CHAPTER: 1

Introduction
1.1. General Introduction

A drug, broadly speaking, is any substance that, when absorbed into the body of a living organism, alters normal bodily function. There is no single and precise definition as there are different meanings in drug control law, government regulations, medicine, and colloquial usage. In pharmacology, a drug is "a chemical substance used in the treatment, cure, prevention or diagnosis of disease or used to otherwise enhance physical or mental well-being." Drugs may be prescribed for a limited duration or on a regular basis for chronic disorders.

Drugs are usually distinguished from endogenous biochemicals by being introduced from outside the organism. For example, insulin is called a hormone when it is synthesized by the pancreas inside the body but if it is introduced into the body from outside, it is called a drug.

Drugs and their metabolites are nowadays an emerging group of organic pollutants due to their enormous use against different diseases in human and veterinary medicine, which are present in trace amount in environmental and biological samples. In many cases, the compounds enter into water when people excrete them or wash them away in the shower. But some are flushed or washed down the drain when people discard outdated or unused drugs. So, a number of states and localities around the country have started discouraging pharmacies, hospitals, nursing homes and residents from disposing of drugs this way. In recent years, concerns have increasingly emerged with regard to the occurrence of pharmaceuticals and personal care products entering into the aquatic environment. One important reason is their potential impact on environment water quality, if the sources supplying natural water to the reservoirs are contaminated by these
compounds [1, 2]. However, the number of methods devoted to the analysis of new groups of substances that have been so-called emerging contaminants [1] is very scarce since these compounds, among which pharmaceuticals and personal care products are included, were not previously considered pollutants. There has been an increasing scientific interest of understanding the consequences of drugs present in the environment on the ecosystem and public health during the last years. Particularly, the study on residues of antibiotics in the different environmental compartments is of special concern [2] mainly due to the associated problem of growing of antimicrobial resistant bacteria.

The presence of antibiotics in food animals has increased human health concerns because it induces pathogen resistance to clinical drugs in humans and side effects of exposure to fluoroquinolones on the central nervous systems of younger children have been also reported [1-3]. Because of the risk of non-compliance, drug interaction and inter individual variability in dose-response, therapeutic drug monitoring (TDM) is of interest to optimise the pharmacological treatment.

Concern for the environment and fear of the pollution that is threatening both human health and existence, creates strong sentiments among the humanity, that it is time to address the issue of pollution. Legislators are being pressured by public opinion to pass laws to regulate and monitor the chemicals being released into the environment. Concurrently the development of analytical instrumentation enabled the detection of these pollutants at trace levels. The basic principle of environmental analysis covers initial planning, sampling, sample pre-treatment and preparation, and finally the measurements of the analyte(s). At every stage, a quality assurance program is essential for a sound analytical protocol to detect and correct problems in the analytical process. The expectations regarding accuracy, sensitivity, precision, reliability, interferences, matrix
effects, limitations, cost, and the speed of the analysis have to be realistic. The ultimate user of the data has to consider every aspect of these issues while evaluating, interpreting, and communicating the results.

Although pharmaceutical analyses have been carried out using various highly efficient analytical instruments, a sample preparation procedure is usually necessary to extract and isolate the analytes of interest from these complex matrixes because most analytical instruments cannot handle the matrix directly. In general, sampling and sample preparation steps account for over 80% of the whole analysis time, and the quality of these steps has a great effect on the success of analysis from complex matrixes. Thus, the development of sample preparation plays a very important role in pharmaceutical analysis, and its final aim should be to isolate and to purify the analyte from those matrixes and to introduce it into the GC, HPLC, CE, or other instruments.

The direct trace determination of pharmaceuticals in environmental matrices by various chromatographic techniques finds increasing use. Water is the matrix of most samples that are studied nowadays in the control of environmental pollution and biotechnological processes, food chemistry, and medicine.

1.2. Classification of drugs

Medications can be classified on the basis of their chemical properties, mode or route of administration, biological system affected, or therapeutic effects. A drug may be classified by the chemical type of the active ingredient or by the way it is used to treat a particular condition. Each drug can be classified into one or more drug classes.

The classification of prescription drugs is an act of distributing medications into classes of similar type. Drugs can also be classified by the source of the active substance,
potential for abuse or by its harmfulness. The standardization of drugs is still evolving. Since technology is rapidly changing the structure and types of drugs, the classification system will continue to undergo refinement.

Classifications are based on:

- Chemical Classification
- Pharmacological Classification
- Potential for Abuse Classification (DEA Drug Schedules)
- Mechanism of action
- Physiologic effects (cellular or molecular interactions)
- Therapeutic effects (Conditions treated)

1.2.1. Chemical Classification

Drugs are often classified according to their chemical structures. Although this is useful for medicinal chemists, it does not provide a meaningful classification scheme for categorizing drug effects. Some compounds with similar chemical structures produce very similar biological effects (e.g. morphine, heroin), but others which belong to the same chemical class often produce much different effects (e.g. apomorphine, nalorphine). Furthermore, compounds which differ in chemical structure often produce similar biological effects (e.g. amphetamine, cocaine). Drugs can also be classified by their chemical characteristics, such as if the molecule is an acid, base or salt.

1.2.2. Pharmacological Classification

This scheme classifies drugs according to their primary pharmacological activity. All compounds produce multiple effects, having the primary effect as well as the
secondary effect. Often the primary therapeutic use of a compound is considered and thus used to classify it pharmacologically.

