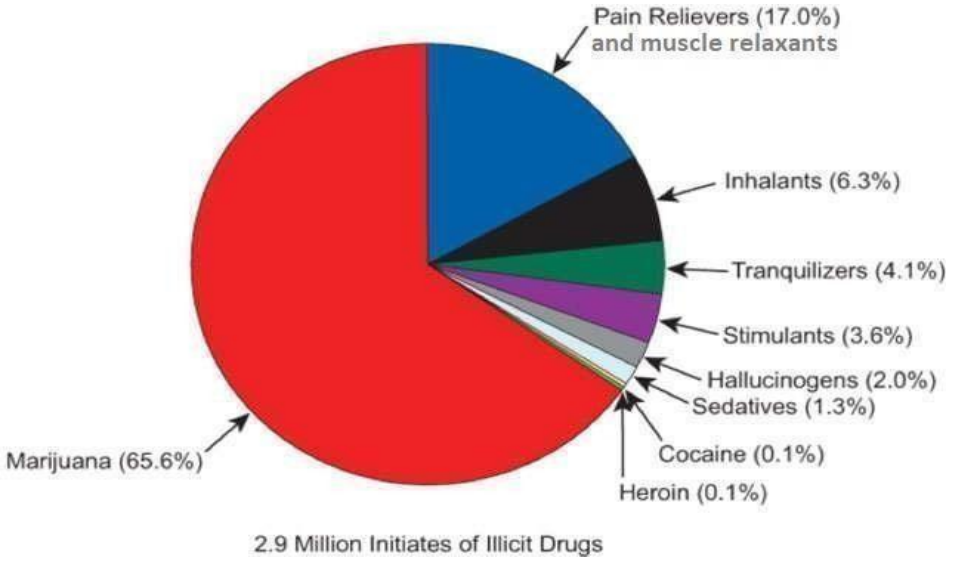


Figure 1.10: Showing the increased use of pain relievers and muscle relaxants

The MIP technology has the potential to make significant advances to the field of forensic research because of its specificity, selectivity and efficiency. Its high specificity makes it suitable for its clear identification, and its effectiveness reduces the amount of time needed. As a result, this method becomes a useful tool in forensic laboratories, facilitating simple extraction and analysis.

Chapter – 2

Literature Review

2.1 Literature Review

Backer *et al.*, (1990), researchers reported a comprehensive investigation of three tragic cases of CSP overdose. In this study, they carefully examined CSP concentrations in various body fluids such as urine, vitreous humor, heart and femoral blood. The first case was of a woman who tragically committed suicide by taking CSP. In a second case, another woman attempted suicide using a combination of verapamil and CSP due to her dire circumstances. The study also drew attention to the tragic death of a depressed man attributed to the interaction of propoxyphene and CSP. Using an advanced analytical method, GC-MS, researchers were able to successfully identify these drugs. The results of this study provide important insight into the effects and circumstances surrounding CSP overdose and highlight the importance of careful monitoring and awareness-raising in the medical community.

Sikdar *et al.*, (1993), carried out a thorough study of a total of 16 carefully documented cases of CSP abuse. These examples were from the Drug Addiction Treatment Centre at the prestigious Graduate School of Medical Education and Research in Chandigarh. Interestingly, the results of this study revealed a disturbing pattern of patients in whom abuse of CSP, originally used as an opiate substitute, emerged as a common solution. This highlights the urgent need to address the underlying issues surrounding CSP abuse and explore better alternative options for efficient extraction and detection techniques. Surveys of patients also revealed a frequent tendency toward poly drug users, in which CSP and different drugs were used concurrently. This multifaceted substance abuse illustrates the complexity of substance abuse, requiring comprehensive strategies to manage the complex web of addictions. By thoroughly documenting and investigating these cases, this study served as a useful resource in understanding the complex dynamics of CSP use, ultimately leading to targeted interventions and thorough help develop a more effective treatment plan.

Mosbach (1994), described how a new technique called molecular imprinting was utilized to create synthetic polymers with specific recognition sites. These polymers exhibited exceptional stereo- and regiospecific selectivity by polymerizing functional monomers in the presence of a particular molecule (template). Their practical usage in a variety of applications, including as the chiral separation of bioactive compounds

and acting as antibody mimics, was made possible by this property. Recent research showed the potential of these custom separation materials, making the possibility of their commercial use conceivable.

Venugopal *et al.*, (2000), in their noteworthy study, documented a compelling case of CSP dependence observed in the outpatient department of a well-known rehabilitation hospital. The patient was a 40-year-old man who had been taking CSP tablets for 7-8 years. A friend of his first turned to this drug for its euphoric effects. To achieve the same satisfactory effect, patients increased their dose over time, resulting in chronic use of CSP. Attempting abstinence resulted in withdrawal symptoms such as restlessness, anxiety, and sweating. This example highlights the need for targeted therapy and support networks for people suffering from drug addiction by highlighting the dangers and difficulties associated with long-term use of CSP. Its research is essential in forensics because of the potential for fatal overdoses from addiction. Studies of its characteristics and effects are helpful in understanding overdose-related mortality. Extensive research on CSP would improve forensics' ability to assess and assign responsibility for death and help develop prevention and treatment.

Anastassiades *et al.*, (2003), presented a low-cost technique for finding pesticide residues in food. A 10 g sample is extracted with 10 mL of acetonitrile, and then 4 g of MgSO4 and 1 g of NaCl were used for liquid-liquid partitioning. Using 150 mg of $MgSO_4$ and 25 mg of PSA sorbent, dispersive solid-phase extraction (dispersive-SPE) was used to remove any remaining water and contaminants. The technique successfully got rid of polar substances such organic acids, pigments, and sugars. For accurate pesticide analysis, GC/MS was utilized. With repeatability below 5% for a variety of pesticides, including polar chemicals, recoveries vary from 85% to 101%. Six samples might be created by one chemist in approximately thirty minutes for a cost of about one dollar each.

Bramness *et al*,. (2004), examined the adverse effects of CSP on drivers. The Department of Forensic Toxicology and Drug Addiction at the Norwegian Institute of Public Health provided researchers with a comprehensive database of 62 cases for analysis. Of note, the only drugs consistently detected in all cases were CSP and meprobamate. Results showed that a significant number (73%) of drunk drivers had higher blood levels of CSP than non-drink drivers (27%). However, there was no

discernible difference in meprobamate levels for any driver. CSP and benzodiazepines had similar clinical effects on clinical impairment tests using the Clinical Impairment Test (CTI), although several different features such as involuntary movements, hand tremors, and tachycardia were present. This study addressed the urgent need to understand how CSP affects driving performance and comprehensive measures to reduce the hazards posed by CSP use while driving.

Caro *et al*,. (2004), using naproxen, a non-steroidal anti-inflammatory medication (NSAID), as the template molecule, a MIP was created. Chromatographic analysis verified the MIP's imprinting action. Then, to selectively extract naproxen, the MIP was used as a selective sorbent in SPE. Additionally, the MIP was used to extract naproxen from urine samples, proving that structurally similar chemicals could be successfully removed from the sample by selective washing with acetonitrile (ACN).

Kim *et al.*, (2005), developed sensitive and precise method using solid-phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS) for carisoprodol (CSP) extraction from human hair sample as CSP was noted to be abused in Korea. The taken linear ranges were 0.5- 10.0 ng/mg for CSP and MPB. This method showed good recoveries (91.5-93.1 % for CSP, 85.5-93.0% for MPB), with LODs of 0.13 ng/mg and 0.12 ng/mg, respectively. These results highlight the significance of advance extraction technique with more precision and specificity.

Ariffin *et al.*, (2007), evaluated new MISPE (Molecular Imprinting Solid Phase Extraction) technique developed to extract diazepam from hair samples. Regarding the extraction of target chemicals, the newly developed approach showed remarkable accuracy, specificity and sensitivity. The results were in agreement to ELISA method and SPE. It is important to note that the MISPE approach showed exceptional accuracy and precision in identifying trace levels of benzodiazepines, their metabolites and diazepam in hair samples. These results highlight the utility of the MISPE technique as a robust and reliable tool for the detection and analysis of benzodiazepines in forensic and toxicology research, and provide insightful information for improving diagnostic and screening techniques.

McCluskey *et al.*, (2007), provided a thorough explanation of the manufacturing of MIPs and looked at possible uses for them in forensics in the future. Modern detection tools used in forensic analysis have built-in drawbacks include reduced specificity,

sensitivity, false positive outcomes, and higher cost rates. As a result, the combination of MIP-based sensors and detection techniques seemed as a game-changing strategy, offering unmatched capabilities for evaluating forensic materials like narcotics and explosives. The accuracy and effectiveness of forensic investigations may be improved by forensic scientists by overcoming current obstacles and ushering in a new era of accurate, sensitive, and cost-effective analysis by taking use of the distinct molecular recognition capabilities of MIPs.

Bramness *et al.*, (2007), conducted a thorough analysis to determine whether long-term use of CSP causes psychomotor disturbances and thereby increases the risk of road traffic accidents. This study involved collecting and analyzing information from the Norwegian Traffic Accident Register on drivers involved in crashes under the influence of alcohol. A potential association was identified by comparing CSP - exposed drivers with those who were not. Surprisingly, the results showed that people taking CSP had a significantly higher risk of road accidents than other drugs such as diazepam and salbutamol. These results emphasize the importance of considering possible psychomotor side effects of CSP before engaging in activities that require the highest possible cognitive and motor function, such as driving, underscores the need for greater caution in assessing the health status of people taking this drug.

Anderson *et al.*, (2008), carried out a study describing the efficiency of two extraction techniques, conventional SPE and a novel molecularly imprinted solid-phase extraction system (MISPE). Comparative analysis of benzodiazepine assays was done in 10 post- mortem scalp hair samples. Subsequently, liquid chromatography tandem mass spectrometry (LC-MS-MS) was used for detection. Both extraction techniques successfully localized all benzodiazepine species in the samples, including diazepam, nordiazepam, oxazepam, temazepam and nitrazepam. But when compared to SPE, the MISPE approach showed better selectivity since it was able to identify benzodiazepines like diazepam, nordiazepam, and oxazepam in many cases when SPE was unable to. Notably, the SPE extraction technique was the only one that could find temazepam. These results highlight the increased selectivity of MISPE over conventional SPE and its potential advantages in the detection of particular benzodiazepines. The work adds to the development of strong and dependable analytical procedures by offering useful insights into optimising extraction methods

for benzodiazepine analysis in forensic investigations.

Gholivand and Khodadadian (2011), made a significant contribution when they investigated the possibility of MIPs as a sorbent for the molecularly imprinted extraction of the muscle relaxant methocarbamol. Despite being widely used, the traditional solid-phase extraction (SPE) method has drawbacks such limited selectivity, poor extraction recovery, and difficulties establishing selective extraction from complicated matrices. The study looked into using MIPs as an alternative sorbent for better selectivity and extraction efficiency to get around these constraints. MIPs provide improved specificity and elevated recovery rates by using the unique imprinting process, enabling the selective extraction of methocarbamol from complicated matrices. This study highlights the potential of MIPs as a useful analytical tool, overcoming the drawbacks of conventional SPE procedures and providing new opportunities for enhanced molecularly imprinted extraction methods in the study of pharmaceutical substances like methocarbamol.

Figueiredo *et al.*, (2011), described how for the direct extraction and quantification of benzodiazepines in human plasma, a combination of SPE, MIP, and electrospray ionization mass spectrometry (ESI- MS) was used. The target analytes are selectively extracted by the MIP and then subjected to direct ESI-MS analysis. This method greatly accelerates analysis speed by reducing ionic suppression during ESI, doing away with the necessity for pre- separation. As a case study, human plasma samples containing medazepam, nitrazepam, diazepam, chlordiazepoxide, clonazepam, and midazolam were used. For medazepam, nitrazepam, diazepam, chlordiazepoxide, and midazolam, the MIP-ESI-MS method demonstrated excellent analytical performance, with calibration curves ranging from 10 to 250 μg L^{-1} ($r > 0.98$), a limit of quantification of 10 μg L^{-1}, and acceptable precision and accuracy in within-day and between-day measurements.

Nebhinani *et al.*, (2013), conducted a thorough investigation to gauge public knowledge of CSP misuse. 34 participants in all, including singles, jobless people, and members of urban nuclear families, participated in the study. It is interesting to note that all participants had a history of substance addiction before taking CSP. The study also found that CSP 's calming effects were dose-dependent. The complicated interaction between a person's personality, prior substance addiction, and the

subjective experiences linked to CSP use is highlighted by these findings, underscoring the necessity of focused interventions and specialized treatment strategies.

Sarafraz-Yazdi and Razavi (2015), preparation of the sample is still an essential stage in the analytical process even with major improvements in analytical instruments. Its goal is to concentrate target analytes while removing interferences. The use of MIPs has improved selectivity during extraction. MIPs have uses in chemical separation, selective extraction, catalysis, and molecular sensing due to their particular affinity for the template molecule. The usage of MIPs in various forms of solid-phase microextraction (SPME) was the main topic of this review. Researchers had increased selectivity and extraction efficiency by using MIPs in SPME. The review sheds light on the many ways MIPs was used to improve SPME method performance.

Li *et al.*, (2016), in order to extract triazine herbicides from grape seeds, their research combined the molecular imprinting technology with MI- DSPE. The synthesis and characterization of MIPs were successful. Through the employment of these MIPs as adsorbents in DSPE, four triazine herbicides could be extracted quantitatively and cleaned up from grape seeds simultaneously. The best washing solvents, washing times, and solvents for eluting were identified. HPLC was used to verify the technique, which demonstrated strong linearity ($r^2 > 0.9993$) in the concentration range of 0.010-5.0 µg g^{-1}. With relative deviations ranging from 1.2% to 10.7%, recoveries at two spiking levels (1.0 and 2.0 µg g^{-1}), varied from 81.2% to 113.0%.

Cela-Pérez *et al.*, (2016), a new technique integrating MISPE and liquid chromatographytandem mass spectrometry (LC-MS/MS) was designed for the identification of Δ9- tetrahydrocannabinol (THC), 11-nor-Δ9- tetrahydrocannabinol carboxylic acid (THC- COOH), cannabinol (CBN), and cannabidiol (CBD) in urine and oral fluid (OF). The MISPE procedure employed cylindrical molecularly imprinted tablets. A non-imprinted polymer library (NIP-library) screening was used to determine the best MIP composition, with acrylamide serving as the functional monomer and ethylene glycol dimethacrylate serving as the cross- linker. The devised technique showed linearity, accuracy, good extraction recovery, process efficiency, and matrix effect. It is the first MISPE-based technique for determining THC, THC-

COOH, CBN, and CBD in urine and OF after the optimised method was successfully used to analyse urine and of specimens.

Yilmaz *et al.*, (2017), studied the present application of MIPs in several areas, including the food industry, environmental monitoring, and medical. MIPs have shown astounding effectiveness in a variety of tasks, including sensing, separation, purification, and sample treatment. This is due to their remarkable identification skills and innate specificity, which make them ideally suited for forensic testing applications. MIPs are especially well-suited for the detection of illegal drugs, doping agents, poisons, and other analytes of forensic relevance because they provide benefits including greater selectivity, shorter analytical times, and increased sensitivity. The thorough assessment highlights how MIPs have the potential to completely transform the area of analytical testing and offers insightful information on their growing uses in forensic research.

Sánchez-Gonzalez *et al.*, (2018), created a MIP that is ideal for cannabis extraction from human urine samples. Micro-solid phase extraction using MIPs (MIP--SPE), which targets the desired analyte specifically, was used for the extraction. After using MIP-SPE to extract the cannabinoids, HPLC-MS/MS was used to analyse the extracted cannabinoids quantitatively. A thorough technique validation revealed outstanding accuracy and recovery rates, confirming the new approach's robustness and dependability. To develop analytical methods in forensic toxicology and drug testing, this novel technology using MIP-SPE provides a viable path for the effective extraction and precise quantification of cannabis from urine samples.

Ebrahimi Rahmani *et al.*, (2018), documented the use of magnetic MIPs to specifically extract morphine from blood and urine samples. This new method facilitates accurate determination of morphine concentrations in biological samples. Extracted morphine in the samples was monitored and quantified using HPLC and UV detection. The use of magnetic MIPs as a selective extraction tool represents a major advance in analytical chemistry by providing a reliable and effective approach for measuring morphine in biological matrices. These findings enable the correct assessment and continuous monitoring of morphine concentrations in patients and have important implications for forensic toxicology and clinical practice.

Ferrari Júnior and Caldas (2018), Concerning the concurrent quantification of 14 pesticides, illicit drugs, prescription medications, and metabolites in human postmortem blood, a d-SPE protocol combined with GC-MS analysis using large volume injection- programmed temperature vaporisation (LVI-PTV) was optimised, obviating the need for derivatization. Excellent repeatability, linearity, moderate accuracy, and recovery were all displayed by the approved technique. It worked well when used to analyse 10 actual postmortem blood samples since it was rapid and easy to put into practice in forensic labs.

Malik *et al.*, (2019), reviewed the wide range of uses for MIPs in analytical chemistry were carefully considered. The authors further divided these applications into three separate categories: chromatography, sensors, and extraction. MIPs were demonstrated to provide effective and selective extraction of target analyte from complicated matrices in the extraction domain. MIPs' potential to deliver sensitive and precise detection of a variety of analyte was proved by their use in sensor technologies. MIPs have proven to be useful stationary phases in the field of chromatography, providing accurate separation and analysis of target chemicals. This thorough study emphasises the versatility of MIPs and their important contributions to the advancement of analytical chemistry, providing fresh opportunities for the creation of specialised extraction methods, sensing platforms, and chromatographic approaches.

Manousi *et al.*, (2019), a newly developed substance known as oxidised graphene oxide (OGO) was created by researchers, and it was utilised to remove rare earth elements (REEs) from water and nuts. To characterise the material, they used a variety of approaches. They identified the variables affecting extraction recovery using Response Surface Methodology (RSM) and optimisation approaches. OGO nanoparticles were successfully used in the sample preparation procedure, producing excellent enrichment factors and extraction recoveries. The technique was successfully used to find minute amounts of REEs in tap and mineral water, as well as in peanuts, almonds, and walnuts.

Lu *et al.*, (2019), Using norfloxacin (NOR) and enrofloxacin (ENR) as templates,precipitation polymerization was used to create dual-template molecularly imprinted polymers (dt-MIPs). For the simultaneous identification and extraction of

the two fluoroquinolones (FQs), these dt-MIPs showed outstanding selectivity and great adsorption capacity. Using HPLC, the efficacy of dt-MIP-based DSPE was improved. The technique produced good results, with recoveries ranging from 80.9% to 101.0% and relative standard deviations ranging from 0.9% to 6.9%. The FQs were effectively recovered from spiked lake, sea, and tap water samples.

Yu *et al.*, (2019), the simultaneous assessment of amide, dinitroaniline, and substituted urea herbicides in bivalve shellfish samples has been examined, leading to the development of a modified rapid, easy, affordable, reliable, and secure sample preparation method known as QuEChERS. In bivalve shellfish extracts, pigments including -carotene, chlorophyll A, and fucoxanthin demonstrated significant group-selective interactions with a MIP made by bulk polymerization. In order to effectively extract matrix components from bivalve shellfish samples, a modified QuEChERS approach based on MIPs and primary and secondary amines was developed. Results showed that 26 amide/dinitroaniline/substituted urea herbicides were effectively identified in bivalve shellfish using the suggested QuEChERS approach in combination with GC-MS/MS.

Wang *et al.*, (2019), for the purpose of finding pyraclostrobin in ginseng samples, a fresh technique was created. HPLC and molecularly imprinted dispersive solid-phase extraction (MIDSPE) are combined. Pyraclostrobin was used as a template to produce MIPs. The MIPs demonstrated significant binding affinity and pyraclostrobin-specific recognition. MIPs produced better recoveries (77.6–93.5%) than C18 sorbent while having lower standard deviations (2.18–6.07%) and relative standard deviations (RSDs) (2.47–6.99%). y=2.09935e- 006x+0.0176235 (R^2=0.998656) served as the quantification equation, and the limit of detection (LOD) was set at 0.01 mg kg^{-1}. Trace pyraclostrobin in ginseng samples was successfully isolated and concentrated using the MIDSPE-HPLC technique. According to analysis, pyraclostrobin levels in the European Union were below the maximum residue limit (MRL) of 0.1 mg kg^{-1}.

Bouvarel *et al.*, (2020), presented the on-line connection of a MIP to nano-liquid chromatography (nano-LC) in order to identify cocaine and its main metabolite, benzoylecgonine, in human plasma and saliva samples. This ground-breaking technique made it simple and accurate to identify the target analytes from these complex biological matrices. Selective extraction and effective separation of cocaine and benzoylecgonine were accomplished using the MIP-nano-LC method,

allowing for sensitive detection and quantification. This method provides a potential tool for forensic and clinical investigation, boosting the capacity to precisely identify and quantify cocaine and its metabolite in complicated biological materials, hence supporting enhanced diagnostic and screening skills.

Mulder and Halquist (2020), raised awareness of the crucial value of precise sample preparation, detection, and identification in the area of forensic toxicology. The authors emphasized that slowness and lack of selectivity are two problems with standard extraction techniques used in forensic sample analysis. The creation and use of MIPs has become a viable new approach for drug extraction in forensic research in order to get around these restrictions. MIPs enable improved target molecule extraction from complicated matrices with increased selectivity, specificity, and efficiency. MIPs can help forensic investigators get better extraction and analysis findings, which makes it easier to identify and quantify drugs and poisons in forensic samples. This innovative strategy has the ability to completely transform the forensic sciences area and improve its skills in forensic toxicology investigations.

Scigalski and Kosobucki (2020), explained how prior to quantitative or qualitative analysis, a target analyte can be extracted from a complicated sample matrix using a process called SPE. Such treatment serves two purposes: removing matrix components that can obstruct the detection process or potentially harm analytical equipment, as well as enriching the analyte in the sample so that it is easily detectable. DSPE, a more recent improvement on the traditional SPE method, have gained popularity due to its amazing simplicity, speedy extraction time, and reduced solvent expenditure requirements, as well as its excellent efficacy and broad application.

Ramanavicius *et al.*, (2021), highlighted the relevance of improving the development of fast affinity sensors. The use of polymer-based semiconducting materials as a key strategy in the field of affinity sensors was thoroughly examined in the review. The authors emphasise the many applications of these materials while highlighting how important it was for them to provide precise and sensitive detection across a range of sensing systems. Researchers‘ have developed very effective and selective affinity sensors by using the special qualities of polymer-based semiconducting materials, such as their adjustable electronic characteristics and variable functionalization. This thorough study is a helpful tool for researchers in the field, illuminating the most

recent developments and promising future directions in the use of polymer-based semiconducting materials for the creation of quick affinity sensors.

Shah *et al.*, (2021), discussed how MIPs have been synthesized in a variety of forms, including beads, particles, membranes, fibres, and composites. The authors emphasised MIPs' critical importance and their numerous applicability across diverse fields. Due to their adaptability, MIPs may be used for a variety of purposes, including as selective sensing, separation, medication delivery, catalysis, and environmental remediation. This work offers important insights into the design and manufacturing of MIP-based materials customised for certain applications by examining the synthesis techniques and various MIP shapes. The thorough knowledge of MIP synthesis and its many forms increases its significance as a flexible tool in several scientific and technical sectors.

Ptotka-Wasylka and Wojnowski (2021), stated that it might be difficult to assess how analytical methods incorporating pre-analytical procedures affect the environment. In order to solve this, the green analytical procedure index (GAPI), which already exists, is being replaced with a new measure called ComplexGAPI. In a hexagon-shaped pictogram, ComplexGAPI adds new fields to depict several facets of the pre-analytical process. If certain conditions are satisfied, each field is highlighted in green. By comparing analytical procedures for detecting pesticides in urine samples, ComplexGAPI's efficacy was established. The chemical community is anticipated to pay attention to, trust in, and adopt ComplexGAPI. Freeware software for creating ComplexGAPI pictograms has been created to make it easier to use.

Maranata *et al.*, (2021), described how in contrast to traditional solid-phase extraction (SPE), MIPs were used as sorbents in molecularly imprinted solid-phase extraction (MISPE). The intricacy of the variables necessitates the optimisation of MISPE conditions. To reach sorption equilibrium and reduce extraction time, variables such contact duration, ion strength, pH, sorbent quantity, flow rate, salt and buffer addition, and solvents need to be optimized. For optimal interactions between sorbent and analyte, each factor's characteristics and dosage must be chosen carefully. The right bonding, sample flow rates, extraction times, salt addition, and sorbent mass all have a role in factors like % recovery. To guarantee good interactions and obtain the best findings, optimizing MISPE in future research should take these

parameters into account and make the necessary adjustments.

Song *et al.*, (2022), discussed MIPs wide utilization in chromatographic and electrophoretic separations. The review emphasized how MIP-based materials may be used in a variety of ways to provide effective and precise separations. MIPs offer enhanced resolution and sensitivity in methods like HPLC, GC, and CE by using their special molecular recognition capabilities. For researchers looking to improve separation science using MIP-based materials, this thorough review offers insightful information.

Arabkhani *et al.*, (2022), the research aimed to provide a feasible and accurate approach for identifying the food adulterant metanil yellow (MY). Prior to analysis, MY was concentrated using a validated UV-spectrophotometric technique and solid-phase extraction. Precipitation polymerization was used to create MIPs for selective MY extraction. The pH, time, capacity, and desorbing solvent of the MIPs were all characterized and optimized. For samples of turmeric with 0.1–10 mg/kg MY, the newly developed MIP solid-phase extraction/UV spectrophotometric technique achieved 88.10–92.76% recovery, effectively determining MY in spiked samples. Even when there was an additional azo dye present, the technique still showed selectivity for MY.

Pilvenyte *et al.*, (2023), in order to identify cancer biomarkers, the authors investigated the manufacture of MIP based sensors. The study investigated the complex manufacturing procedure for MIP-based sensors with an emphasis on their use in identifying and measuring cancer-related biomarkers. These sensors make use of the MIPs' innate selectivity and sensitivity to accurately and consistently identify cancer biomarkers in intricate biological samples. This study advances personalised medicine and early detection techniques for better patient outcomes by highlighting the development and application of MIP-based sensors in the field of cancer diagnosis.

Jain *et al.*, (2023), for the detection of favipiravir in biological, pharmaceutical, and forensic materials, a novel technique was created by combining fabric phase sorptive extraction (FPSE) with GC-MS. Favipiravir was successfully extracted using FPSE, and then following derivatization with N, O-bis (trimethylsilyl) trifluoroacetamide

(BSTFA), it was analysed using GC-MS. Key FPSE elements were chosen and optimised using Placket-Burman Design (PBD) and Central Composite Design (CCD). PEG produced by sol-gel technology has the maximum extraction efficiency. According to GC-MS results, the method's linear range was 0.01–10 µg mL^{-1}, and its low limits of detection (LODs) and quantification (LOQs) were 0.001-0.0026 µg mL^{-1} and 0.003-0.0086 µg mL^{-1}, respectively. The approach identified and measured favipiravir in human urine, whole blood, plasma, and forensic materials with acceptable precisions. The ComplexGAPI index was also used to assess the method's environmental effect, emphasizing its potential for becoming green.

Daniele *et al.*, (2024), designed Molecularly Imprinted Polymer (MIP) based voltammetric sensor for quick and selective detection of synthetic cannabinoids. Optimization was execute via employing a non-psychotropic compound as the template. Electrochemical characterization and differential pulse voltammetry (DPV) were used for quantification, acquiring a detection limit of around 0.01 mM and linearity up to 0.8 mM. The MIP based sensor showed better selectivity and reproducibility compared to non-imprinted and bare electrodes. The study highlights the need of new advanced techniques which are more rapid, specific and highly selective.

2.2 Research Gap

CSP is a centrally acting muscle relaxant that has been increasingly abused and misused, which has resulted in a number of pernicious outcomes including drug overdoses, deaths, and cases involving impaired driving. According to recent research, more people are abusing CSP, ingesting it excessively, mixing it with other medications, and acquiring it via falsifying prescriptions. It is necessary to create a more effective, specific, and quick approach for the extraction and detection of CSP since forensic laboratories routinely deal with instances involving drug misuse overdose, and driving while intoxicated. Despite the rise in incidences and negative effects linked to CSP usage, there aren't enough quick, precise, simple and cost-effective ways to extract and detect drugs for more effective and efficient detection. The ability of forensic laboratories to detect CSP abuse cases properly and effectively must be improved in order to improve prevention, intervention, and forensic investigations relating to CSP usage and its repercussions.

2.3 Objectives

1.Designing of molecularly imprinted polymers for carisoprodol recognition.

2.Extraction, binding and elution of carisoprodol by molecularly imprinted polymers.

3.Development of sensitive analytical procedure for detection of carisoprodol in forensic matrices.

4.Comparison between MIPs and non MIPs for extraction of carisoprodol

Chapter – 3

Synthesis, Extraction, Binding and Elution of Carisoprodol by Molecularly Imprinted Polymers

3.1 Introduction

Skeletal muscle relaxants (SMRs) are a distinct class of medications that are frequently prescribed to address two kinds of basic medical situations: spasticity caused by upper motor neuron syndromes and also muscular spasms caused by peripheral musculoskeletal state [Chou *et al.*, 2004]. SMRs are often prescribed, but there is little laconic data at hand about their pharmacology. They contribute to an important part of the assemblage of therapies for usual medical conditions like lower back pain. Like other muscle relaxants, carisoprodol (CSP, N-isopropyl-2 methyl-2-propyl-1, 3- propanediol dicarbamate) marketed as Soma has been used for decades. In addition to this, CSP was first introduced during the late 1950s, becoming a non-scheduled SMR during those years. However, Soma now comes under schedule IV-controlled substance. Even though the action mechanism is unclear, studies suggest that CSP may impede interneuron function in the descending reticular formation and spinal cord [Harden and Argoff, 2000; Reeves *et al.*, 2004]. Its pharmacological properties exhibit to be a white crystalline compound with a light, distinguishing odour and a bitter flavour. It tends to come in a quantity of 250 mg and 350 mg in the form of white tablets. These are prescribed for administration three to four times a day. The CNS is primarily affected by the adverse effects of CSP, showing signs of dizziness, drowsiness, ataxia, tremor, blurred vision, and headache [Bramness *et al.*, 2000; Reeves and Burke, 2010]. Moreover; studies have revealed that it is attributed to drug abuse and psychomotor impairment leading to high risk of traffic accidents [Zacny *et al.*, 2011]. Different studies have shown that consumption of CSP leads to impairment effects followed by a high risk of road accidents [Bramness *et al.*, 2004; Bramness *et al.*, 2007]. Abuse potential of CSP is similar to benzodiazepines and users leisurely consume it for its sedating, calming, and relaxing effects. Furthermore, due to its narcotic potentiating effects, it is frequently misused following opioid drugs. It is also employed to enhance sexual performance and avoid withdrawal symptoms in opioid addicts [Reeves *et al.*, 1999; Reeves and Carter *et al.*, 1999; Nebhinani *et al.*, 2013] Even though the tendency of tolerance, dependence, and abuse, all are class effects, CSP abuse remains on the rise far more than the other available SMRs [Boothby *et al.*, 2003]. Therefore, it is crucial to extract and determine CSP from different biological samples.

Several procedures of analysis were published for the identification of CSP. Most common of them are LC-MS [Miksa and Poppenga, 2003; Skinner *et al.*, 2004], GC-MS [Downey *et al.*, 2009], reversed-phase high- performance liquid chromatography (RP-HPLC), and electro spray ionization used with massspectrometry (ESI-MS) [Acharya *et al.*, 2016]. In addition to this, the SPE method is utilized for sample pre-concentration, extraction, and clean-up before most of the above- mentioned analytical techniques [Coulter *et al.*, 2012]. Although SPE's first experiment-based applications date back fifty years, it wasn't until the middle of the 1970s that this technique was applied to analytical tasks [Liška, 2000]. However, SPE is a traditional technique with concerning demerits. It is a multi-step and time-consuming method as the sample containing the target analyte goes through different stages; like clean-up, pre-concentration, and fractioning which may correspond to the significant drop of target analytes throughout these stages [Šafařı´ková and Šafařı´k, 1999; Wierucka and Biziuk, 2014; Andrade-Eiroa *et al.*, 2016]. Additionally, lack of selectivity results due to the co-extraction of unwanted analytes during sample preparation from complex samples. The usage of extensive volumes of toxic organic solvents for elution and washing of SPE adsorbents also poses a negative impact on the environment as well as on analysts. This also warrants an added pre-concentration step of the last extract before further evaluation.

In intend to minimize the adverse outcome of analytical processes upon nature, the idea of GAC has emerged which emphasizes the usage of least or zero amount of toxic organic solvents and minimal generation of organic waste. Therefore, a future analytical procedure has to be established considering the 12 principles of GAC [Baggiani *et al.*, 2007; Andrade- Eiroa *et al.*, 2016]. These concerns have paved the way to evolve new methods, such as DSPE which is a miniaturized configuration of SPE. It functions by direct inclusion of a sorbent within the sample solution continued by dispersion facilitated by vortex agitation or ultra- sonication to facilitate sorbent-analyte contact [Jain and Singh, 2021]. After finishing the dispersion process, the sample is subjected to centrifugation and the sorbent is collected in a separated vial where adsorbed analytes on sorbent are eluted or back extracted in a little amount of organic solvent. In contrast to conventional SPE techniques, DSPE is rapid; offers high sample throughput, is cost-effective and greener [Han *et al.*, 2014; Islas *et al.*, 2017]. DSPE is not only used in toxicological extractions but it has shown significant

extractions from beverage matrices and fruits with the help of different adsorbents [Tuzen *et al.*, 2022; Altunay *et al.*, 2022]. This technique has also been used recently to extract and measure triazole from fruit juice samples [Khosrowshahi *et al.*, 2022].

The nature of samples encountered in forensic science laboratories is extremely complex which requires effective as well as sensitive procedures to identify drugs and toxins at trace levels [Telepchak *et al.*, 2004]. Therefore, selective and sensitive sample preparation protocols are necessary to get well-grounded and accurate results. Since the introduction of molecularly imprinted polymer (MIP) technology by Wulff and Sarhan in the 1970s, analyte extraction has been improved efficiently by synthesizing and using MIPs as adsorbents [Wulff *et al.*, 1973; Theodoridis and Manesiotis, 2002; He *et al.*, 2007]. Many researchers explored MIPs in sample preparation as it has many advantages including high specificity, selectivity, robustness, stability, cost-effectiveness, and high shelf life [Turiel *et al.*, 2007; Tamayo *et al.*, 2007]. The process of polymerization is used to synthesize MIPs; selected template molecules and monomers are added in the presence of cross-linkers and initiators followed by the polymerization process. Further, this template is eliminated from this polymer leaving binding sites that are complementary to the functional groups of the template molecule; these sites are competent to again bind with the target molecule [Haupt and Mosbach, 2000; Vasapollo *et al.*, 2011]. Various fields have and currently are using MIPs in various other applications [Ahmadi *et al.*, 2015; Nawaz *et al.*, 2020].

Through the current work, for the first time, we report a novel sample preparation strategy based on DSPE utilizing tailor-made MIP for CSP and its analysis by GC-MS in complex matrices like urine, blood, and pharmaceutical formulations. The greenness of the recommended method has been assessed with ComplexGAPI.

3.2 Experimental

3.2.1 Chemicals and Reagents

Unless otherwise specified, all chemicals and reagents utilised in this study were of analytical grade. CSP (purity >99%) was acquired from Indian Pharmacopoeia Commission (IPC, Ghaziabad, India). Methacrylic acid (MAA) (purity >99%), ethylene glycol dimethacrylate (EGDMA) (purity >97%), and 2, 2-azobis-

isobutyronitrile (AIBN) (purity >98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were further utilized in the synthesis without purification. All the solvents used in the present research including analytical grade acetonitrile (ACN, purity >99%) and methanol (MeOH, purity >99%) were collected from Thermo Fisher Scientific (Waltham, MA, USA). MeOH was utilized to make stock solutions of CSP at a concentration of 3000 µg mL^{-1}. This stock solution was then diluted according to the required arrangement of working standard solutions.

3.2.2 MIP Preparation

MIP for CSP was synthesized using the bulk polymerization method. CSP (0.14 mmol) was mixed in 10 mL of ACN as the template. MAA (0.56 mmol) was added to this and sonicated (LabQuest, India) for 15 min. The solution was then sonicated for another 20 min after adding EGDMA (2.8 mmol) and 300 µL of AIBN to ensure they were fully dissolved.

Following sonication, the formed solution was purged with nitrogen for 5 min to eliminate any remaining oxygen in the reaction mixture followed by sealing the round bottom flask. The polymerization was carried out in an oil bath at 75ºC for 26 hours. After the termination of the polymerization process, the reaction flask was taken out and left to cool down at room temperature. The synthesized polymer was crushed, grinded, and sieved for several cycles in mortar and pestle as shown in figure 3.2; to attain a particle size as low as 158 nm for this Zeta potential and particle analyzer (Zetasizer Lab, Malvern) were used to determine the average diameter of MIP and NIP as shown in figure 3.3. This was followed by washing the powdered MIP with MeOH to remove the template; CSP. The washing procedure was recapitulated until the template was fully eliminated from the MIP and could no longer be detected by GC-MS. Subsequently, these MIP particles were desiccated for later use. Likewise, by using the same procedures and methods described above, non-imprinted polymer was also synthesized excluding the addition of a template molecule in it.

Template dissolved in acetonitrile

↓

Add MAA (monomer) to it and sonicate for 15 mins

↓

Add EGDMA (crosslinker), AIBN (initiator) to it and sonicate for 20 mins The

↓

solution was purged with N_2 for 5 mins

↓

Seal the round bottom flask

↓

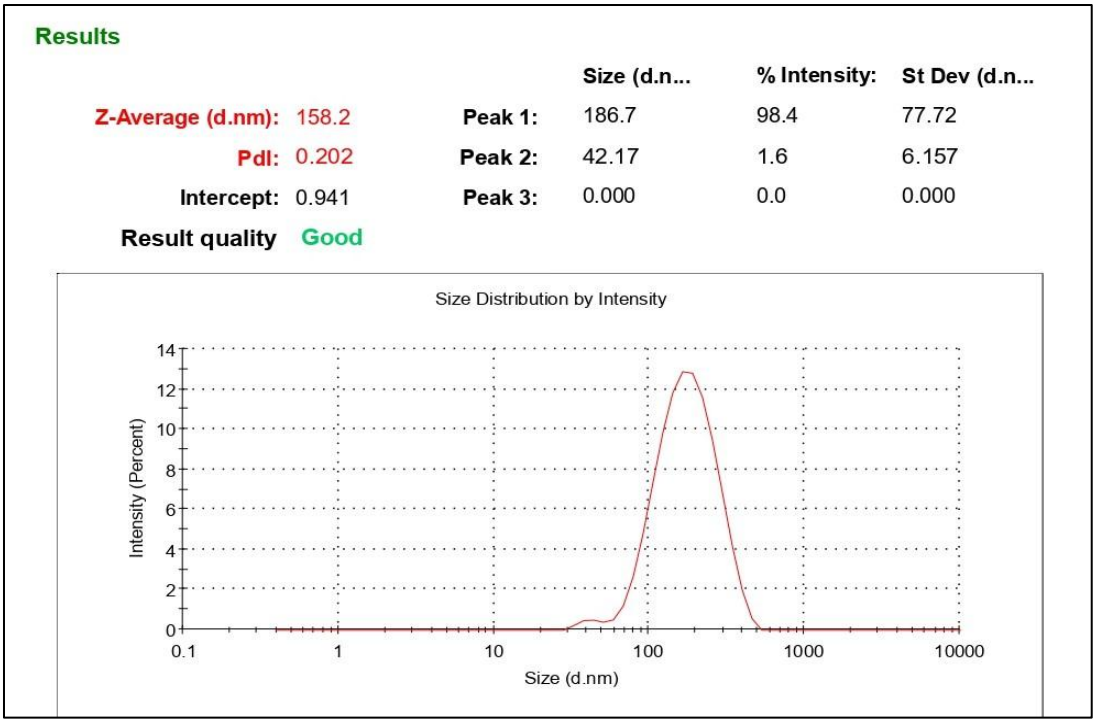
Place it in an oil bath at 75℃ for 26 hours

Figure 3.1 Stepwise process of MIP and NIP (without template) preparation reaction

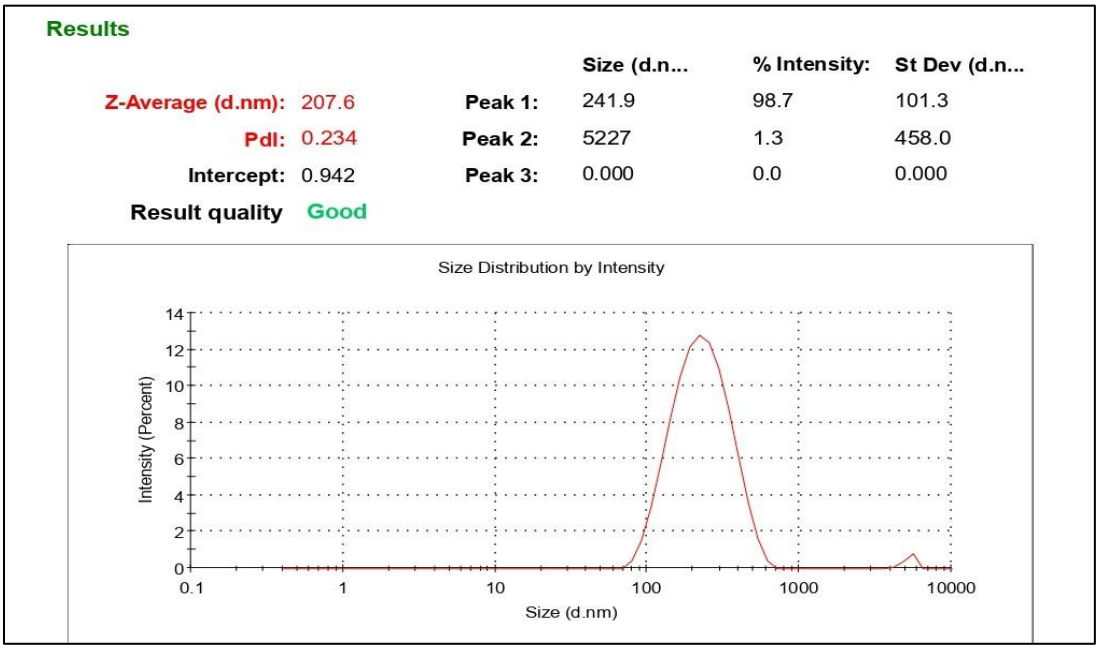
Molecularly Imprinted Polymer

Non Imprinted Polymer

Figure 3.2 Showing synthesized MIP (left) and NIP (right)



a) Z-Average of MIP



b) Z-Average of NIP

Figure 3.3 Showing Z-Averages of MIP and NIP

3.2.3 Characterization of MIP and NIP

MIP and NIP samples were characterized by FT-IR with Diamond ATR and pellet accessories (Perkin Elmer Spectrum 2) in the wave number range of 400–4,000 cm^{-1}, Field Emission Scanning Electron Microscope (FE-SEM; JEOL JSM-7610F Plus EDS: Oxford EDS LN2free, Au Coater: JEOL Smart Coater) was used to examine the surface morphology of the polymer particles and particle size of the polymers were also checked by Malverrn Zetasizer Nano ZS90.

3.2.4 Binding Affinity

Equilibrium adsorption experiments were executed to assess the binding affinity of CSP on imprinted polymers. In the mentioned experiment, 2 mL of CSP solution at different concentrations – 1, 10, 30, 50, 80, and 100 μgmL^{-1} were mixed with 20 mg of MIP and NIP particles in different vials. This mixture was further vortexed for 24 hours at room temperature succeeded by centrifugation at 4000 rpm for 5 min. The collected supernatant was lastly analyzed with GC-MS.

3.3 Results and Discussion

3.3.1 MIP Preparation and Binding Performance

In this work, MIP was synthesized by bulk polymerization approach, with the use of CSP as the template and MAA as the most commonly used functional monomer. Attributed to the reason that MAA is an acidic monomer, it can form hydrogen bonds with the target molecules. Moreover, EGDMA was used as a cross-linker due to its ability to stabilise the MIP network and also to keep functional monomers connected at a specific location after the template was removed. The initiator and porogen used were AIBN and ACN respectively [Wang *et al.*, 2019]. The MIP was synthesized by combining a template, monomer, and crosslinker in 1:4:20 ratios. The robustness and amount of interaction seen between the functional groups of template and monomer are significant elements that regulate the recognition and binding efficiency of the imprinted polymer [Mudiam *et al.*, 2012].

3.3.2 Removal of Template from MIP

Took a centrifuge tube and added methanol and MIP in it, followed by vortex for 5

mins, and then centrifuged for next 5 mins. This supernatant was then collected in an eppendorf tube and marked this sample as 1. This process was repeated 20 times. The same procedure was repeated for NIP. These samples were further analysed under UV-vis spectrophotometer (Figure 3.4) and GC-MS (Figure 3.5), where reduced MIP spectra and lower template peaks signify the removal of template respectively.

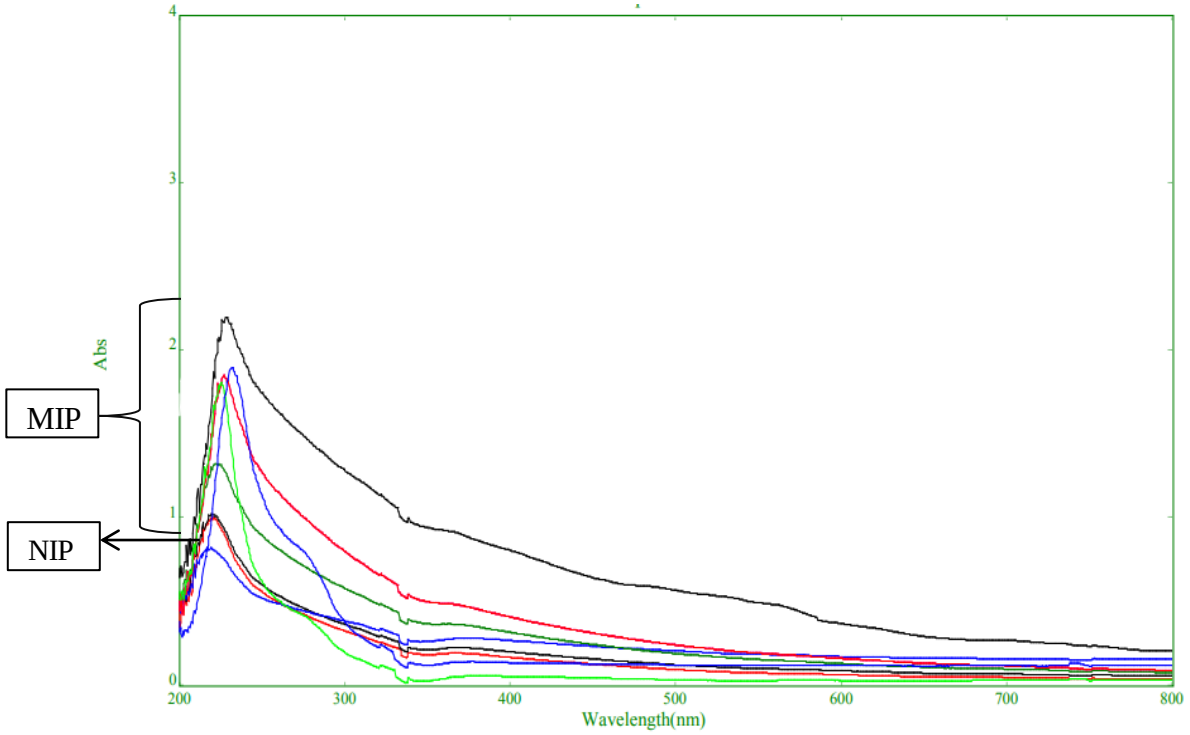
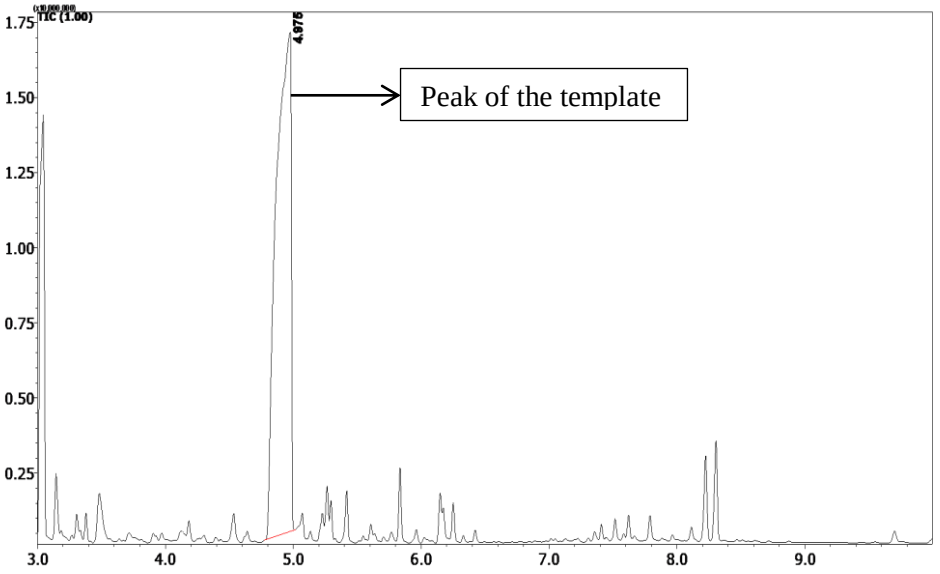
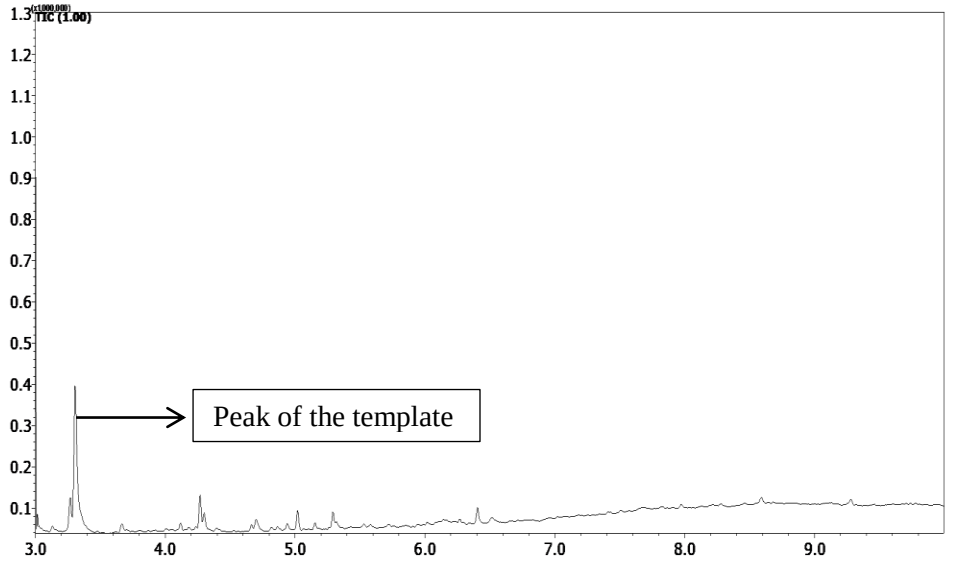


Figure 3.4 Showing UV-vis Spectroscopy for MIP and NIP washings



a) Template removal (First washing)



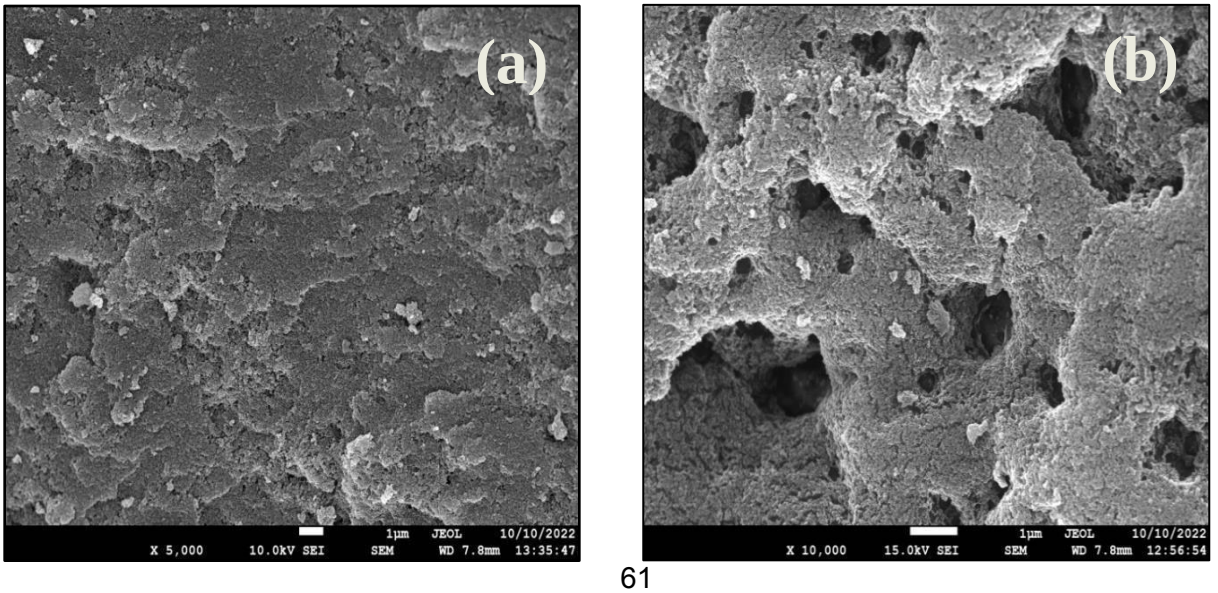
b) Template removal (Last washing)

Figure 3.5 Showing a) First washing and b) Last washing in GC-MS

3.3.3 Characterization of MIP and NIP

Both MIP and NIP were characterized by FE-SEM and FTIR. FE-SEM was used to observe their morphological elucidation which can be seen in Figure 3.6. Imprinting effect of the template creates the morphological difference between MIP and NIP; resulting in irregular, rough, and relatively larger surface area of MIP particles as compared to NIP, which had regular and smooth shape.

MIP (X 5000) **MIP (X 10,000)**



NIP (X 5000) **NIP (X 10,000)**

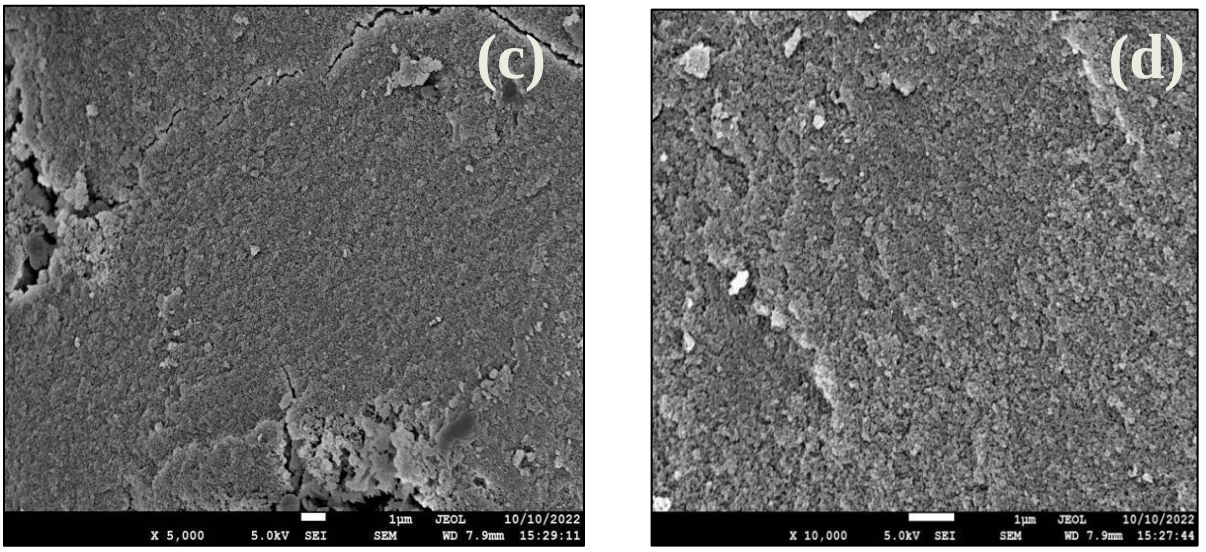


Figure 3.6 The scanning electron microscopy of MIP (a), (b), and NIP (c), (d)

The infrared spectra of (a) CSP, (b) MAA, (c) MIP, and (d) NIP obtained by FTIR are displayed in Figure 3.6. FTIR spectrum of MIP at 2948 cm^{-1} was due to stretching vibrations of the C-H group. In the spectra of MIP; the IR absorption band at 1722 cm^{-1} of the C=O group and C-O stretching at 1127 cm^{-1} shows the existence of EGDMA. In addition, the two absorption bands at 1449 cm^{-1}, and 1388 cm^{-1} correspond to C-H bending in the methylene group. The absorption bands at 1259 cm^{-1} were caused by C-O-C symmetric and asymmetric stretching vibrations, derived from the monomer involved in the polymerization. The absorption band at 937 cm^{-1} shows the plane bending vibration for the C-H bond. Moreover, the out-of-plane wagging vibration for the C-H bond was denoted by the 863 cm^{-1} absorption band. The absorption band spectrum of MIP and NIP does not have notable differences between them. A low amount of the drug added in comparison to the polymer is the possible explanation for this phenomenon which has been given by the researchers in their previous studies [D'Urso *et al.*, 2019]. To verify this assumption, the powdered drug was physically mixed with the NIP particles. The ratio of drug and NIP in this mixture was kept the same as in the MIP. It can be seen in the NIP+CSP band spectra shown in Figure 3.5(e) that CSP absorption bands were not detectable from this physical mixture.

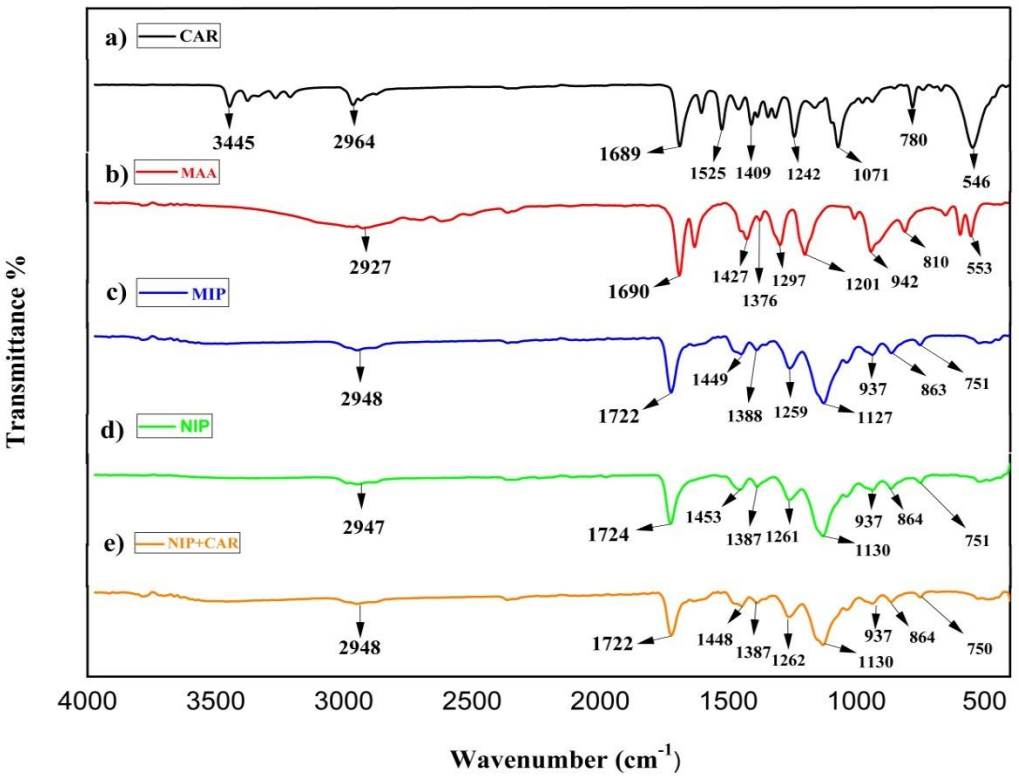


Figure 3.7 FTIR spectra of: a) CSP, b) MAA, c) MIP, d) NIP, e) NIP+CSP

3.3.4 Binding Affinity

The binding affinities of both MIP and NIP were assessed in triplicate (n=3) using an equilibrium adsorption experiment. The quantity of CSP bound to MIP initially increased at the binding equilibrium and eventually reached the saturation point as the CSP concentration increased. The binding percentage (% bound), absolute quantity (μg/mg), and imprinting factor (α) at different concentrations for polymers were calculated as given below:

$$\% \text{ bound} = Q \text{ bound}/Q \text{ initial} \times 100$$

$$\alpha = \text{amount bound in MIP}/\text{amount bound in NIP}$$

Here, Q denotes the quantity of bounded analyte with the polymer whereas Q initially symbolises the added quantity of the analyte before the polymer binding. The symbol α denotes the imprinting factor; the amount bound to MIP and the amount bound to NIP represents the amount of analyte bound to MIP and NIP respectively [Ahmadi *et al.*, 2015]. In this experiment, the binding affinity of MIP was compared to that of NIP for their % bound, absolute quantity, and imprinting factor. The analyte was taken in

six different concentrations (1, 10, 30, 50, 80, and 100 µg); the highest % bound was found to be 73.92% and the imprinting factor was 2.29. This clearly shows that the binding affinity for CSP is higher in MIP as compared to the binding affinity of NIP as the pattern in figure 3.7 resulted to be non- linear. The imprinting effects of the binding sites contributed to the increased affinity of MIP.

Table 3.1 Showing absolute quantity (µg/mg) and imprinting factor for MIP and NIP

Concentration in µg/mL	% Bound		Absolute quantity in µg/mg		Imprinting factor (α)
	MIP	NIP	MIP	NIP	
1 µg	69.43	17.08	0.069	0.017	4.06
10 µg	73.92	32.19	0.739	0.321	2.29
30 µg	72.82	36.57	2.184	1.097	1.99
50 µg	68.46	34.62	3.423	1.731	1.97
80 µg	57.79	25.9	4.623	2.072	2.23
100 µg	48.28	21.46	4.828	2.146	2.24

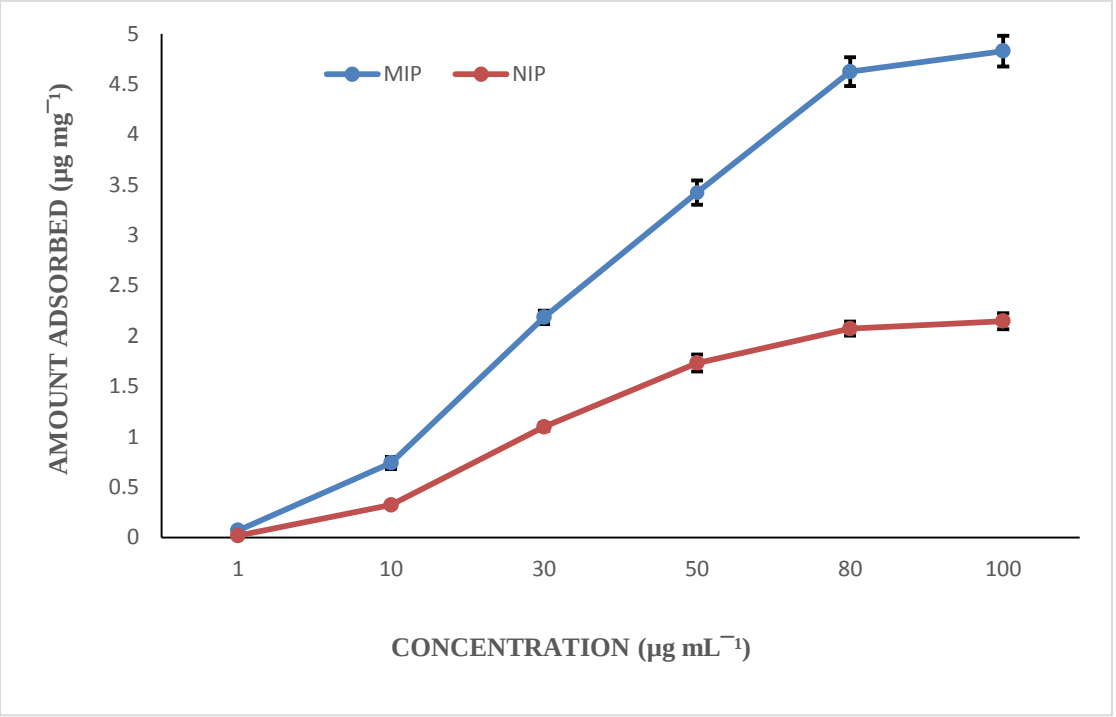


Figure 3.8 Binding isotherm study of CSP for MIP and NIP

3.4 Conclusion

MIP and NIP were successfully prepared by bulk polymerization technique, and their in- depth characterization utilizing FTIR and FE-SEM investigations was then completed. FTIR peaks and images of FE-SEM showed successful polymer formation. Results showed clear differentiation between MIP and NIP formation. After polymer formation, the particle size of the polymer was also checked. Moreover, the binding isotherm study revealed higher binding affinity of CSP towards MIP as compared to NIP. The application of MIP-DSPE (Molecularly Imprinted Polymer Dispersive Solid-Phase Extraction) technique to extract CSP from both biological matrices and pharmaceutical materials can be used for further optimization and validation studies. These extensive studies sought to improve the extraction procedure, precision, accuracy, and dependability of the analytical method utilizing the MIP- DSPE methodology. Overall, the effective MIP formation showed high binding properties with CSP as compared to NIP.

Beyond initial polymerization and characterisation, the features of MIP and NIP were thoroughly investigated. Further studies were conducted to examine the thermal stability and reusability of the polymers, and the results showed that MIPs maintained both their binding effectiveness and structural integrity even after being used repeatedly. This feature emphasizes how sustainable and affordable it is to use MIPs in real-world applications. Additionally, the selectivity investigations confirmed that MIPs had exceptional selectivity, allowing them to separate CSP from compounds with similar structures, in addition to their strong affinity for CSP. For applications in complicated matrices, where specificity is critical, this selectivity is essential. According to the thorough research, there is room for improvement in the MIP-DSPE method's throughput and efficiency of CSP extraction. This would make it a feasible alternative for regular analytical operations in clinical diagnostics and pharmaceutical quality control. High binding affinities, selectivities, and reusability of MIPs demonstrate their strong performance, which sets them apart from traditional polymer-based extraction methods.

Chapter – 4

Development of Sensitive Analytical Procedure for Detection of Carisoprodol in Forensic Matrices

4.1 Sample Collection

Biological samples (urine and blood) were acquired from the authors of the proposed study between the ages of 25-40 years with ethical permission. All these collected samples were stored at ~4ºC under refrigeration. Pharmaceutical tablets named Carisoma compound of white color with a combination of generics, namely: CSP (muscle relaxant) (175 mg), caffeine (brain stimulant) (32 mg), and paracetamol (mild analgesic and fever reducer) (325 mg) were purchased from Mohali, Punjab.

4.2 Sample Preparation

Blank blood samples (0.5 mL) donated by healthy volunteers were fortified with CSP at 1 µg mL^{-1} of concentration. In intend to mimic drug-protein binding at physiological conditions; these samples were then incubated at 37 °C for 30 min [D'Urso *et al.*, 2019]. Further, 0.5 mL of MeOH was added to deproteinized the blood samples succeeded by centrifugation at 3000 rpm for 5 min to sediment the cellular debris and get a clear supernatant.

Similarly, urine samples were initially centrifuged at 3000 rpm for 5 min to eliminate any extra debris from them. The supernatant thus obtained was spiked with CSP at1 µg mL^{-1} and subjected to the MIP-DSPE procedure described later.

Before grinding the pharmaceutical tablets into a fine powder, the average weight of five tablets containing 175 mg CSP each was computed. The content of this fine powder was dissolved in 10 mL of MeOH and then sonicated for 10 min. This fine powder‘s content is proportionate to the computed average weight of tablets.

4.3 MIP Dispersive-Solid Phase Extraction (MIP-DSPE) Procedure

4.3.1 Pharmaceutical Formulations

The experiment commenced by weighing five tablets with a dosage of 175 mg each and determining their average weight. Subsequently, the tablets were crushed into a fine powder. The powder, equivalent to the average weight of the tablets, dissolved in 10 mL of MeOH and subjected to 10 min sonication followed by centrifugation for 5 min; followed by filtration. To achieve a concentration of 1 µg mL^{-1}, the filtrate diluted with ultrapure water. Under the optimal conditions, 1 µg mL^{-1} of CSP was spiked into 8 mL of ultrapure water at a pH of 3, utilizing a 0.1 M HCl solution. Then, 50 mg of MIP added to the solution, followed by vortex agitation (LabQuest, India) at

2000 rpm for 20 min to ensure thorough dispersion throughout the sample. The mixture was then subjected to centrifugation (Labman Scientific Instruments, India) at 3000 rpm for 5 min to allow the dispersed MIP to settle in the tube. The resulting supernatant was discarded, and the elution process was initiated. To accomplish this, 1 mL of MeOH was introduced into the tube and vortexed for 2 min, followed by micro-centrifugation (Labman Scientific Instruments, India) for 5 min. The obtained supernatant, consisting of 1 mL of MeOH, was transferred into a GC- MS vial for subsequent analysis as shown in figure 4.1.

4.3.2 Biological Samples

Initially, biological samples i.e., urine and blood were spiked with CSP at a concentration of 1 µg mL^{-1}. To deproteinize the blood samples, 0.5 mL of MeOH was added, followed by centrifugation at 3000 rpm for 5 min to separate the cellular debris and obtain a clear supernatant. Similarly, urine samples were also centrifuged at 3000 rpm for 5 min to remove any debris. The resulting supernatants from the biological samples (urine and blood sample) were diluted to a volume of 8 mL using ultrapure water at pH 3. Subsequently, the samples underwent the MIP-DSPE procedure as described above. After completion, the MIPs were washed, dried, and reused for further experiments.

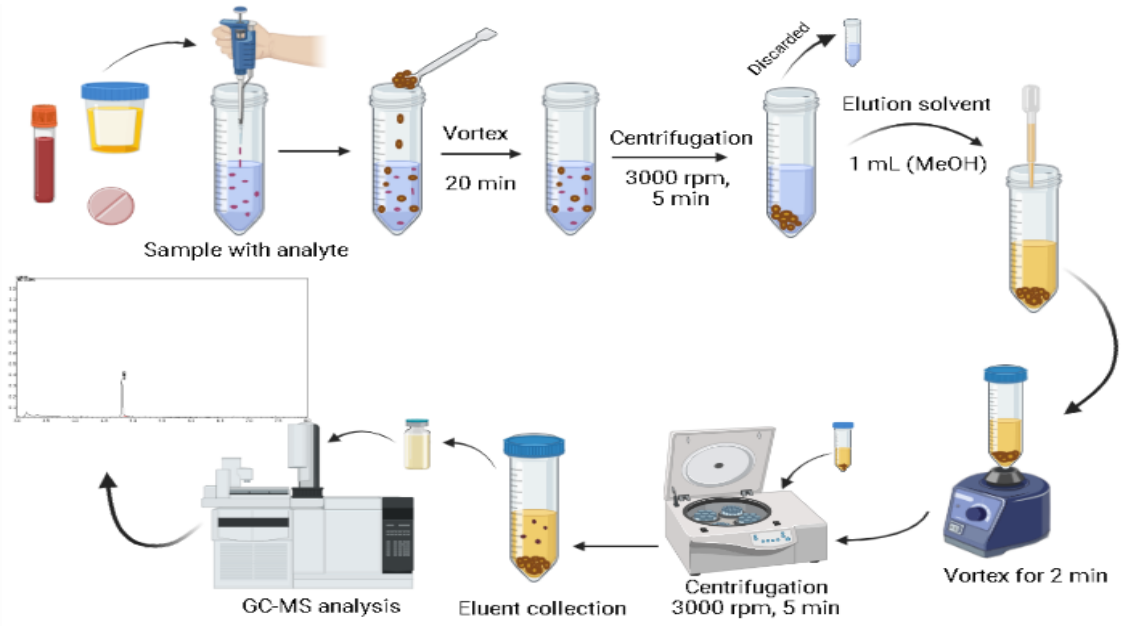
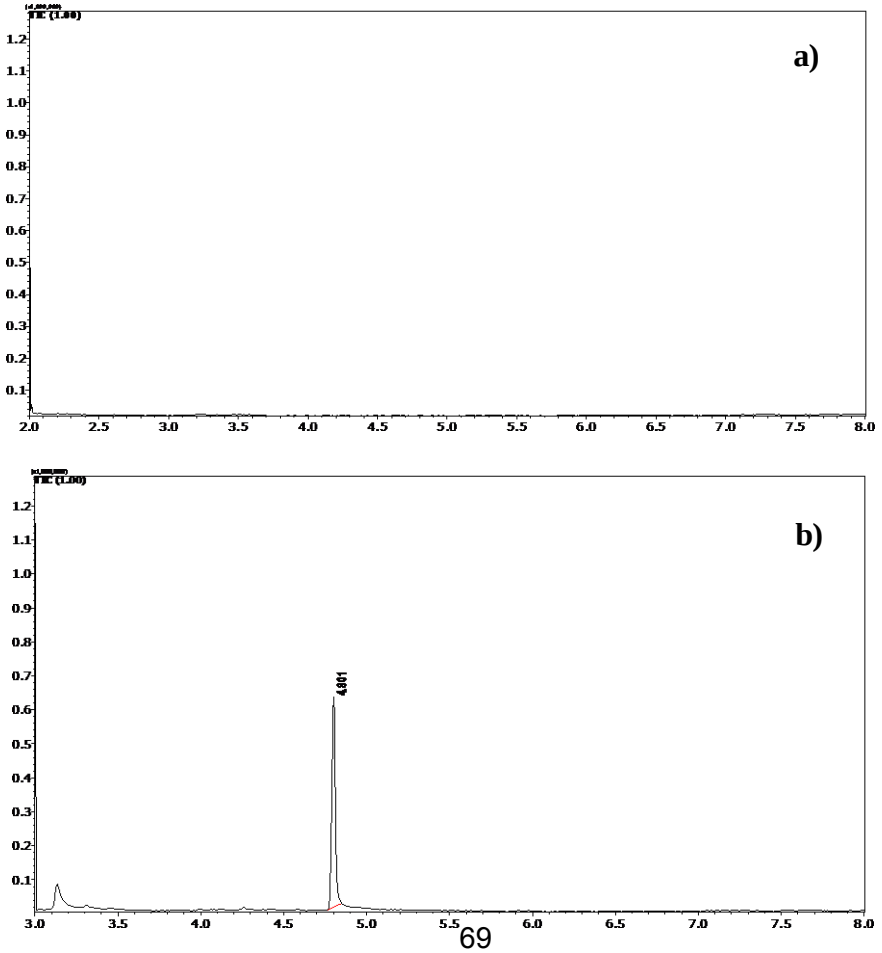


Figure 4.1 Schematic representation of proposed MIP-DSPE Procedure for CSP analysis by GC-MS.

4.4 Gas Chromatography-Mass Spectrometric Conditions

CSP was analysed after carrying out MIP-DSPE experiments on a Shimadzu Nexis GC-2030 hyphenated with a QP-2020 NX mass spectrometer. The GC-MS system was equipped with an AOC-20i auto-injector. Exactly 1 µL of the extract was injected into the GC-MS injection port at 250°C (split value 10) which was connected with an SH-Tri-5Sil MS capillary column (30 m length x 0.25 mm internal diameter x 0.25 µm film thickness with the stationary phase of 5 % phenyl and 95 % di-methylpolysiloxane). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was initially kept at 150 °C and gradually increased up to 250°C at a rate of 20°C/min and finally kept hold for 7 min; resulting in a total run time of 8 min. The temperatures of the transfer line and ion source were set at 250 and 200°C, respectively. Ionization of CSP was performed at electron energy of 70 eV in positive ionization (+EI) mode with a mass range of 50 – 500 amu. CSP was quantified in selected ion monitoring (SIM) mode with quantifier ion of *m/z* 55 and qualifier ions of *m/z* 97, 158, and 245. Figure 4.2 shows the chromatograms of standard CSP; CSP in urine and blood samples after extraction with the MIP-DSPE method.



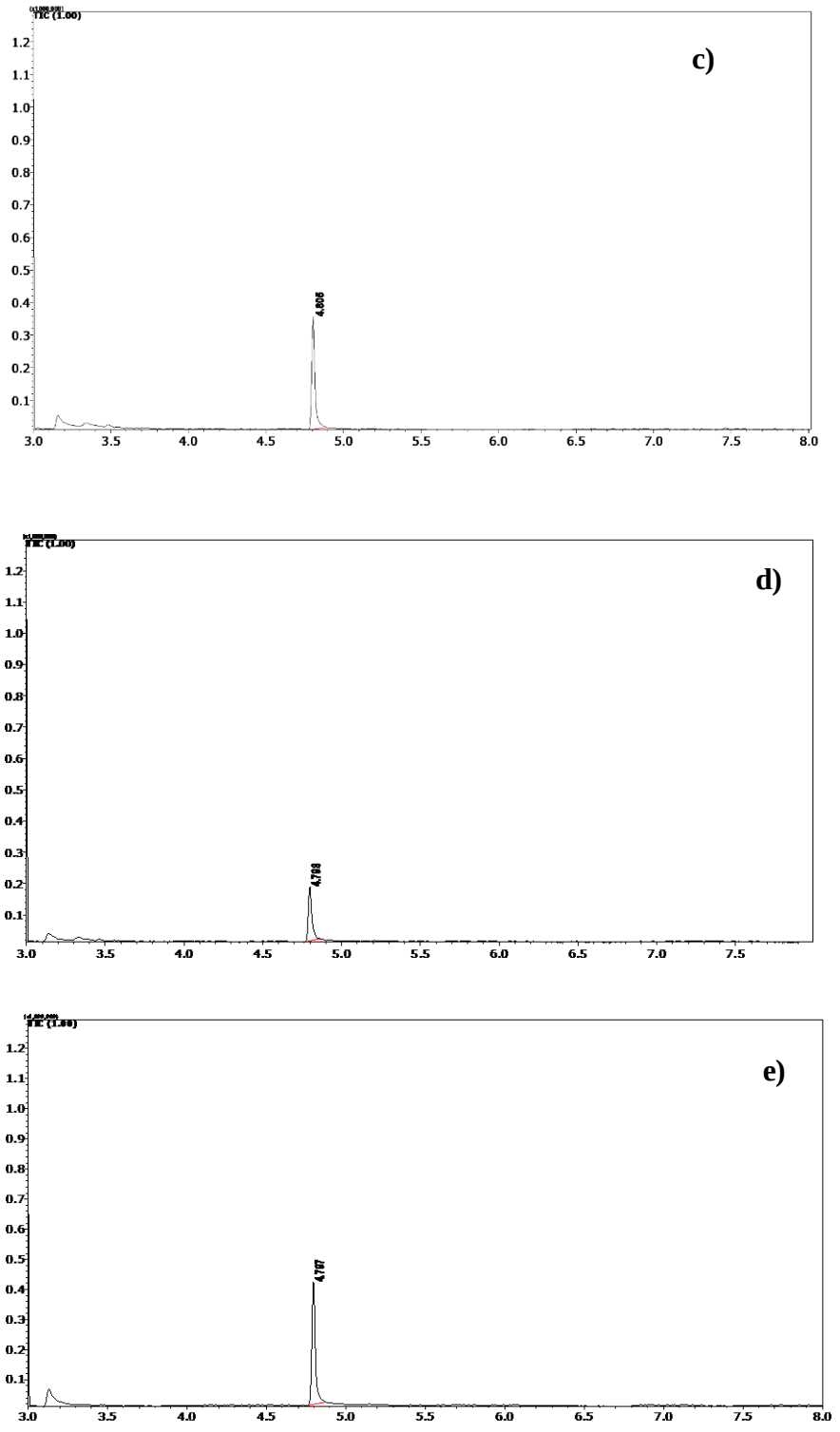


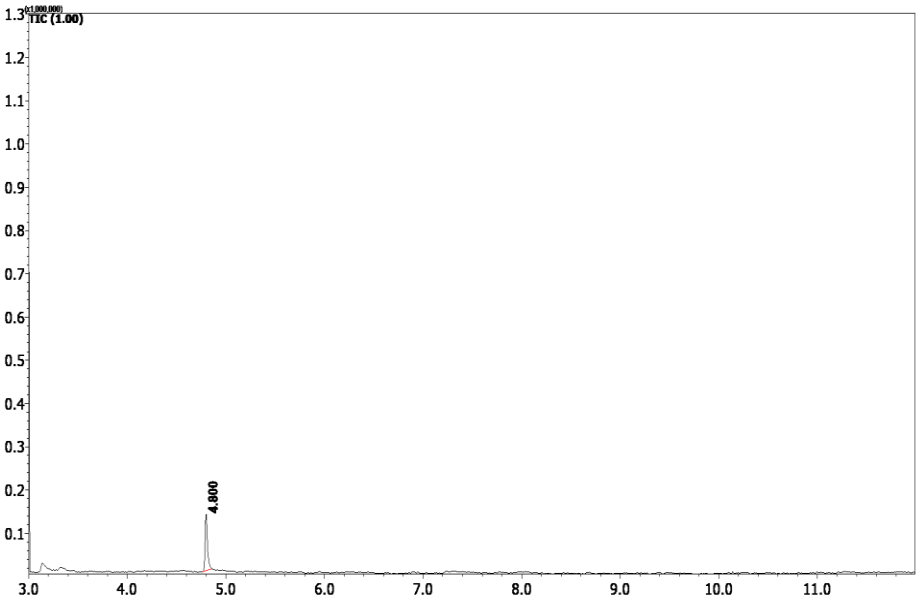
Figure 4.2 Showing GC-MS chromatogram of (a) blank, (b) standard CSP at 1 µg mL^{-1}, (c) CSP in urine, (d) CSP in blood, and (e) pharmaceutical sample, after extraction with MIP- DSPE method

4.5 Optimization of MIP-DSPE Parameter

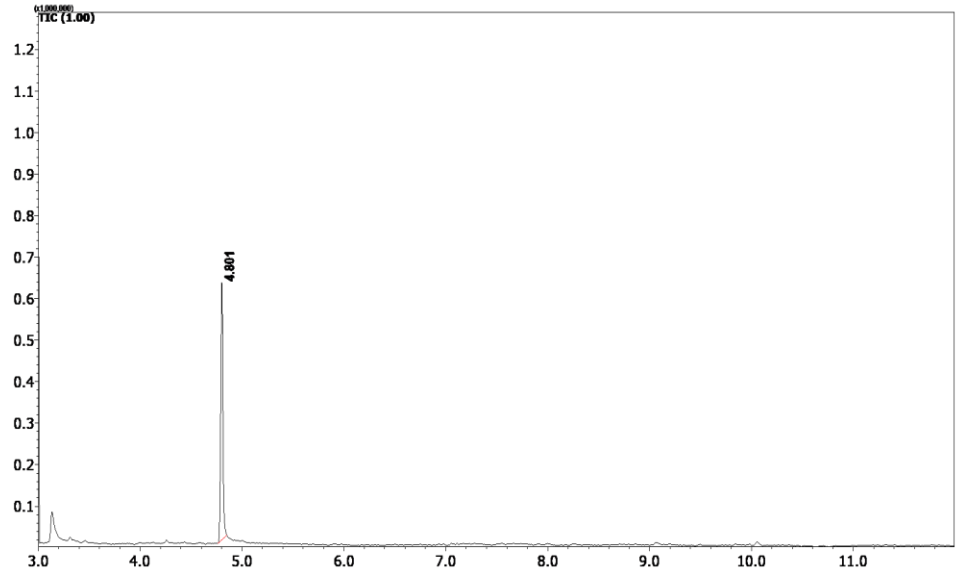
To perform efficient dispersive SPE with MIP as a sorbent, it is obligatory to optimize the different factors that may impact the final extraction efficiency. Factors such as volume of sample taken, amount of MIP used as sorbent, vortex agitation time, type of elution solvent, optimization of desorption time, sample pH and ionic strength; and sample volume were optimized to achieve maximum extraction efficiency.

4.5.1 Optimization of the Amount of MIP

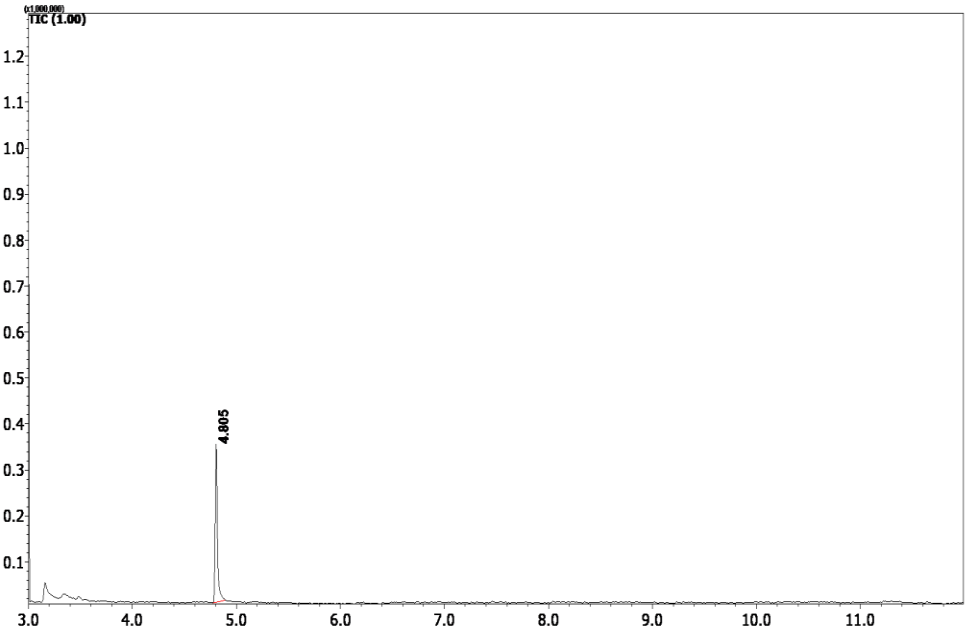
The role of MIP is the selective extraction of the drug from the biological samples through the DSPE procedure. To optimize the dose of MIP for DSPE, different amount of polymer in the range of 20–300 mg (20, 50, 100, 150, 200, and 300 mg) (figure 4.3) was added to the sample containing CSP at 1 µg mL^{-1}, keeping all other variables constant. Figure 4.4 A indicates that the highest extraction efficiency was found to be with 50 mg of the MIP and recovery did not increase by adding more amount of adsorbent to the sample.



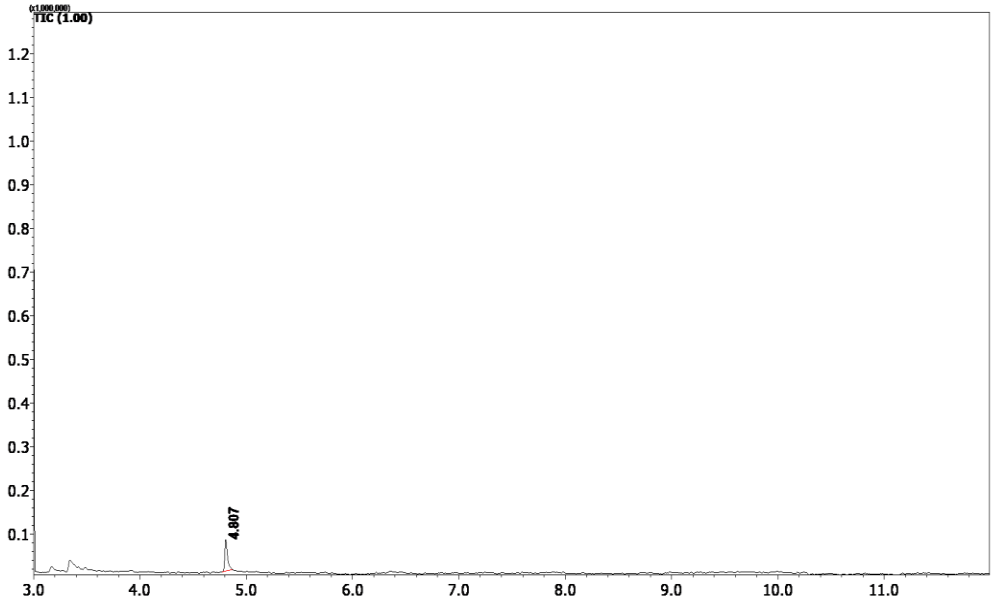
a) 20 mg MIP



b) 50 mg MIP



c) 100 mg MIP



d) 200 mg MIP

Figure 4.3 Showing optimization chromatogram at different amounts of MIP a) 20 mg MIP b) 50 mg MIP c) 100 mg MIP d) 200 mg MIP

Table 4.1 Analytical attributes for amount/dose of MIP

Dose of sorbent(mg)	Area			Average	SD	RSD %	EF %
20	213996	204421	183710	200709	15480.46	7.712888	6.346211
50	904963	984125	894230	927772.7	49096.73	5.291892	29.33521
100	511976	442516	491236	481909.3	35656.88	7.399084	15.23747
150	276191	244996	285834	269007	21345.79	7.935033	8.505723
200	126675	146390	135378	136147.7	9880.01	7.256834	4.304848
300	47795	44628	51936	48119.67	3664.802	7.616017	1.521494

4.5.2 Vortex Agitation Time

Vortex agitation facilitates the mass shift of analyte from the aqueous phase to the adsorbent, thus improving the extraction efficiency of the procedure. To optimize the vortex agitation time, samples were vortex agitated for different time durations (5, 10, 15, 20, 25, and 30 min), keeping other parameters as their constant values. The findings depicted in Figure 4.4 B indicate that the extraction efficiency exhibited a noticeable improvement as the vortex agitation time was extended up to 20 min. However, extending the extraction time beyond 20 min did not lead to any significant increase in the peak areas of the target analyte. This suggests that after 20 min, the system reached an equilibrium state where further extraction did not yield an additional increase in the peak area. As a result, an extraction time of 20 min was chosen for subsequent experiments due to its optimal extraction efficiency.

Table 4.2 Analytical attributes for vortex agitation time

Extraction time (min)	**Area**			**Average**	**SD**	**RSD %**	**EF %**
5	437369	406193	417472	420344.7	15785.27	3.755317	22.14715
10	359732	339375	369187	356098	15234.61	4.278207	18.76211
15	475027	455938	495734	475566.3	19903.48	4.185217	25.05667
20	534060	554493	584284	557612.3	25256.88	4.52947	29.37952
25	521451	561837	571038	551442	26377.26	4.783324	29.05441
30	570502	521047	550292	547280.3	24864.67	4.543315	28.83514

4.5.3 Selection of pH

Different interactions including hydrogen bonding, ionic interactions, and hydrophobic interactions occurring between the analyte and MIP are influenced by the pH of the sample. Sorbent adsorptions towards the analyte are influenced by the pH of the sample [Du *et al*., 2012]. The formed bond decides how effectively an analyte is

absorbed into the sorbent, and as a result, pH can affect how productive the extraction procedure is [Sadeghi and Jahani, 2013]. Therefore, the pH of the sample solution is a crucial parameter that significantly influences the behaviour of the CSP with a pKa value of 4.85. To examine the impact of sample pH on extraction efficiency, a range of pH values from 3 to 9 was investigated. Figure 4.4 C presents the results, revealing that the lowest extraction efficiency of CSP using DSPE occurred at a pH of 9, while the highest efficiency was observed at pH 3. This suggests that CSP predominantly exists in its molecular form, and the optimal extraction efficiency is achieved at a pH below its pKa value. Therefore, to maintain consistent and effective extraction, the sample pH was set at 3 for all subsequent experiments.

Table 4.3 Analytical attributes for selection of pH

pH	Area			Average	SD	RSD %	EF%
3	572859	567263	588642	576254.7	11086.63	1.923911	29.78699
5	492504	515628	489514	499215.3	14292.19	2.862931	25.80478
7	413348	444782	426385	428171.7	15792.98	3.688469	22.13248
9	347483	336483	323547	335837.7	11981.04	3.56751	17.35967

4.5.4 Optimization of Desorption Conditions

For effective elution of the analyte from the MIP, a series of desorption solvents were screened including ACE, ACN, MeOH, and EtOH. The result in Figure 4.4 D shows that CSP was best eluted by MeOH followed by ACN, EtOH, and ACE. Considering the above observation, MeOH was selected for back-extraction of CSP from MIP. Optimization of elution solvent was followed by a series of variable desorption times. For this, the samples were vortex agitated for different time intervals in the range of 2 – 10 min (2, 5, 8, and 10 min). The results in Figure 4.4 E showed that a desorption time of 2 min showed marginal high extraction efficiency compared to the rest of the

time intervals as analyte extraction efficiency tends to decrease with increasing time intervals. This demonstrated that a longer desorption period would make a small volume of the desorption solvent splash on the centrifuge tube wall, resulting in a decline in extraction efficiency. As a result, it was concluded that desorption time of 2 min is the most effective time for achieving a high extraction efficiency of CSP.

Table 4.4 Analytical attributes for selection of desorption solvents

Elution solvent	**Area**			**Average**	**SD**	**RSD%**	**EF %**
ACE	488635	463173	495663	482490.3	17094.38	3.542947	21.36554
ACN	526893	505172	534173	522079.3	15087.84	2.889952	23.11861
EtOH	501022	483194	522518	502244.7	19690.49	3.920498	22.2403
MeOH	608611	627316	599472	611799.7	14193.23	2.319915	27.09159

Table 4.5 Analytical attributes for selection of desorption time

Desorption time (min)	**Area**			**Average**	**SD**	**RSD %**	**EF %**
2	635678	664496	659965	653379.7	15496.59	2.371759	29.62883
5	582522	563825	542183	562843.3	20187.41	3.586684	25.52327
8	585091	562049	616284	587808	27219.39	4.630661	26.65535
10	581690	592027	531831	568516	32187.81	5.661725	25.78051

4.5.5 Effect of Ionic Strength of the Sample

The optimization of the ionic strength performed using three different concentrations of NaCl sample – 0%, 5%, and 10%. As the number indicates, 0% solution had no NaCl, 5% and 10% solution had 0.4 g and 0.8 g of NaCl for 8 mL of sample respectively. The results in Figure 4.4 F show that the sample with 0% of ionic strength gave the best results. In addition to this, extraction efficiency decreased with the increased amount of NaCl. According to the theoretical aspects, increasing ionic strength could have two opposing influences on the effectiveness of the extraction – first is the growing salting-out effect, which favours the extraction efficiency; but our results showed decreasing extraction efficiency with increasing salt addition due to the second condition which is increased ionic competition between Na+ and HMAMP+ to build ionic interaction with a group of carboxylate ion in MIP matrix [Djozan *et al.*, 2011; Maranata *et al.*, 2021].

Table 4.6 Analytical attributes to check the effect of ionic strength

Ionic strength	Area			Average	SD	RSD %	EF %
0%	5810014	6037824	5737163	5861667	156844.8	2.675771	29.48725
5%	5703859	5927431	5549374	5726888	190077.7	3.31904	28.80924
10%	5008816	4827394	5198273	5011494	185454	3.700573	25.21043

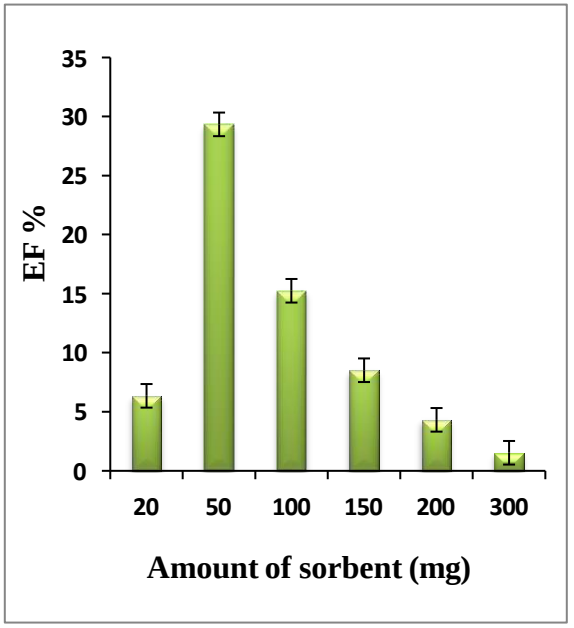
4.5.6 Volume of Sample

Finally, the outcome of the sample volume studied for the DSPE procedure. For the present work, different sample volumes were taken in the range of 2 – 10 mL in five separate centrifuges. The results depicted in Figure. 4.4 G demonstrate that the optimal extraction efficiency was observed at a sample volume of 8 mL. However, the addition of ultrapure water to the MIP resulted in sample dilution, leading to a decrease in the concentration of the target analyte which can adversely affect the extraction efficiency. This is primarily attributed to the binding sites on the MIP not being fully occupied by the target analyte, resulting in reduced interactions and a

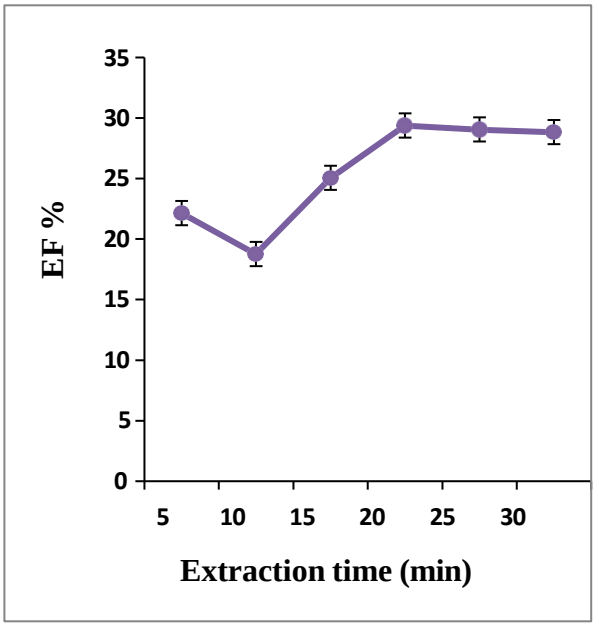
subsequent decrease in overall extraction efficiency. Therefore, a sample volume of 8 mL was used for further experiments.

Table 4.7 Analytical attributes for selection of volume of sample

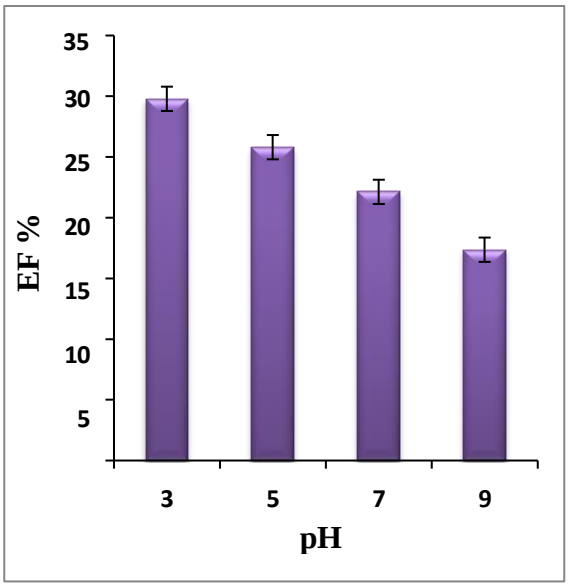
Volume (mL)	Area			Average	SD	RSD %	EF%
2	242884	263746	216384	241004.7	23736.86	9.84913	12.59303
5	441523	468263	432273	447353	18689.88	4.177882	23.49797
8	554896	574129	581029	570018	13542.85	2.375863	29.94116
10	503204	484628	520182	502671.3	17782.98	3.537696	26.40366



A



B


35
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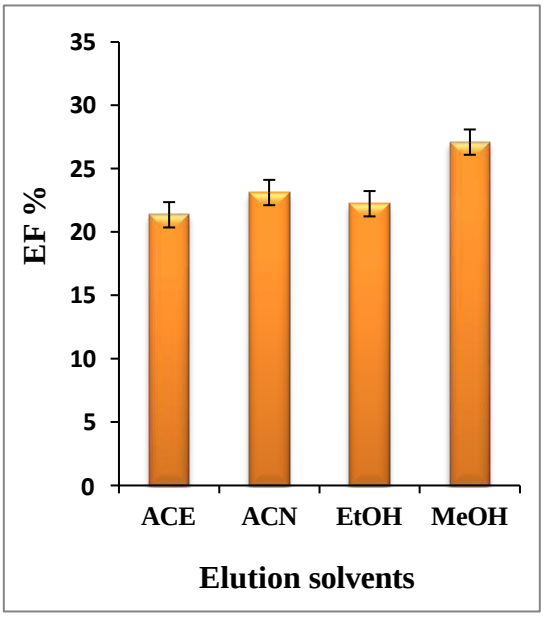
C


35
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5
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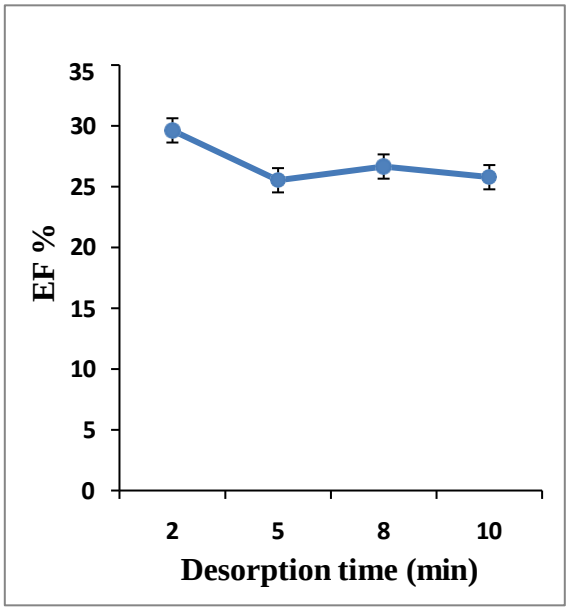
EF %
ACE
ACN
EtOH
MeOH
Elution solvents

D


35
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EF %
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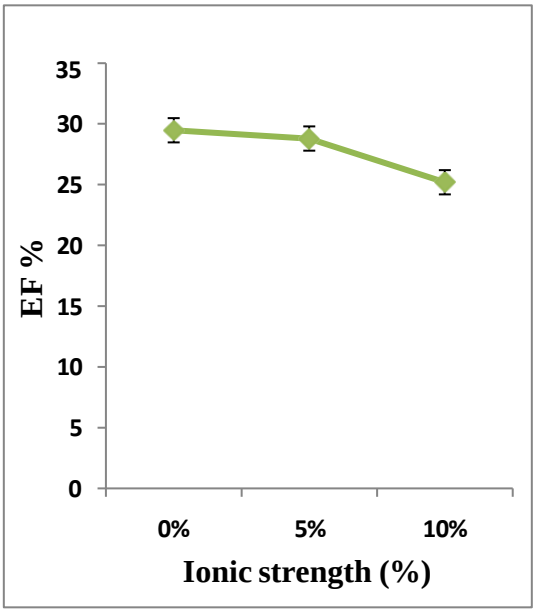
Desorption time (min)

E


35
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EF %
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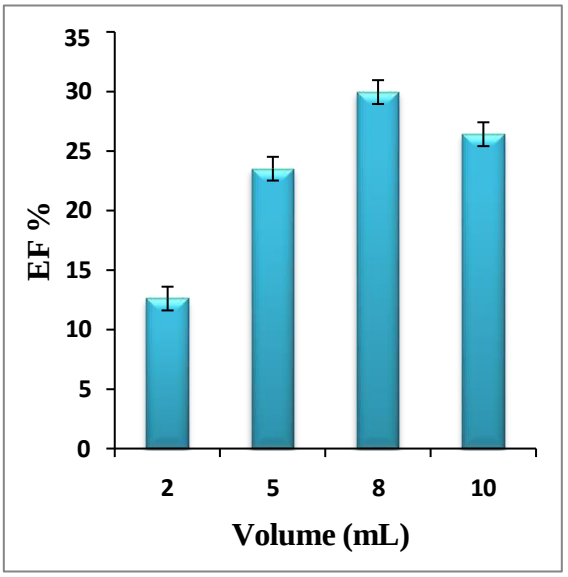
10%
Ionic strength (%)

F



G

Figure 4.4 Optimization of MIP-DSPE parameters: A) amount of MIP, B) Extraction time, C) selection of pH, D) desorption solvent, E) desorption time, F) optimization of ionic strength, G) volume of sample

4.6 Validation Assay

The introduced technique validated in terms of linearity, precision, recovery, the LOD, and the LOQ. The blank urine sample, blood sample, and pharmaceutical tablets were spiked with increasing concentrations of CSP (0.1, 0.3, 0.5, 0.8, 1, 5, 10 µgmL^{-1}) to get a seven-point calibration curve following the optimization of all MIP, and DSPE parameters. Recovery assay of MIP-DSPE was achieved by spiking CSP in urine, blood, and pharmaceutical samples at three different concentrations (0.1, 1, 10 µgmL^{-1}). Five injections of CSP at three different concentrations (0.3, 1, 10 µgmL^{-1}) in urine, blood, and pharmaceutical samples in one day and for five consecutive days were carried out to estimate intra-day and inter-day precisions along with blank sample analysis.

4.6.1 Validation for Urine Samples

Table 4.8 Showing calibration (Linearity and accuracy) for urine samples

Concentration (ppm)	Area	Calculated	Accuracy or absolute recovery %
0.1	3655	0.227238679	227.2386789
0.3	3720	0.271501532	90.50051073
0.5	3985	0.45195778	90.39155601
0.8	4505	0.806060606	100.7575758
1	4585	0.860537964	86.05379639
5	10861	5.134286687	102.6857337
10	17930	9.94804222	99.4804222

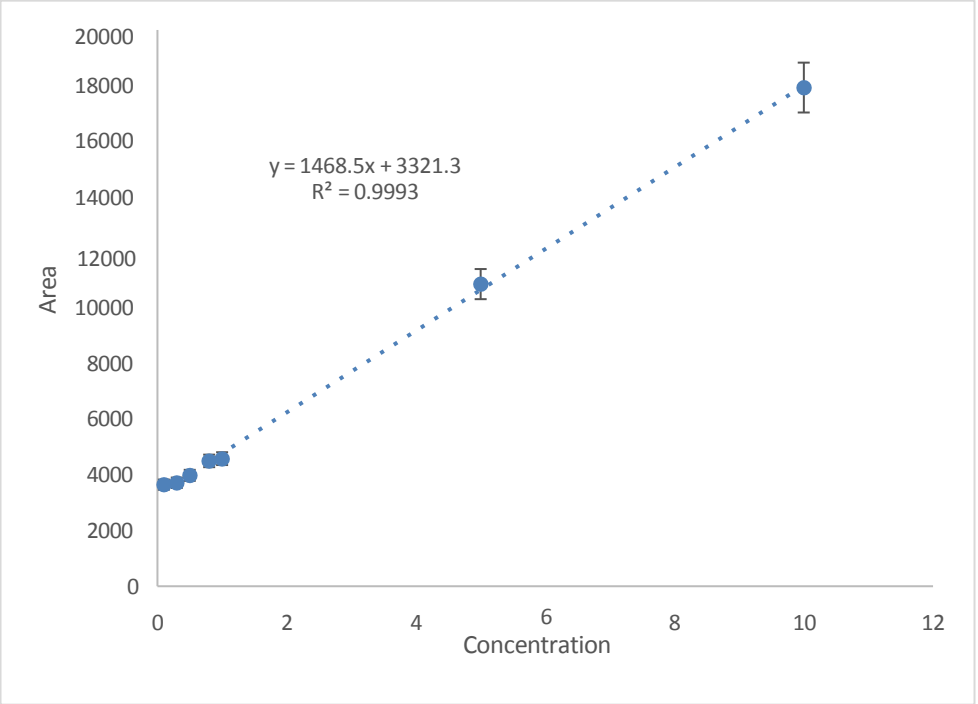


Figure 4.5 Showing linear regression graph for urine samples

Table 4.9 Showing intraday precision for urine samples

Concentration (ppm)	Area					Average	SD	RSD%
	1st	2nd	3rd	4th	5th			
0.3	3043	2865	2903	2720	3172	2940.6	173.0789	5.885836
1	3528	3309	3413	3785	3482	3503.4	177.7113	5.072538
10	14572	14854	15697	15846	14552	15104.2	622.9849	4.124581

Table 4.10 Showing interday precision for urine samples

Concentration (ppm)	Area					Average	SD	RSD%
	1st	2nd	3rd	4th	5th			
0.3	2874	2756	3165	3284	3267	3069.2	240.1243	7.823679
1	3396	3495	3176	2935	3267	3253.8	215.7886	6.631894
10	14290	13224	14102	13015	14865	13899.2	768.8184	5.531386

4.6.2 Validation for Blood Samples

Table 4.11 Showing calibration (Linearity and accuracy) for blood samples

Concentration (ppm)	Area	Calculated	Accuracy or absolute recovery %
0.1	2326	0.163816451	163.8164513
0.3	2446	0.256674147	85.55804896
0.5	2769	0.506616111	101.3232222
0.8	3204	0.843225257	105.4031572
1	3199	0.839356187	83.93561866
5	8775	5.154143775	103.0828755
10	14954	9.935541283	99.35541283

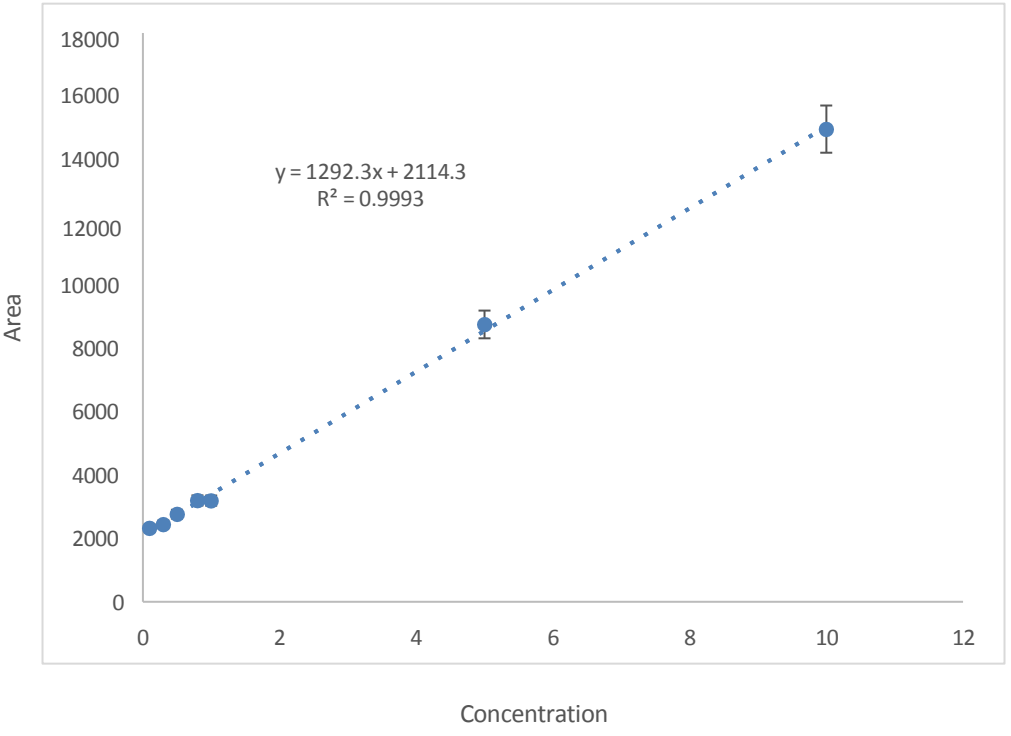


Figure 4.6 Showing linear regression graph for blood samples

Table 4.12 Showing intraday precision for blood samples

Concentration (ppm)	Area					Average	SD	RSD%
	1st	2nd	3rd	4th	5th			
0.3	1730	1879	1964	1986	1845	1880.8	102.4876	5.449147
1	2248	2385	2419	2231	2169	2290.4	106.7136	4.65917
10	13892	13476	12894	13531	12769	13312.4	469.2625	3.525003

Table 4.13 Showing interday precision for blood samples

Concentration (ppm)	Area					Average	SD	RSD%
	1st	2nd	3rd	4th	5th			
0.3	1625	1857	1754	1689	1953	1775.6	131.0946	7.383117
1	2085	2174	2293	1943	2158	2130.6	128.7257	6.041757
10	13636	12894	14269	13325	14669	13758.6	714.1269	5.190404

4.6.3 Validation for Pharmaceutical Tablet

Table 4.14 Showing calibration (Linearity and accuracy) for pharmaceutical tablets

Concentration (ppm)	Area	Calculated	Accuracy or absolute recovery %
0.1	5332.5	0.169134372	169.1344
0.3	5730	0.339727909	113.2426
0.5	6127.5	0.510321445	102.0643
0.8	6757.5	0.780696107	97.58701
1	7177.5	0.960945882	96.09459
5	16291.5	4.872365993	97.44732
10	28395	10.06677825	100.6678

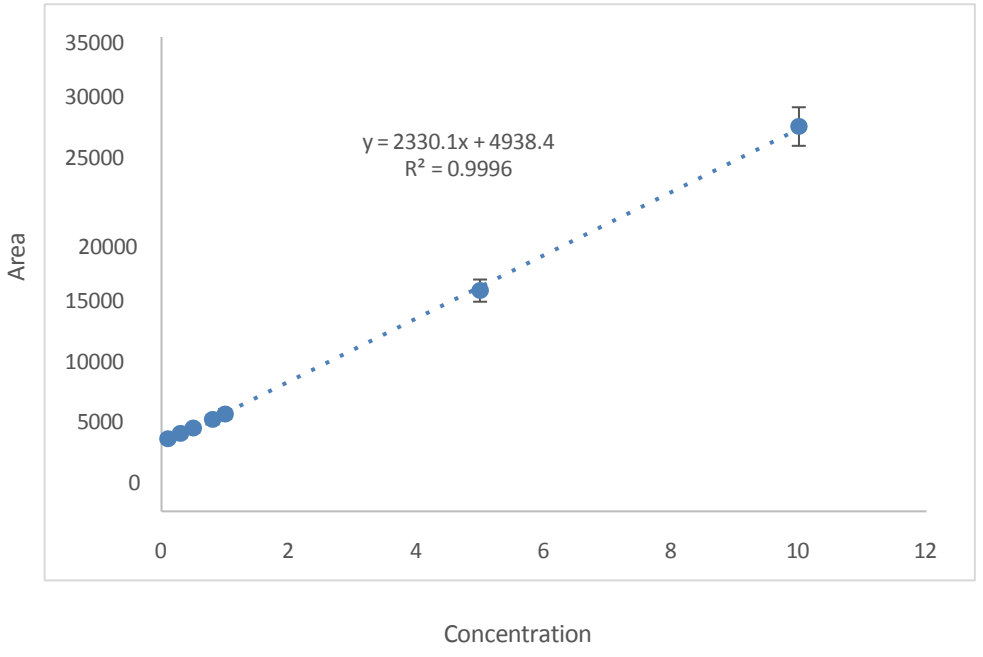


Figure 4.7 Showing linear regression graph for pharmaceutical tablet

4.7 Method Validation Parameters

Linearity, accuracy, recovery, precision, LOD, and LOQ were evaluated under optimum conditions for the proposed method. Matrix-matched calibration curve in urine and blood was plotted for CSP in the concentration range of 0.1-10 µg mL^{-1} as shown in table 4.8 and 4.11. A good correlation between the concentration and response was obtained in the said linear range for urine, blood, and pharmaceutical formulation (R^2= 0.9993, 0.9993, and 0.9996 respectively) as shown in figure 4.5, 4.6 and 4.7. LOD and LOQ for CSP in all three matrices were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively. LOD for pharmaceutical, urine, and blood were calculated to be in the range of 0.0050, 0.0052, and 0.0076 µg mL^{-1} respectively. Similarly, LOQ for pharmaceutical, urine, and blood were calculated to be in the range of 0.0165, 0.0171, and 0.0250µg mL^{-1} respectively.

Intra-day and inter-day precisions were shown as per cent relative standard deviation (% RSD) and were assessed at three different concentration levels of calibration graphs (low QC, middle QC, and high QC) for all matrices (n = 5). In all cases, intra-day and inter-day precisions were found to be lesser than 7 % and 11 %, respectively (Table 4.15). Recovery (accuracy) studies in urine and blood were executed at three different concentrations and were observed to be in the range of 86-99.4% in urine and 83.9-99.3% in blood showing an insignificant matrix effect. Recoveries in the pharmaceutical samples were calculated by added – found method and shown in Table 4.15. Moreover, the pre-concentration factor for the presented method was calculated to be 16 (volume of sample 8 mL, the final volume of the extract is 0.5 mL), therefore, enrichment recoveries (ER%) in urine, blood, and pharmaceutical samples were found to be 118.06 %, 103.68 %, and 120.25 %, respectively as mentioned in Table 4.15.

Table 4.15 Analytical attributes of the MIP-DSPE-GC-MS method for CSP

Matrices	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	R^2	Linearity (µg mL^{-1})	Calibration curve	Precision (% RSD)					
						Intra-day (µg mL^{-1})			Inter-day (µg mL^{-1})		
						0.3	1	10	0.3	1	10
Urine	0.0052	0.0171	0.9993	0.1-10	y = (1468.5 ± 17.6) x + (3321.3 ± 75.2)	5.8	5.0	4.1	7.8	6.6	5.5
Blood	0.0076	0.0250	0.9993	0.1-10	y = (1292.3 ± 15.8) x+ (2114.3 ± 67.3)	5.4	4.6	3.5	7.3	6.0	5.1
Pharmaceutical	0.0050	0.0165	0.9996	0.1-10	y = (2330.1 ± 13.0) x + (4938.4 ± 55.6)	6.3	5.2	4.5	8.4	7.0	5.8

Table 4.16 Absolute and enrichment recovery of the proposed method

Matrices	Recovery % (µg mL^{-1})			EF	ER %
	0.3	1	10		
Urine	90.5	86.0	99.4	18.89	118.06
Blood	85.5	83.9	99.3	16.59	103.68
Pharmaceutical	113.2	96.0	100.6	19.24	120.25

4.8 Comparison with Previous Methods

The proposed method has been compared with previously reported analytical methods for the determination of CSP in biological matrices and the comparison has been shown in Table 4.16. It is apparent from the table that there are limited published methods available for this particular analysis. In this study, a pioneering technique employing DSPE with a tailor-made MIP and subsequent GC-MS analysis has been introduced for CSP determination. This study represents the application of such a novel methodology. Significantly, the MIP-DSPE- GC- MS approach achieves lower

LODs compared to previously reported methods [Kim *et al.*, 2005; Downey *et al.*, 2009; Coulter *et al.*, 2012]. This improvement in LODs can be attributed to the enhanced sample enrichment obtained through the sample preparation procedure employed in this study, which differs from the SPE-based extraction utilized in the prior methods. Moreover, the precision of the proposed method is comparable to that reported in other studies [Kim *et al.*, 2005; Downey *et al.*, 2009; Coulter *et al.*, 2012], as evidenced by similar precision values obtained using the MIP-DSPE-GC-MS method.

Table 4.17 The proposed method for analysing target analyte in biological samples is compared with previously reported methods

Sample Matrix	Extraction Method	Technique	Precision (RSD %)	LOD (µg mL^{-1})	References
Oral fluid	SPE	LC-MS-MS	1.5-4.8	-	[Coulter *et al.*, 2012]
Whole blood	SPE	GC-MS	3.2-4.0	0.2	[Downey *et al.*, 2009]
Human hair	SPE	GC/MS	1.5-9.3	0.13	[Kim *et al.*, 2005]
Urine, whole blood, and pharmaceutical tablet	DSPE	GC-MS	3.5-8.4	0.0050-0.0076	Present study

4.9 Estimation of the Greenness of the Proposed Method

In the last few years, numerous metrics have emerged for estimating the greenness of analytical methods. The most thorough and rapidly obtaining popularity method amongst all is Complex Green Analytical Procedure Index (ComplexGAPI). Starting with the initial phase of MIP synthesis till the final evaluation of analyte, the proposed work has been assessed for its greenness with the help of ComplexGAPI [Jain *et al.*, 2022; Jain *et al.*, 2022; Jain *et al.*, 2023]. GAPI software was used to enter the values in different parameters to obtain a pictogram as shown in Figure 4.8, which is

composed of five pentagrams at the top with one hexagonal field at the bottom. Different parts of the pictogram represent different aspects of the analytical method e.g., sample preparation, different reagents used, instrumentation, collection of the samples and their preservation; and lastly in the center; the general type of method used. The color scale is divided into three different levels green, yellow, and red denoting low, medium, and high impact on the environment respectively. Each of the above- mentioned parameters of the analytical methods are assessed using this color scale. Moreover, the hexagon at the pictogram‘s bottom represents the influence of pre- analytical processes on the environment. By looking at Figure 4.8, it may be asserted that the presented analytical technique is reasonably green and has no significant adverse environmental impact, because the pentagrams are majorly yellow or green, indicating compliance with the concept of GAC. Here, red colored areas in the pictogram and hexagonal field correspond to different parameters used in the proposed method like 1 – sample collection (offline), 3 –transportation(required), 5 – the type of method used (extraction), 7 – the type of solvent used (MeOH), 12 – the amount of energy consumed (>1.5 kWh/hour for GC-MS) and II (hexagon)– temperature (>1 hour).

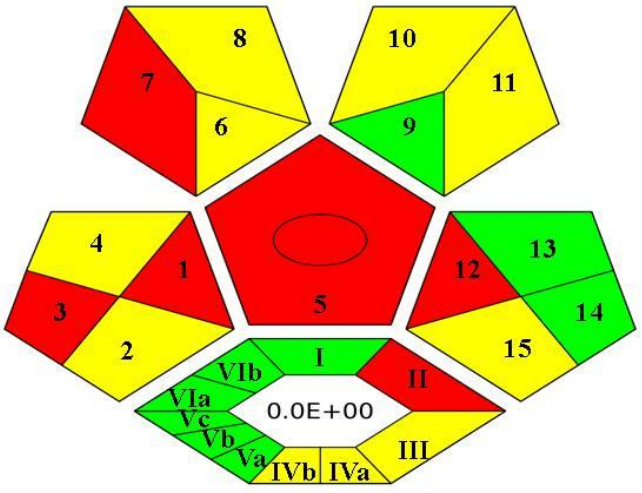


Figure 4.8 ComplexGAPI pictogram for the proposed method

4.10 Polymer Reusability and Stability

The synthesized MIP was utilized for extracting CSP from aqueous solutions in a consecutive manner for ten cycles. For this purpose, a fixed concentration of CSP (10 μg mL^{-1}) was extracted using the developed DSPE-MIP protocol and eluted in 1 mL of MeOH. In each cycle, the MIP was desorbed, dried, and re-employed for the subsequent extraction step under the optimized conditions. Even after seven consecutive cycles, over 90% of CSP was still successfully extracted. The consistent extraction efficacy observed after seven cycles indicates the potential for reusing the MIP, which is a critical factor for promoting the environmentally friendly and cost-effective application of adsorbent materials. Further, the stability of the synthesized MIP for its extraction efficiency at various temperatures was studied in the range of 50 to 100ºC. For this purpose, 50 mg of MIP incubated at different temperatures in a glass vial and then used for extraction. Results were then compared with non-incubated MIP; the MIP retained its affinity towards CSP and no decrease in the extraction efficiency was observed with increasing temperature, suggesting that synthesized MIP is stable in elevated temperature conditions also [Svenson and Nicholls, 2001].

4.11 Conclusion

In the current novel work, MIP synthesized using CSP as the template by bulk polymerization that showed significant specificity and affinity towards the target drug when applied as an adsorbent for DSPE. A simple and rapid MIP-DSPE method combined with GC-MS was optimized for selective recognition and extraction of CSP from biological and pharmaceutical samples. In comparison to the conventional extraction techniques, MIP- DSPE is easy to use, rapid, and specific. The optimized method is justified to be rapid, selective, greener, sensitive, low cost with higher shelf life, and simple for the determination of CSP from complex matrices. A pharmaceutical sample also used for the validation of the optimized method; showing promising extraction and recoveries. Moreover, MIP-DSPE excelled in analytical performance for notable operating ease and significantly reduced process time, less solvent, sorbent, and sample amount needed. The proposed MIP-DSPE- GC-MS method also displayed significant recoveries, repeatability, and reproducibility in both biological and pharmaceutical samples. Furthermore, the requisites defined by GAC

principles were also satisfied in the present research as the MIP-DSPE method remarkably reduced the utilization of organic solvents and reagents leading toa greener study. The developed method could be used routinely to analyze CSP in biological samples of both clinical and toxicological relevance.

Chapter – 5

Synthesis of Molecularly Imprinted Polymer based on Polyethylene Glycol and Polyvinyl Alcohol

5.1 Introduction

CSP is a muscle relaxant that acts centrally to alleviate musculoskeletal pain. It is also known to be used illicitly which have further led to fatality sometimes [Reeves *et al.*, 2012]. Although all medications in the SMR class have the characteristics of dependence, tolerance, and abuse, CSP abuse has reportedly increased more than that of other SMRs [Boothby *et al.*, 2003]. The increased likelihood of traffic accidents brought on by impaired driving is a key worry associated with CSP abuse. This is causing car accidents, and in some cases, fatalities. Therefore, it is crucial for forensic toxicologists to gather samples and find the abuse-causing drug [Bramness *et al.*, 2004].

MIPs are synthetic polymers that are created with specialized molecular recognition and binding sites. Due to their great selectivity, stability, and affordability as compared to other molecular recognition materials, they are widely employed in a variety of domains, including separation research, drug delivery, and sensing applications. In the presence of a template molecule, functional monomers and cross-linking agents are polymerized to create MIPs [BelBruno, 2018]. Following polymerization, the template molecule is eliminated to produce particular cavities or imprinted sites with great selectivity for recognizing and binding to the target molecule [Yan and Ho Row, 2006]. A synthetic polymer with exceptional film-forming abilities, polyvinyl alcohol is easily cross- linked using a variety of cross-linking agents. Polyethylene glycol (PEG), a hydrophilic and biocompatible polymer, can offer an adaptable framework for the imprinted spots. It has been shown in the previous studies that the addition of polyvinyl alcohol (PVA) and and PEG increases the binding affinity and selectivity of MIPs for a variety of target molecules, including medications, insecticides, and proteins [Foroutan and Zibaseresht, 2019].

Several methods have been reported over the years for the synthesis of MIPs using PVA and PEG, including precipitation polymerization, emulsion polymerization, and surface imprinting. Precipitation polymerization involves the addition of a cross-linking agent to a mixture of functional monomers and template molecule in a solvent followed by the addition of a non-solvent to precipitate the polymer. Emulsion polymerization involves the emulsification of a mixture of functional monomers, cross-linking agent, and template molecule in water followed by polymerization.

Surface imprinting involves the formation of a thin layer of MIP on the surface of a support material [Kanao *et al.*, 2020].

The synthesis of MIPs using PVA and PEG as functional monomer has several advantages over traditional MIP synthesis methods. The hydrophilic nature of PVA and PEG allows for the preparation of MIPs in aqueous media, making them more environmentally friendly. Additionally, PEG can provide a more flexible and accessible framework for the imprinted sites, resulting in improved binding affinity and selectivity [Piperno *et al.*, 2011; Kanao *et al.*, 2020;].

Several examples of the synthesis of MIPs using PVA and PEG as functional monomers have been reported in the literature. Ampicillin-imprinted polymers was prepared using PVA and PEG by precipitation polymerization. The resulting MIPs showed high selectivity and binding affinity for ampicillin in aqueous solutions [Tang *et al.*, 2017]. MIPs with PVA as the template have been used for the selective recognition and separation of biologically active molecules, such as adrenaline and histamine [Huang *et al.*, 2018; Cai *et al.*, 2019]. Meanwhile, MIPs with PEG as the template have been used for the selective extraction and detection of environmental pollutants, such as bisphenol A [Zhao *et al.*, 2018; Huang *et al.*, 2019].

The experiment was set up for the synthesis of MIP based on PEG and PVA. The basic aim of the work was to successfully synthesize the MIPs and further use it for extraction of CSP. In the process, different instruments have also been used for the analysis purpose.

5.2 Experimental

5.2.1 Materials and Methods

Complete synthesis was carried out in a standard laboratory setting. Starting materials HEMA (TCI), PVA (LOBA), PEG (LOBA), 4-Toluenesulfonyl chloride (TsCl) (Aldrich), Triethylamine (Et_3N) (SDFCL), Dichloromethane (LOBA), 1, 4-Dioxane (LOBA), CSP (TCI) had been used exactly as received. Various spectroscopic techniques were used to characterise the synthesized compounds, namely, IR spectra obtained with a SHIMADZU FTIR-8400S spectrometer and Bruker Avance Neo FT NMR spectrometer used for multinuclear NMR (^{1}H, ^{13}C) spectra. In NMR (1 H and 13C), CDCl3 was used as an internal standard, and the chemical shifts mentioned were comparable to tetramethylsilane (TMS).

5.2.2 Synthesis with Hydroxyethylmethacrylate

5.2.2.1 Synthesis of Tosyl Functionalized HEMA

50 mL Dichloromethane (solvent) was mixed with 5.7 g of p-Toluenesulfonyl chloride and 5 mL of hydroxyethylmethacrylate (HEMA) in the presence of 5 mL of Triethylamine. The mixture was constantly heated and stirred at 50℃ (monitored constantly with laboratory thermometer) for 5 hours as shown in figure 5.1. The mixture was composed of unreacted TsCl, HEMA-Ts and Et_3N. TLC was taken of this reaction. After 5 hours, this solution was separated in DCM and water to remove unreacted products. This separated solution was further concentrated for FTIR characterization. Figure 5.2 is showing the complete stepwise reaction of the synthesis.

Figure 5.1 Showing synthesis of Tosyl Functionalized HEMA (HEMA-Ts) in reflux reaction.

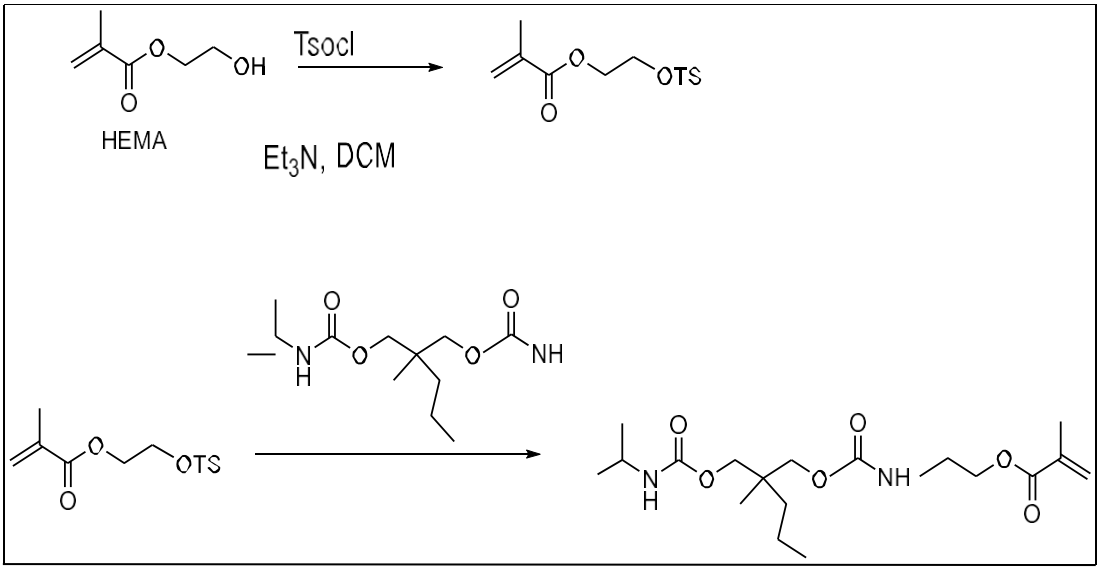


Figure 5.2 Showing stepwise reaction of synthesis with HEMA

5.2.2.2 TLC of HEMA-Ts:

For thin layer chromatography (TLC), silica gel G was used. The mobile phase used was Ethyl acetate (3): Hexane (7). Three samples were taken for spotting on TLC: the left spot was of HEMA diluted with DCM, the right spot was of the reaction solvent diluted with DCM and the spot on the center was the combination of both. The retention factor (Rf) value of spot 1 (left) is 0.5 and the Rf value of spot 2 (right) is 0.6. The center combination spot indicates spotting of both left and right mixture in figure 5.3 therefore clearly indicating the formation of HEMA-Ts.

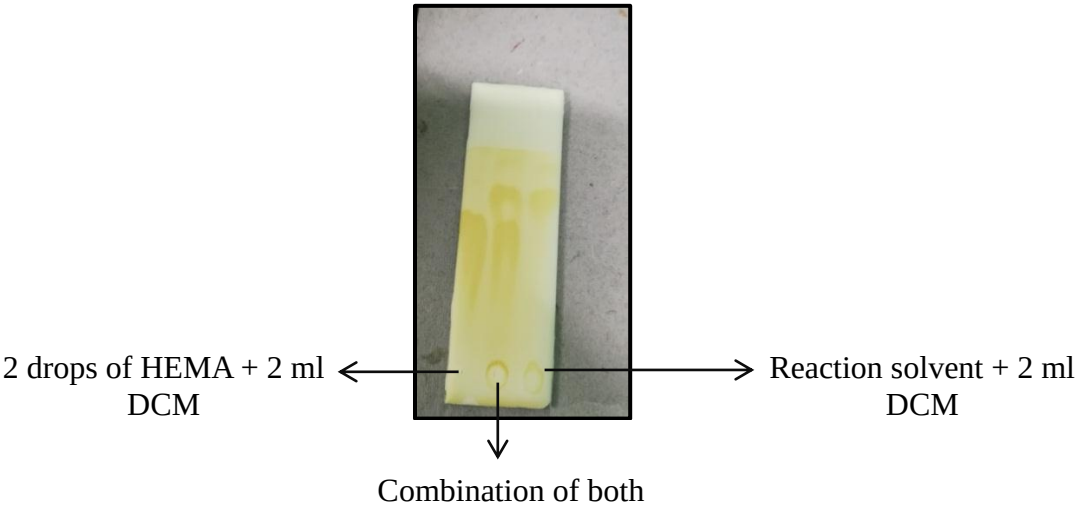


Figure 5.3 TLC of HEMA-Ts

5.2.2.3 FTIR Spectroscopy-

The FTIR spectrum of HEMA-Ts was obtained. The corresponding r values for the absorbance peak at wavenumbers of 1155, 1296, 1377, 1670, 1718, 2955 and 3745 cm^{-1} were analyzed. We can clearly see the presence of a peak at 3745 cm^{-1} which is linked to the stretching of O-H from the intramolecular and intermolecular hydrogen bonds. The peaks observed at 2955 cm^{-1} is related to the symmetric and antisymmetric stretching vibrational of C-H from alkyl groups. The peak at 1670 cm^{-1} may be assigned to the C=O stretching group. The peaks at 1377 cm^{-1} and 1155 cm^{-1} can be related to the S=O group as shown in figure 5.4.

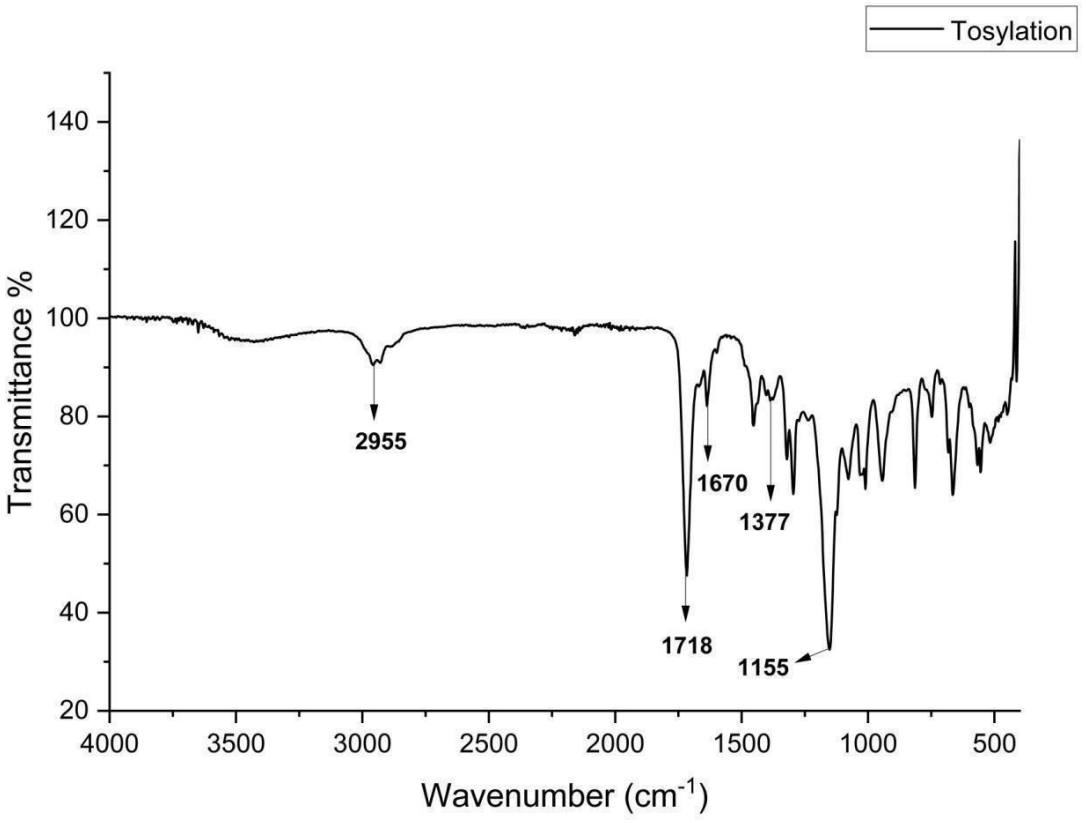


Figure 5.4 FTIR spectrum of HEMA-Ts

5.2.3 Synthesis with Polyvinyl Alcohol

5.2.3.1 Synthesis of Tosyl Functionalized Polyvinyl Alcohol

Deionized water was mixed continuously for two hours at 80 °C with 10 % (w/v) of polyvinyl alcohol (PVA) in it. To this solution, 4-Toluenesulfonyl chloride (4.33 g) and triethylamine (10.4 mL) were added; the combination was made up of unreacted

TsCl, PVA- Ts, and Et3N after 24 hours of stirring at room temperature. This solution was further filtered and solvent was eliminated with vacuum evaporation (Figure 5.5).

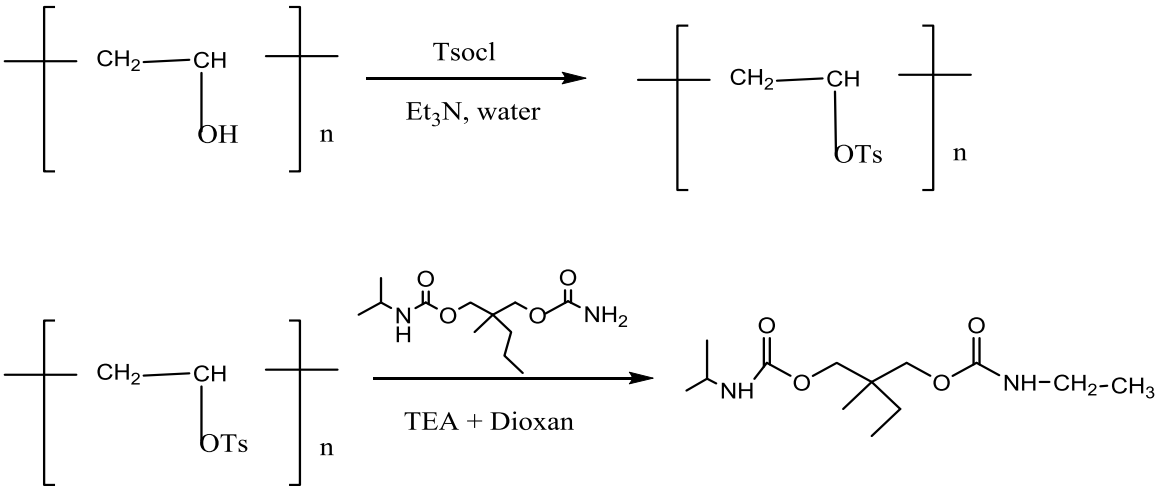
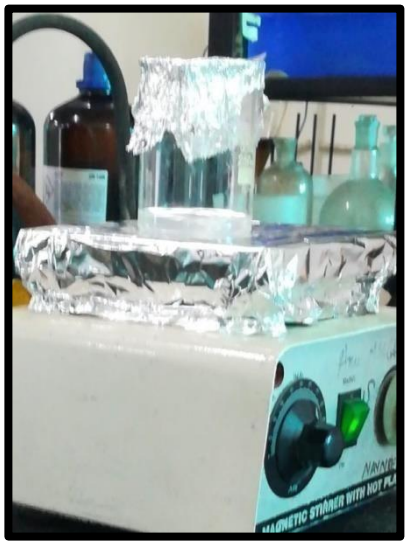


Figure 5.5 Schematic illustration of tosylation reaction followed by amidation (PVA)



PVA being stirred at 80 ℃ in deionised water

PVA + TsCl in presence of Et_3N

Concentrated PVA-Ts

Figure 5.6 Showing stepwise synthesis of Tosyl Functionalized PVA (PVA-Ts)

5.2.3.2 Tosyl Chloride Formation

Color/texture: orange coloured gel-like formation; IR (neat, cm^{-1}) 3348, 1638,1476,1398 and 1177 as shown in figure 5.7; ^{1}H NMR (400 MHz, DMSO): δ 1.19 (t), 2.3 (s), 2.51 (dd),3.07 (qd), 3.70 (s), 3.74 (d), 7.14 (d), 7.49 (d); ^{13}C NMR (125 MHz, DMSO): δ 21.25 (s), 39.26 (s), 40.09 (s), 46.03 (s), 125.95 (s), 128.62 (s), 138.52 (s), 145.37 (s).

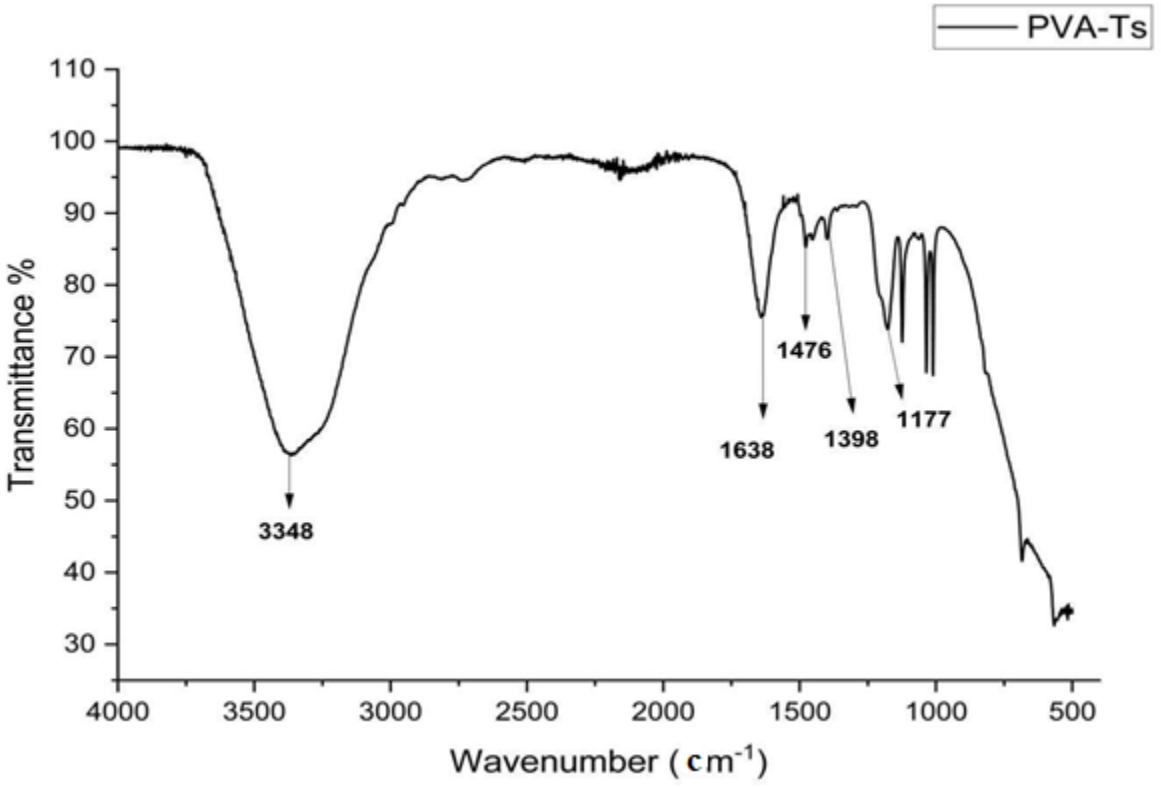


Figure 5.7 FTIR spectrum of PVA-Ts

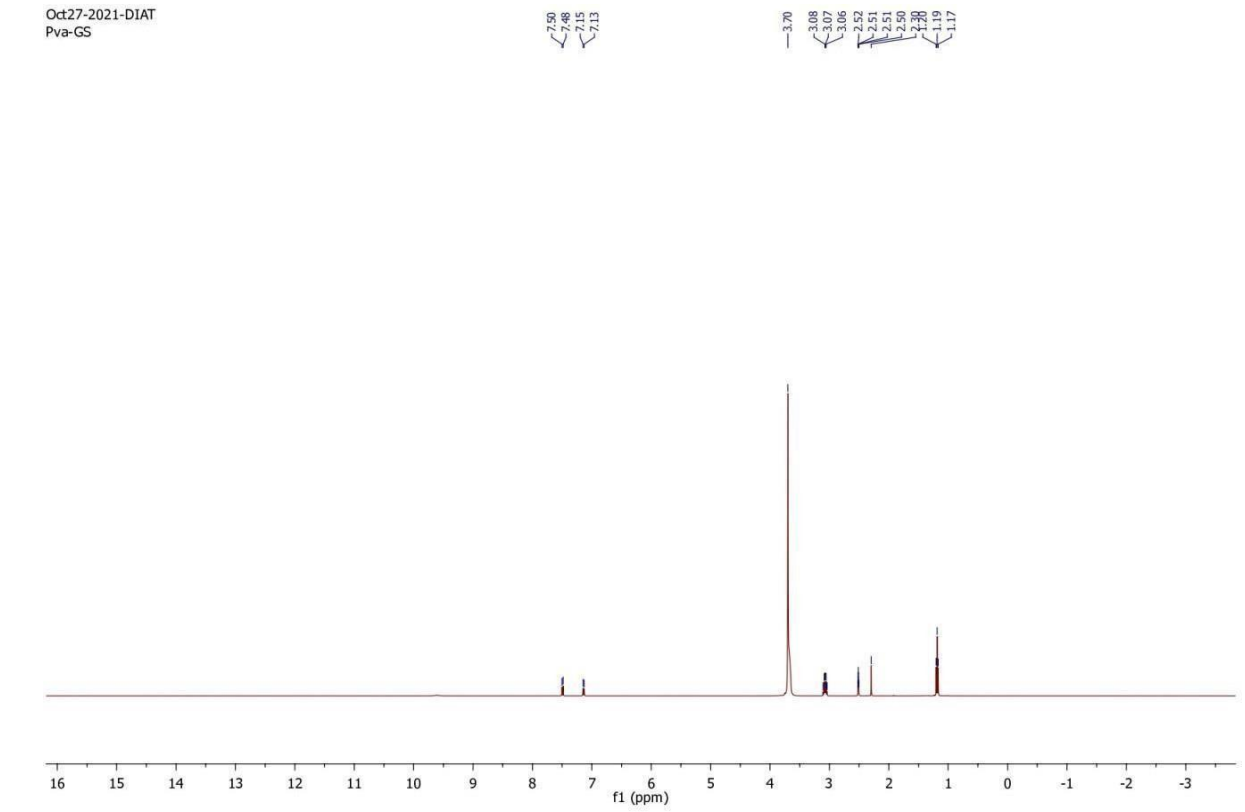


Figure 5.8 Showing ^{1}H NMR of PVA-Ts

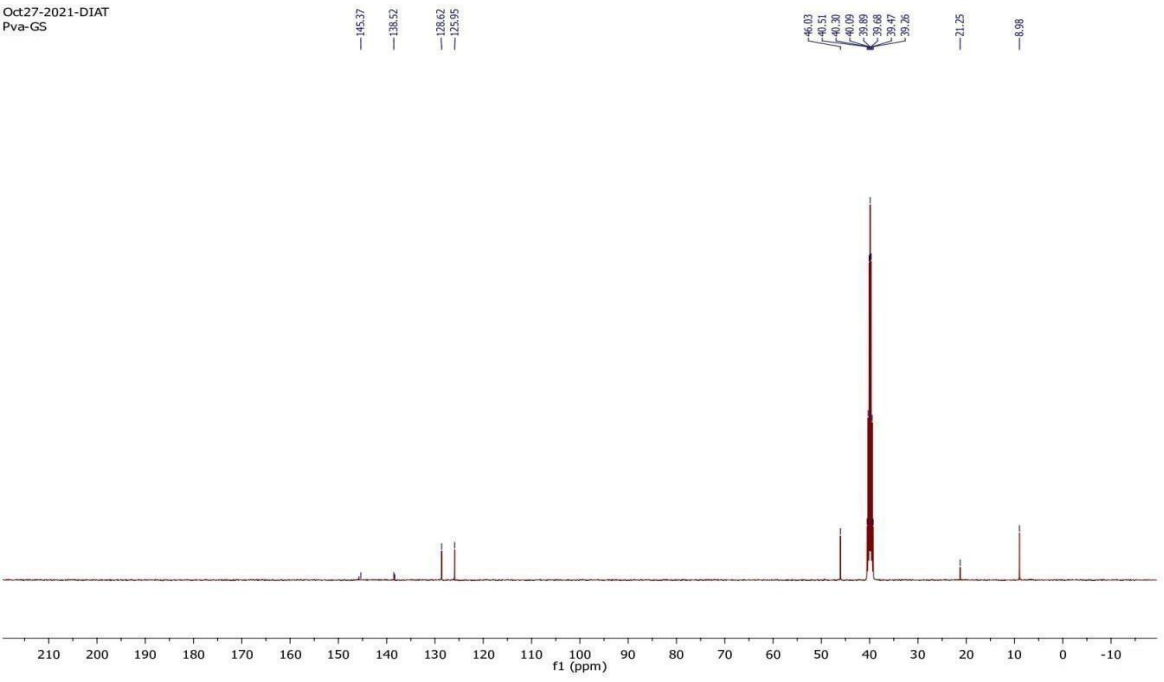


Figure 5.9. Showing ^{13}C NMR of PVA-Ts

5.2.3.3 Reaction of PVA-Ts with Carisoprodol

For this reaction, 0.3 g Tosylate (PVA-Ts) was added to 40 mL of 1, 4 – Dioxan (solvent) along with 3 mL of Et_3N. 0.33 g; CSP was also added to this solution. For 6 hours, the reaction mixture was stirred at 80 ℃. Ethanol was added to this solution followed by reflux for 3 hours to get reddish brown precipitates as shown in the figure 5.8. Further, vacuum evaporation was used to remove the solvent from this solution. These precipitates were dried at room temperature, crushed and dried in dessicator.

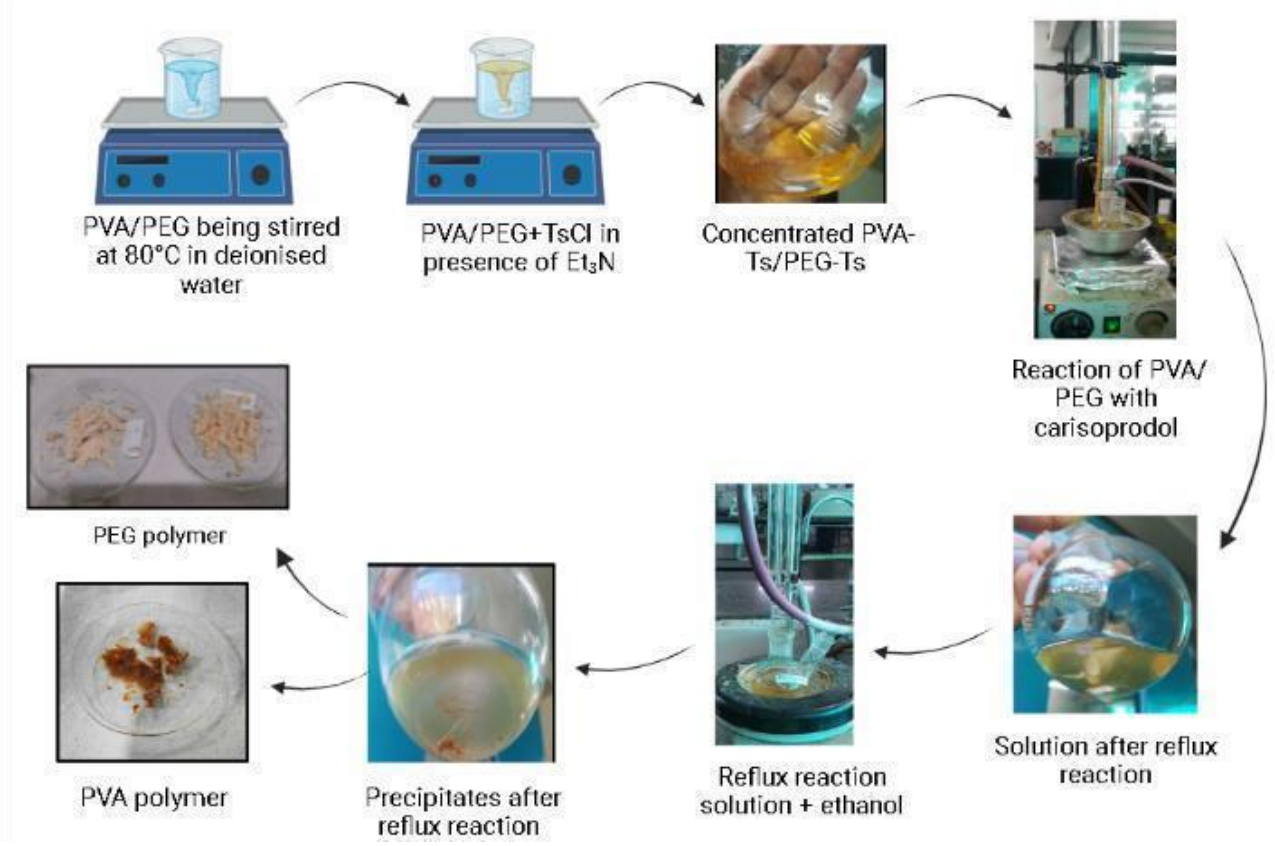


Figure 5.10.Diagrammatic representation of the complete synthesis or PVA/PEG

5.2.3.4 Amidation

Color/texture: dark brown; IR (neat, cm^{-1}) 3346, 2942, 1696, 1368, 1329 and 1234 cm^{-1} as shown in figure 5.11; the NMR spectroscopy was also performed at a frequency of 400 MHzusing $CDCl_3$ as the solvent. 1H NMR (400 MHz): δ 0.90 (s), 1.20 (m), 1.39 (t), 1.80 (s), 2.36 (s), 3.15 (q), 3.89 (d), 4.57 (s), 4.72 (s), 7.18 (d), 7.27 (s), 7.78 (d);

^{13}C NMR (125 MHz): δ 8.62 (s), 14.89 (s), 16.37 (s), 18.95 (s), 23.01 (s), 36.92 (s), 37.44 (s), 43.04 (s), 46.01 (s), 67.95 (s), 68.67 (s), 76.7 (s), 77.03 (s), 77.3 (s), 125.9 (s), 128.81 (s), 156.09 (s), 156.97 (s).

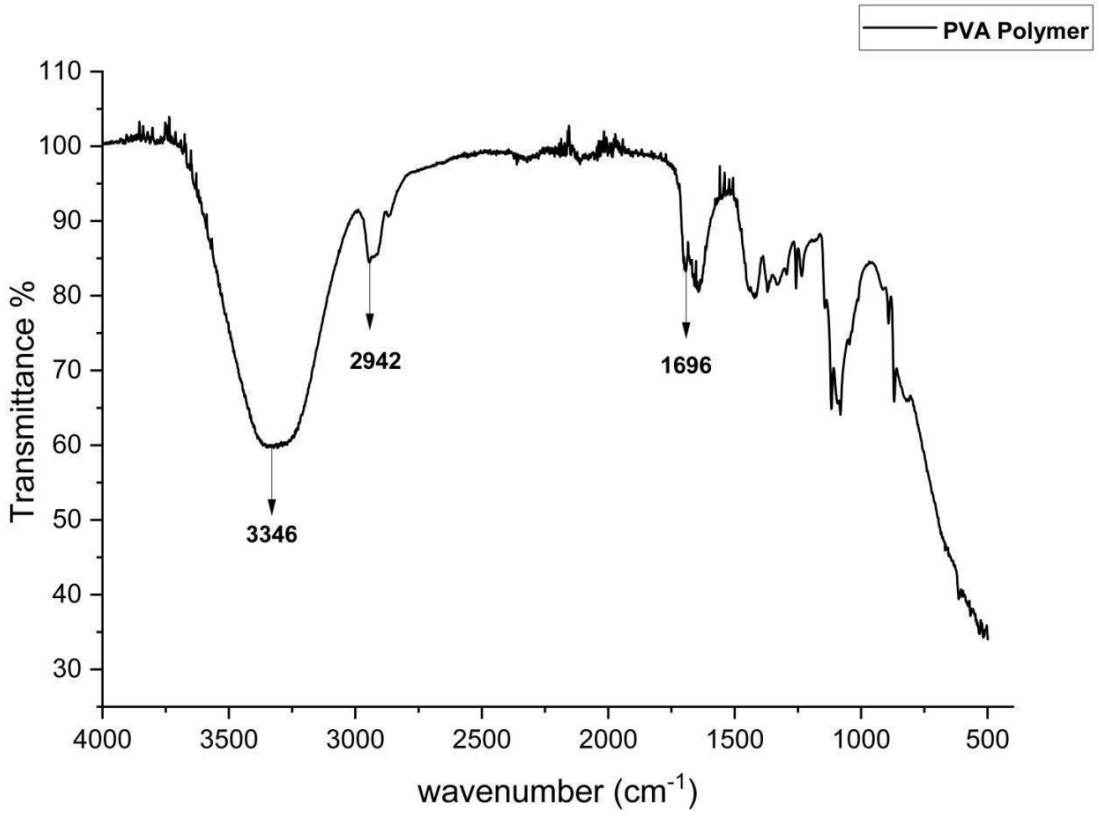


Figure 5.11 FTIR spectrum of amidation (PVA)

5.2.4 Synthesis with Polyethylene Glycol

5.2.4.1 Synthesis of Tosyl Functionalized PEG (4000, 8000, and 1500)

10 % (*w/v*) of polyethylene glycol (PEG) (4000, 8000, and 1500) solution was added in deionized water kept at 80 ℃ under continuous stirring for a period of 2 hours. To this solution, 4-Toluenesulfonyl chloride (4.33 g) and triethylamine (10.4 mL) were added; the combination was made up of unreacted TsCl, PEG-Ts, and Et3N after 24 hours of stirring at room temperature. After the completion of the above reaction, this solution was further filtered and solvent was eliminated with vacuum evaporation as shown in Figure 5.8. Schematic illustration of tosylation reaction followed by amidation reaction as is shown in figure5.12.

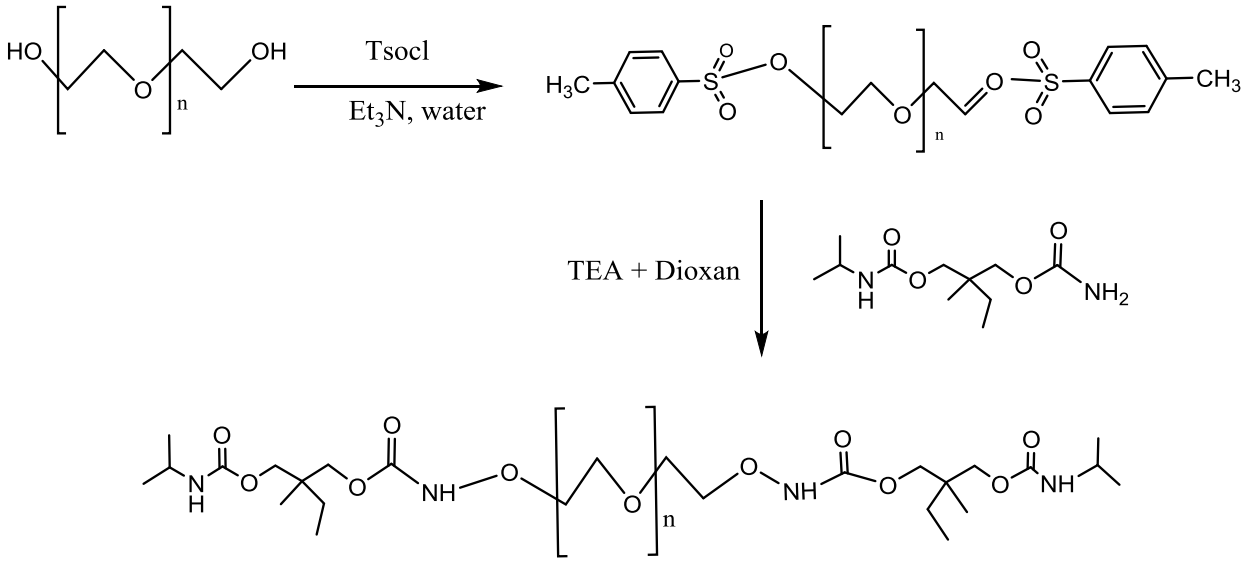


Figure 5.12 Schematic illustration of tosylation reaction followed by amidation (PEG)

5.2.4.2 Tosyl chloride formation For PEG-Ts (4000)

Color/texture: orange; IR (neat, cm^{-1}) 2888, 1466, 1343, 1280, and 1174 cm^{-1} as shown in figure 5.13; the NMR spectroscopy at frequency of 400 MHz using $CDCl_3$ as the solvent. ^1H NMR (400 MHz): δ 2.15 (s), 2.35 (s), 3.46 (s), 3.64 (s), 7.18 (d), 7.29 (s), 7.77 (d); ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.32 (s), 70.51 (s), 76.75 (s), 77.07 (s), 77.39 (s), 125.86 (s), 128.18 (s), 139.96 (s), 142.34 (s).

For PEG-Ts (8000)

Color/texture: orange; IR (neat, cm^{-1}) 2886, 1473, 1343, 1280, and 1171 cm^{-1}; the NMR spectroscopy was also performed at a frequency of 400 MHz using $CDCl_3$ as the solvent. ^1H NMR (400 MHz): δ 2.34 (s), 3.12(d), 3.43 (d), 3.63 (d), 7.17 (d), 7.29 (s), 7.75 (dd); ^{13}C NMR (125 MHz): δ 21.31 (s), 46.07 (s), 70.50 (s), 76.78 (s), 77.10 (s), 77.42 (s), 125.83 (s), 128.77 (s), 139.96 (s), 142.36 (s).

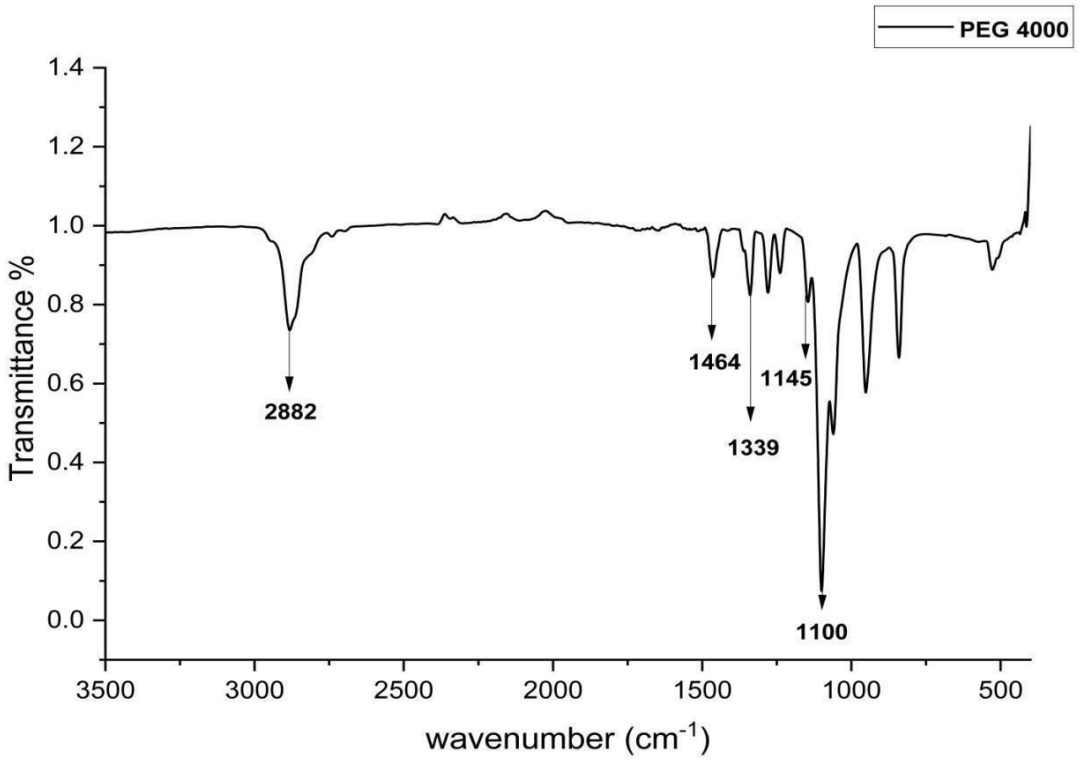


Figure 5.13 FTIR spectrum of PEG-Ts (4000)

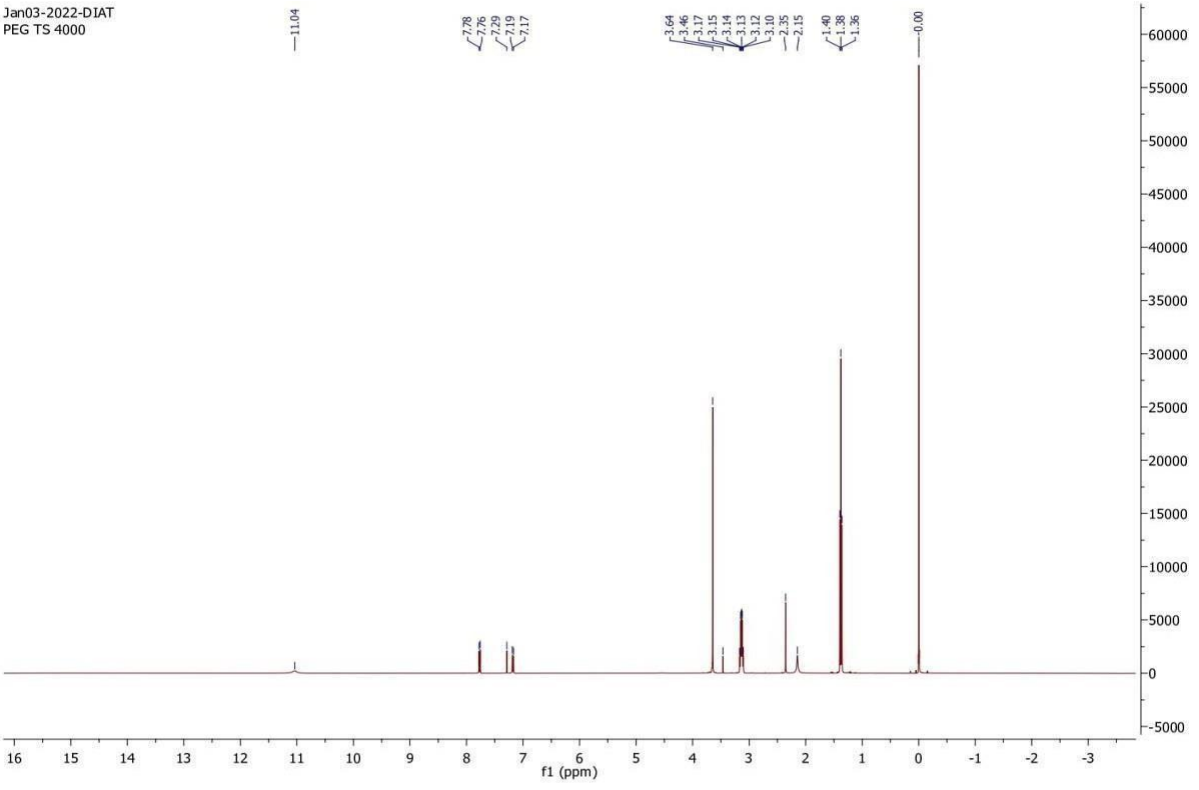


Figure 5.14. Showing ^{1}H NMR of PEG-Ts (4000)

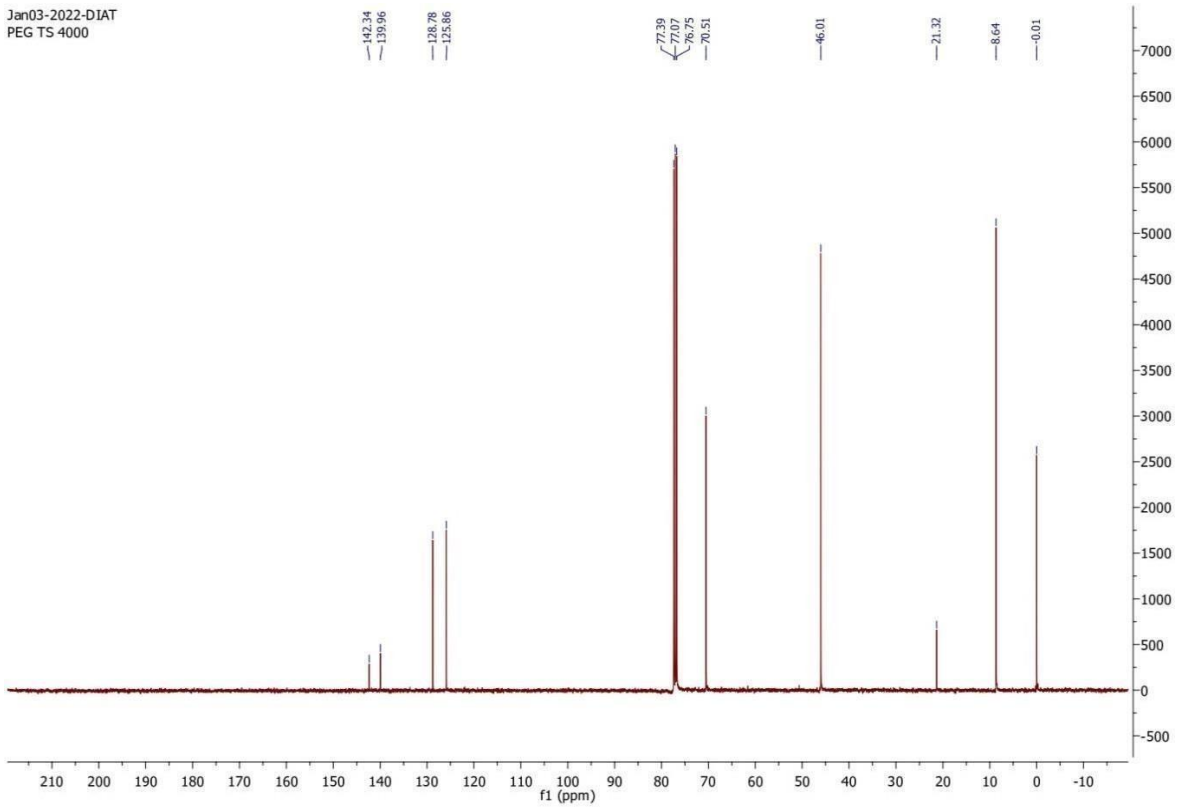


Figure 5.15. Showing ^{13}C NMR of PEG-Ts (4000)

For PEG-Ts (1500)

Color/texture: orange; IR (neat, cm^{-1}) 2886, 1474, 1341, 1282 and 1172 cm^{-1}; the NMR spectroscopy was also performed at a frequency of 400 MHz using $CDCl_3$ as the solvent. ^1H NMR (400 MHz): δ 1.25 (dd), 1.41(d), 1.9 (s), 2.37 (s) 3.16 (s), 3.66 (s), 7.19 (d), 7.29 (s), 7.79 (d); ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.34 (s), 23.02 (s), 77.04 (s), 67.95 (s), 68.66 (s), 77.36 (s), 125.89 (s), 128.80 (s), 139.99 (s), 142.51 (s).

5.2.4.3 Reaction of PEG-Ts (4000, 8000, and 1500) with Carisoprodol

0.5 g Tosylate (PEG-Ts) was added to 50 mL of 1, 4 – Dioxan (solvent) along with 5 mL of Et_3N. 0.57 g; CSP was also added to this solution. For continuous 6 hours, the reaction mixture was stirred at 80 ℃. Ethanol was added to this solution followed by reflux for 3 hours to get reddish brown precipitates as shown in the figure 5.8. Further, vacuum evaporation was used to remove the solvent from this solution. These precipitates were dried at room temperature, crushed and dried in dessicator.

Amidation

For PEG polymer (4000)

Color/texture: brown colored formation; IR (neat, cm^{-1}) 3416, 2961, 1698, 1469, 1387, and 1241 cm^{-1} as shown in figure 5.16, ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.96 (d), 1.15 (s), 1.22 (s), 1.40 (t), 2.37 (s), 3.16 (s), 3.77 (s), 3.9 (s), 4.28 (d), 7.20 (d), 7.29 (s), 7.80 (d); ^{13}C NMR(125 MHz, $CDCl_3$): δ 8.63 (s), 21.57 (s), 16.3 (s), 14.90 (s), 46.00 (s), 70.54 (s), 76.72 (s), 77.03 (s), 77.35 (s), 125.91 (s), 128.99 (s).

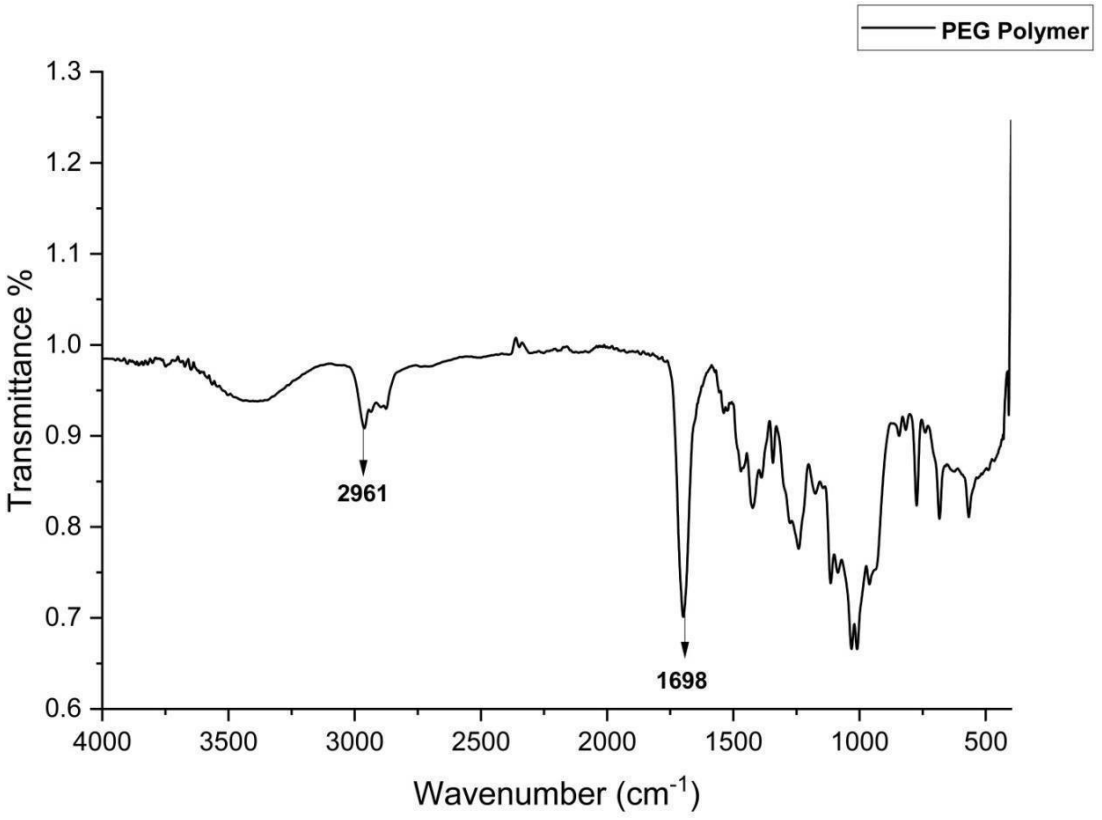


Figure 5.16. FTIR spectrum of amidation (PEG)

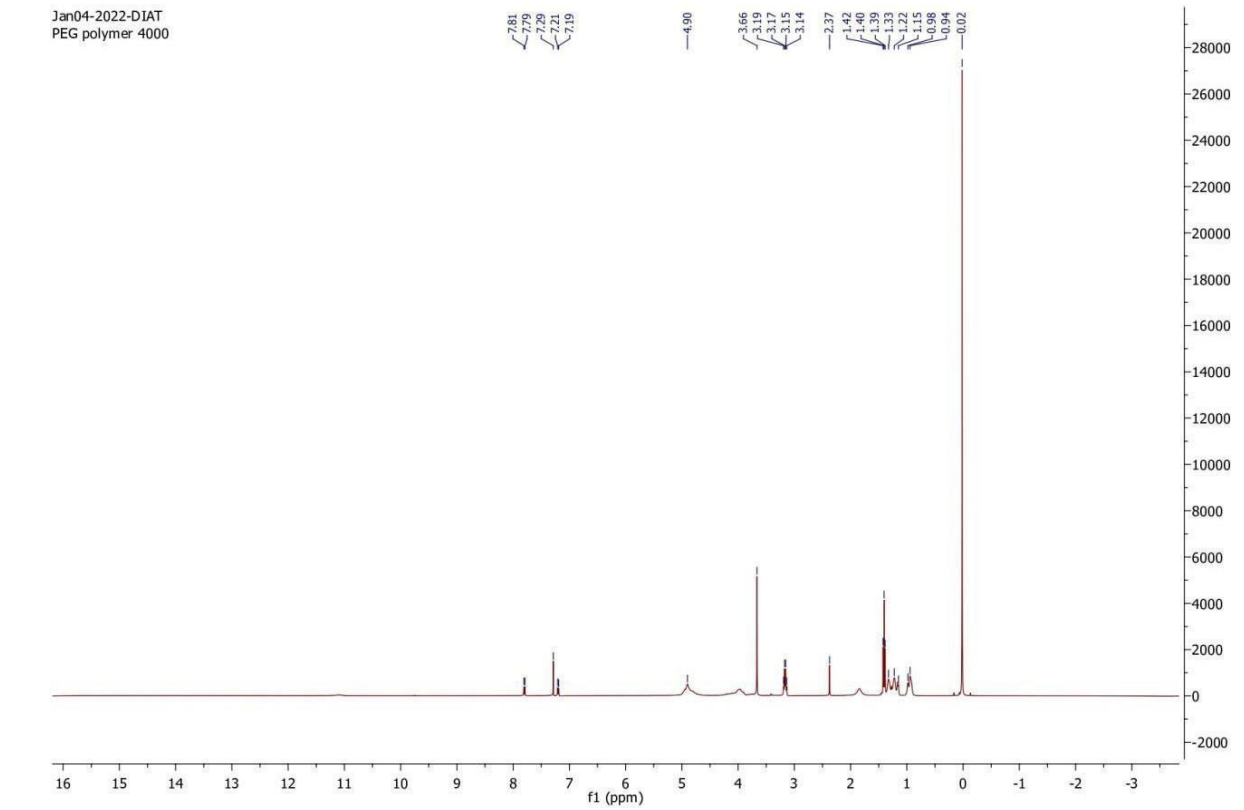


Figure 5.17. Showing ^{1}H NMR of PEG Polymer (4000)

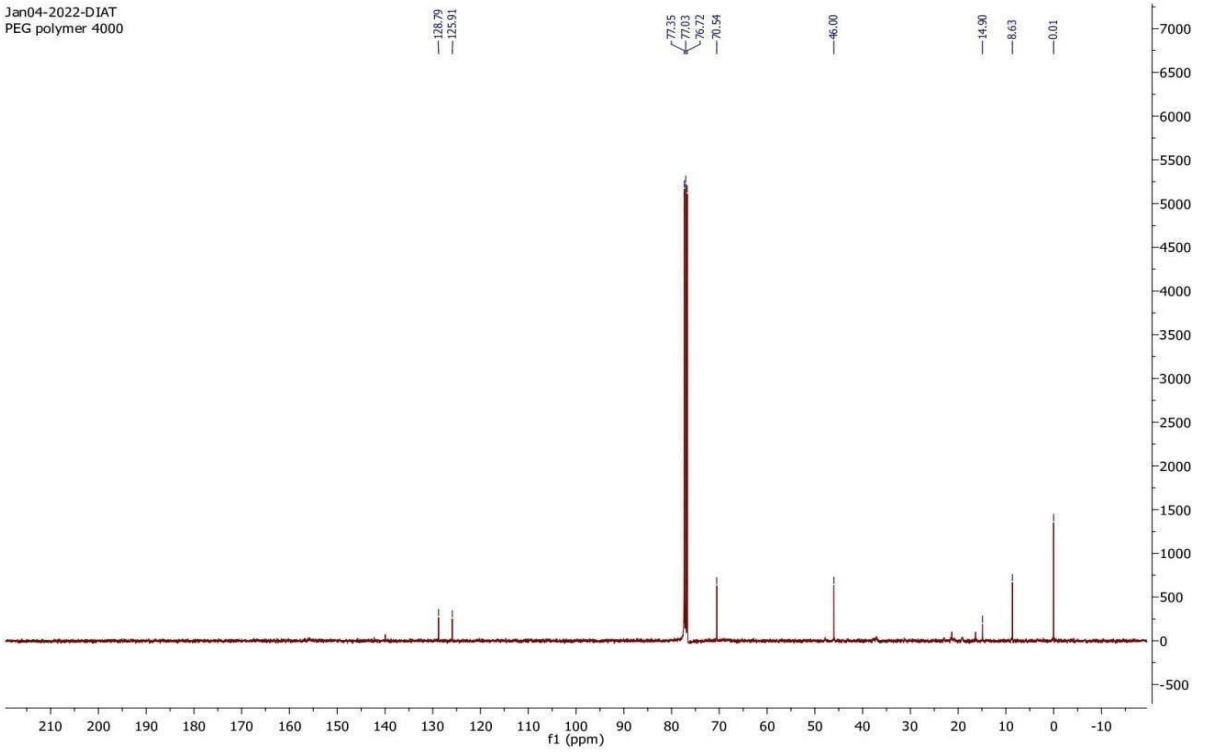


Figure 5.18. Showing ^{13}C NMR of PEG Polymer (4000)

For PEG polymer (8000)

Color/texture: whitish coloured sticky texture formation; IR (neat, cm^{-1}) 3447, 2965, 1692, 1460, 1345, and 1243 cm^{-1}; ^{1}H NMR (400 MHz): δ 0.9 (s), 1.16 (s), 1.25 (dd), 1.41 (d) 2.37 (s), 3.16 (s), 3.66 (s), 3.9 (d), 7.19 (d), 7.29 (s), 7.79 (d); ^{13}C NMR (125 MHz): δ 18.96 (s), 21.34 (s), 23.02 (s), 36.92 (s), 37.44 (s), 46.02 (s), 77.04 (s), 70.53 (s), 67.95 (s), 68.66 (s), 76.72 (s), 77.36 (s), 125.89 (s), 128.80 (s), 139.99 (s), 156.96 (s).

For PEG polymer (1500)

Color/texture: whitish coloured solid texture formation; IR (neat, cm^{-1}) 3446, 2966, 1692, 1462, 1345, and 1243 cm^{-1}; ^{1}H NMR (400 MHz): δ 0.90 (s), 1.16 (d), 1.32 (d), 1.39 (d), 2.09 (s), 2.36 (s), 3.15 (q), 3.65 (s), 3.89 (s), 4.80 (s), 7.18 (d), 7.29 (s), 7.77 (d); ^{13}C NMR (125 MHz): δ 8.62 (s), 18.93 (s), 21.32 (s), 36.90 (s), 37.44 (s), 46.02 (s), 67.94 (s), 68.63 (s), 70.51(S), 76.44 (s), 76.90 (s), 77.38 (s), 125.88 (s), 128.80 (s).

5.3 Results and Discussion

a. Synthesis

PVA polymer was synthesized via a two-step process; initially tosyl chloride was formed in presence of triethylamine, followed by amidation with the addition of CSP. Similarly, PEG polymer was also synthesized by tosylation reaction followed by addition of CSP. The intermediate products and final polymers were characterized using spectroscopic methods such as FTIR, ^{1}H NMR, and ^{13}C NMR.

b. Spectroscopic analysis FTIR Spectroscopy

The IR spectroscopy data collected for tosylation and amidation in the range 4000-500 cm^{-1} were in line with anticipated results. The peaks observed for tosylation (PVA-Ts) were at 3348 cm^{-1} related to the stretching of O-H from the intramolecular and intermolecular hydrogen bonds, the peak at 1638 cm^{-1} may be assigned to the C=C stretching group, 1464 cm^{-1} may be related to C-H bending, 1398 cm^{-1} and 1177 cm^{-1}can be linked to the group of S=O which confirms the formation of tosylate. The

peaks observed for amidation (PVA-Ts + CSP) were at 3346 cm^{-1}, 2942 cm^{-1} shows C-H stretching. The peak at 1696 cm^{-1} is showing amidation; this may indicate the introduction of amide function on the PVA backbone as shown in figure 5.19.

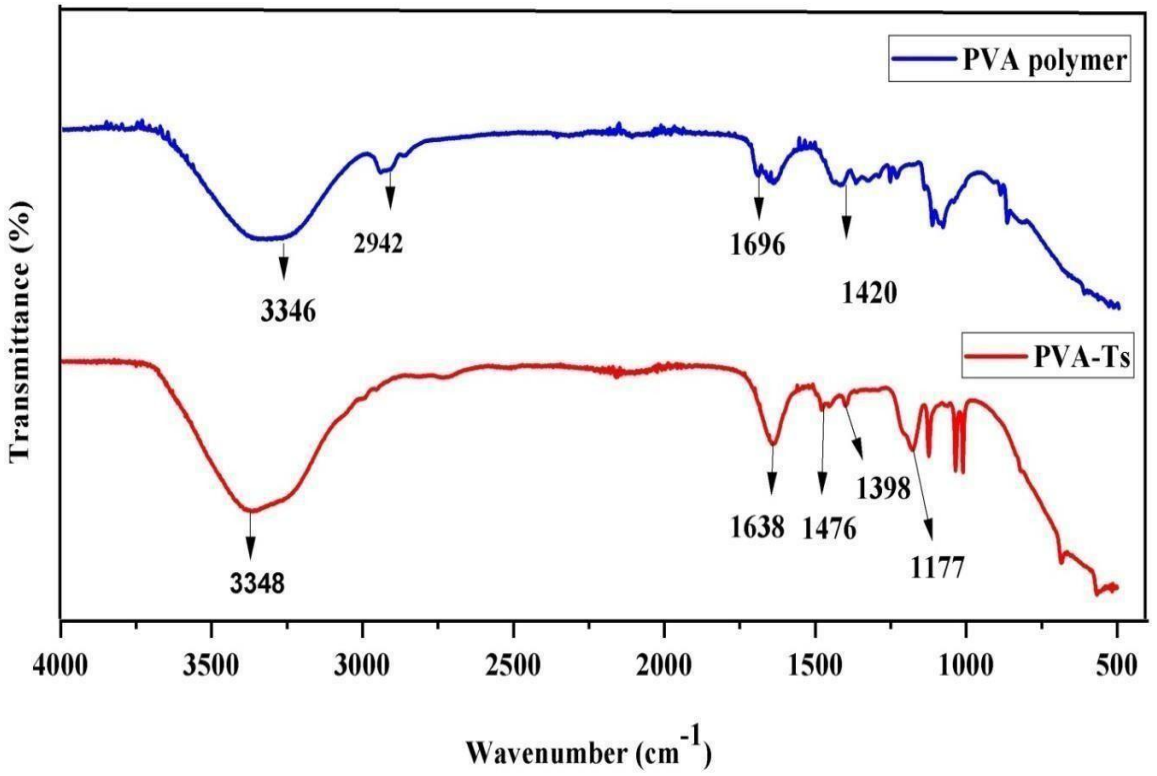


Figure 5.19.Showing FTIR of PVA-Ts and PVA polymer

The peaks observed for tosylation (PEG-Ts 4000) were at 2888 cm^{-1} which is linked to the C- CH_3 bonds. The peaks at 1466 cm^{-1} may be related to C-H bending. The peaks at 1343 cm^{-1} and 1174cm^{-1} can be linked to the group of S=O. The peaks observed for amidation (PEG-Ts + CSP) were at 3416 cm^{-1}, 2961 cm^{-1} shows C-H stretching. The amidation is shown by the peak at 1698 cm^{-1}; this may indicate the introduction of amide function on the PEG backbone. Similarly, IR spectroscopy was done for PEG 8000 and PEG 1500; they gave similar results as PEG 4000 as shown in figure 5.20.

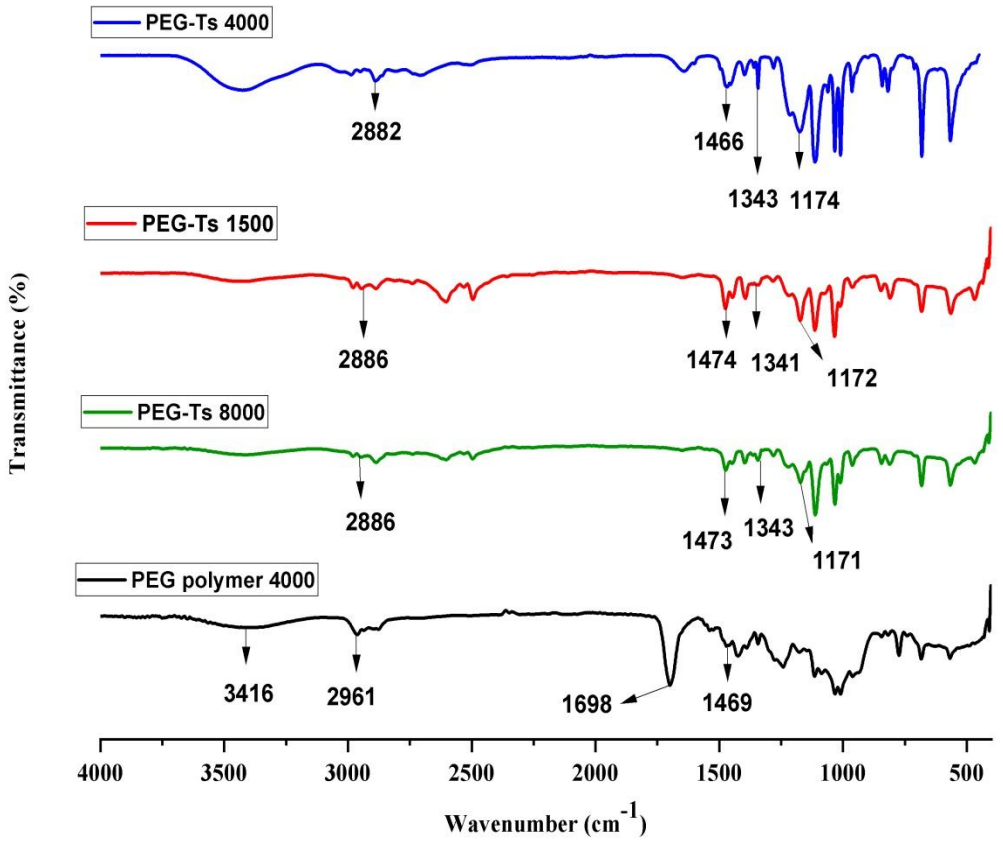


Figure 5.20.Showing FTIR of PEG-Ts and PEG polymer

NMR Spectroscopy

The complete reaction of tosylation and amidation (PVA) was confirmed via NMR spectra (^{1}H and ^{13}C). For tosylate, in ^{1}H NMR; the triplet at δ 1.19 ppm indicated the presence of methyl (CH_3) protons, the quadruplet at δ 3.70 showed the presence of –CH_2 protons. Additionally, δ 2.3 was the peak of CH_3 protons that were attached to aromatic group. Peaks in the region δ 7.49-7.14 showed the presence of aromatic ring. In ^{13}C NMR spectrum, peak appearing at δ 21.25 and δ 39.26 was due to CH_2 – CH_3 carbon. The peak at δ 46.03 showed the presence of –CH_3 carbon that was attached to aromatic group. Peaks in the region δ 125.95, δ 128.62, δ 138.52, and δ 145.37 were related to aromatic carbons.

For amidation, in ^{1}H NMR; the triplet at δ 1.39 ppm indicated the presence of two methyl group (CH_3). . The peak appearing at δ 3.15 showed the proton of CH_2 attached to NH group, δ 4.57 indicated the protons of CH attached to the NH group and δ 3.89 was due to protons of –O-CH_2-C attached to isopropyl group. In addition to

that, δ 0.9 was due to protons of isopropyl group. The peak appearing at δ 7.18 and 7.78 were due to proton of NH group attached to CH and C=O respectively. In ^{13}C NMR spectrum, peak appearing at δ 23.01 were due to two methyl group carbons, δ 37.44 and 14.89 showed the presence of CH_2 – CH_3 carbon attached to NH respectively. The δ 46.01 was due to carbon of CH attached to NH. The peak at δ 67.95 and 68.67 indicated the presence of –O-CH_2-C. Peaks in the region δ 156.09, δ 156.97 were due to carbons of C=O. Similarly, PEG also had similar NMR spectrum indicating the formation of the polymer.

5.4 Conclusion

In an effort to produce MIPs for extraction purposes, the study used HEMA, PVA, and PEG. Unfortunately, the resultant polymer lacked the necessary stability as it failed to remove the template drug from the polymer formed. As we know, the removal of template from the polymer is an important step to get the complimentary binding sites which are used for further extraction. Therefore, the resultant polymers were found to be inappropriate for the proposed extraction procedure. As a consequence, the researchers used a different strategy and created a novel MIP using methacrylic acid (MAA). As clearly explained in chapter 3, this newly created MIP was put through extensive extraction, optimization, and validation procedures. In the context of extraction applications, the move to MAA-based MIP indicates a promising path that offers prospective advantages and the same has been explained in the previous chapters. The polymers synthesized with HEMA, PVA and PEG was not used further for extraction purpose. These findings can be helpful in selecting the polymer synthesizing techniques for future.

Chapter – 6

Conclusion

The present work has made an effort to address the issues in the specific extraction and detection of trace amount of CSP from biological matrices like urine, blood and pharmaceutical samples. The MIP-DSPE method has proven to be efficient in extracting and detecting CSP in trace amounts as well. The developed method plays a crucial role in criminal investigations and court cases; forensic science has grown even more important in identifying and analyzing drugs and poisons in biological samples for medico- legal purposes. This study explores the topic of forensic toxicology, providing light on cutting-edge analytical techniques that might have a big influence on the forensics industry and improve our knowledge of drug usage and its effects. Comprehensive measures are urgently needed to address the developing problems of substance dependency and addiction in light of the growing concern about drug misuse, particularly among young people.

This study is focused on the misuse of muscle relaxants, particularly CSP, which was once thought to have low potential for abuse but has now been discovered to have both sedative and euphoric effects. As a result of the widespread intoxication, dependence, and even mortality caused by CSP usage, forensic toxicologists are now required to identify and examine drugs of abuse in biological samples. Their contributions provide insightful information about the social and medical effects of drug usage, assisting in the development of preventive, intervention, and public health programs. The focus of this study on synthesis of MIP, a novel forensic science strategy, is remarkable. MIPs can be applied in forensic laboratories for the precise and quick identification and analysis of substances like CSP because of their high specificity and efficiency. This technique offers significant prospects for a range of uses, with the potential to revolutionize molecular recognition techniques and advance a number of forensic science domains.

The study also highlights the intricate relationships between forensic toxicology, drug addiction, and muscle relaxants, emphasizing the need for vigilant prescription medication monitoring and the implementation of efficient control strategies. In order to effectively address the complex issues raised by drug addiction and death associated with muscle relaxants like CSP, joint efforts including medical professionals, law enforcement agencies, and forensic specialists is needed. Together, these parties can successfully combat drug misuse and advance public safety by

combining their knowledge and resources. Overall, by offering important insights into drug misuse and forensic analysis, this work can makes a substantial contribution to the area of forensic science. In the context of forensic science and public health, the emphasis on effective and targeted detection techniques promotes a deeper understanding of drug-related issues, enables the creation of evidence-based prevention strategies for drug abuse, and ensures the safer use of prescription drugs.

The study also explores the production and use of MIPs made from various functional monomers, such as PVA and PEG. Despite early difficulties, a new MIP employing methacrylic acid (MAA) was able to synthesize as to their modifications and persistence. This adaptability in strategy highlights the promise of MIP- based technologies for several applications outside of forensic science, such as medication delivery, sensing, and separation research, and also shows the dynamic character of scientific investigation. The study also demonstrates the enormous potential of MIPs in addressing the abuse of CSP and its impairment effects on traffic safety. The on-going development and improvement of extraction techniques offer up new applications for MIP-based techniques, which have the potential to revolutionize drug analysis and detection in forensic science and other fields.

Lastly, the proposed analytical method's robustness, sensitivity, and environmental friendliness were successfully validated for the detection of CSP in biological samples using MIP-DSPE in conjunction with GC-MS. This method's precision, accuracy, and eco- friendliness make it a promising tool for routine analysis in clinical and forensic laboratories, underscoring its potential to impact public health and safety positively In conclusion, this research amalgamates diverse facets of forensic science, including forensic toxicology, drug abuse, and MIP technique. By synergizing these elements, this study enriches our understanding of drug-related issues and equips forensic science with innovative tools and methodologies to tackle drug abuse challenges effectively. Through continued interdisciplinary collaboration and unwavering commitment to scientific advancement, the future of forensic science holds immense potential to revolutionize criminal investigations and safeguard public welfare.

Limitations and future scope

MIP is specific in nature therefore; it will not extract meprobamate which is a metabolite of CSP. The highly specific material called as MIPs are made to recognize and bind to certain target molecule. The parent medication, CSP, appears to be the target of this MIPs apparent design. Meprobamate, a metabolite of CSP, cannot be extracted by this method.

CSP extraction cannot be done in completely metabolized sample. After the medication has been completely metabolized, CSP cannot be extracted from a sample. The process by which a medication is broken down into its metabolites inside the body is referred to as metabolism. Meprobamate, a metabolite of CSP that may no longer be present in its original form, may have completely transformed CSP in this situation.

Template bleeding is also one of the major challenges. In the future studies, meprobamate can be used as a template in MIP synthesis. Other microextraction techniques can be explored for the analysis of CSP. Researchers might investigate several extraction methods created especially for the separation of meprobamate from complicated matrices. Even in samples that have been fully metabolized, these approaches could enhance the recovery and detection of the metabolite.

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Appendix A

Institutional Ethics Committee
Punjab Institute of Medical Sciences
Garha Road, Jalandhar (Punjab) 144001 India

Ph No. 0181-660600 Ext- 6035, 4005 (Email: iec@pimsj.com)

Regd. No. ECR/1413/Inst/PB/2020 Regd.No. Regd.No. EC/NEW/INST/2023/PB/0275

Reference No: IEC/PIMS/24/01

Decision

Protocol no. IEC/24/02 **Dated: 03/01/24**

Name and designation of the Applicant: Sakshi Manhas, Dr. Saurabh Shukla

Title of the research proposal reviewed: Evaluation performance of molecularly imprinted polymers for carisoprodol recognition in forensic matrices.

Along with protocol, other documents reviewed: Patient information sheet, informed consent form, Study Proforma

List of IEC members who attended the meeting- clear description of their role

Dr. Sandeep Kaushal (Chairman), Dr. Jagminder Kaur Bajaj (Member Secretary), Dr. H.S Bains, Dr Megha Sood, Dr. Tania Moudgil, Dr. Bavneet Kaur Dang (Scientific Members) Mrs Parminder Berry (Socially aware member) and Mr Simratpal Singh (Socially aware member)

A Clear statement of decision reached: Approved

Principal Investigator has to submit progress Report every _____months

Any advice by the IEC to the applicant:

In case of conditional decision, any requirement by IEC, including suggestions for revision and the procedure for having the application re-reviewed: NA

In case of rejection of the proposal, reason(s) for the rejection will be clearly stated: NA

Sandeep Kaushal

Signature of the Chairman/member secretary with date

Chairman
Institutional Ethics Committee
PIMS, Jalandhar

List of Publications

1. **Manhas S.**, Bajaj A., Jain B., Kumar D., Singh J., Shukla S., and Jain R. Molecularly imprinted polymer-based dispersive solid-phase extraction for the selective determination of carisoprodol in biological and pharmaceutical samples. New Journal of Chemistry. 2023, 47, 14436-14446.
2. **Manhas S.**, Kumar A., Shukla S., Kumar D., and Singh J. Detection of trace elements by molecularly imprinted polymers in forensic science. AIP Conference Proceedings. 2023, 2800, 020050.
3. Gani Mir T., **Manhas S.**, Wani K. A., Akhtar N., Shukla S., Prakash A. Alterations in microbiome of COVID-19 patients and its impact on forensic investigations. Science and Justice. 2024, 64 (1), 81-94.
4. Kumar A., Kumar D., Goyal A., **Manhas S.**, Kumar A., and Sharma Ajit. Trends, technology, and future prospects of bioceramic materials. Advanced Ceramics for Versatile Interdisciplinary Applications, Elsevier. 2022, 251-277.

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List of Conferences

1. **Oral presentation** in 4th International conference on ―Recent Trends in Bioengineering‖ at MIT Pune, Maharashtra, 12th – 13th Feb, 2021 and secured **second position** at the conference.
2. **Poster presentation** in 3th International conference on ―Recent Advances in Fundamental and Applied Sciences at Lovely Professional University, Phagwara, India, 25th – 26th June, 2021 and secured **first position** in poster presentation.
3. **Oral presentation** 1st International Forensic Science E-Conference on ―Empowering Forensic Sciences‖ organized by National Forensic Sciences University, 10th – 11th July, 2021 and secured **fourth position** at the conference.
4. **Oral presentation** in International conference on ―Materials for Emerging Technologies (ICMET-21) organized by Department of Research Impact and Outcome, Division of Research and Development, Lovely professional University, Punjab held on February 18th – 19th , 2022.
5. **Oral presentation** at National conference on Forensic Advancements and Technological Explorations (FATE 2022): transcending disciplines organised by School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, India, 25-26 November, 2022.
6. **Poster presentation** in the 4th International conference on ―Recent Advances in Fundamental and Applied Sciences (RAFAS 2023) organized by Lovely Professional University, Punjab held on 24th – 25th March, 2023. Secured **third position** in poster presentation.
7. **Oral presentation** in Annual International Conference on ―Forensic Sciences (AICFS 2023) organized by Geeta University, Naultha, Haryana held on 27th – 29th April, 2023. Secured **first position** in oral presentation.