The classification scheme given below focuses on each compound's main psychotropic effects but in some cases, drugs are classified differently from its primary pharmacological effect. For example, pseudoephedrine is a popular decongestant that has mild stimulatory properties. Pseudoephedrine's decongestant effect might be considered its primary effect, while its stimulatory effect would be considered a secondary side-effect. However, from a psychopharmacological perspective, pseudoephedrine's stimulatory effect is its primary effect and its decongestant action is a secondary (although therapeutically more useful) effect. Therefore, pseudoephedrine is classified below as a mild stimulant like caffeine and nicotine.

Table 1.2.1: Classification of drugs on the basis of their pharmacology

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Primary Effects/Approved Medicinal Uses</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Opiates/Opioids/Narcotic Analgesics</strong></td>
<td>Analgesia, cough suppression, anti-diarrhea, suppression of opiate withdrawal, sedation; currently used therapeutically for the first four effects</td>
<td>Opium, morphine, codeine, heroin (diacetyl morphine), fentanyl, methadone, meperidine, L-alpha-acetylmethadol (LAAM)</td>
</tr>
<tr>
<td><strong>Narcotic/Opiate Antagonists</strong></td>
<td>Block the effects of narcotics; used to treat opiate overdose</td>
<td>Naloxone, naltrexone</td>
</tr>
<tr>
<td><strong>Psychomotor Stimulants</strong></td>
<td>Stimulate psychological and sensory-motor functioning; used therapeutically to treat ADHD and narcolepsy, sometimes as an appetite suppressant, occasionally antifatigue, formerly for asthma and for sinus decongestion</td>
<td>Amphetamine, methamphetamine, cocaine, methylphenidate</td>
</tr>
<tr>
<td><strong>Other Stimulants</strong></td>
<td>Similar to psychomotor stimulants but with much less efficacy; various</td>
<td>Caffeine, nicotine, ephedrine,</td>
</tr>
<tr>
<td>Category</td>
<td>Description</td>
<td>Examples</td>
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<td>--------------------------------</td>
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<tr>
<td>Therapeutic effects</td>
<td>Including caffeine compounded with aspirin in some OTC pain relievers, ephedrine in OTC asthma medicines, pseudoephedrine in OTC sinus decongestants and OTC appetite suppressants</td>
<td>pseudoephedrine</td>
</tr>
<tr>
<td><strong>Barbiturates</strong></td>
<td>General decrease in CNS arousal/excitability level; used therapeutically for anesthetic, anticonvulsant, sedative, and hypnotic effects</td>
<td>Thiopental, secobarbital, pentobarbital, phenobarbital</td>
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<tr>
<td><strong>Minor Tranquilizers</strong></td>
<td>General decrease in CNS arousal/excitability level, but low dose are somewhat selective for anxiety and much less sedative than barbiturates; used therapeutically as anxiolytics, benzodiazepines also as anesthetics and anticonvulsants</td>
<td>Includes two subclasses: benzodiazepines (e.g., diazepam, chlordiazepoxide, flunitrazepam [Rohypnol]) and muscle relaxants (e.g., meprobamate)</td>
</tr>
<tr>
<td><strong>Major Tranquilizers</strong> (antipsychotics/neuroleptics)</td>
<td>General sedation at high doses, with selective antipsychotic activity at lower doses; used therapeutically to treat schizophrenia and other major psychotic disorders</td>
<td>Haloperidol, pimozide, flupenthixol, chlorpromazine, spiroperidol, clozapine</td>
</tr>
<tr>
<td><strong>Antidepressants</strong></td>
<td>No perceptible CNS effects in normals, but effectively alleviate depression in many depressives; used therapeutically to treat depression</td>
<td>Includes three subclasses: monoamine oxidase inhibitors (e.g., pargyline), tricyclic antidepressants (e.g., amitriptyline, desmethylimipramine), and selective serotonin reuptake inhibitors (SSRIs: e.g., sertaline)</td>
</tr>
<tr>
<td><strong>Antimanic</strong></td>
<td>Dampens extreme mood swings in some people; used to treat manic-depressive (bipolar) disorders</td>
<td>Lithium</td>
</tr>
<tr>
<td>Substance</td>
<td>Description</td>
<td>Example</td>
</tr>
<tr>
<td>--------------------</td>
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<tr>
<td>Alcohol</td>
<td>General decrease in CNS arousal/excitability level; no current therapeutic uses, but formerly used as an anesthetic and a sedative</td>
<td>Ethyl alcohol (other alcohols have similar actions but are associated with very toxic effects, e.g., methanol)</td>
</tr>
<tr>
<td>Volatile Anesthetics</td>
<td>General decrease in CNS arousal/excitability level; used therapeutically for anesthesia</td>
<td>Nitrous oxide, halothane, ether</td>
</tr>
<tr>
<td>Volatile Solvents</td>
<td>Produce feelings of intoxication, can produce hallucinations at high doses; no therapeutics uses (all can cause marked brain damage in moderately low concentrations)</td>
<td>Toluene, benzene</td>
</tr>
<tr>
<td>Psychogenics</td>
<td>Produce altered states of consciousness; hallucinogenics produce hallucinations sometimes reported as &quot;mystic&quot; experiences; cannabinoids usually produce increased feelings of &quot;well being&quot; and &quot;mellow&quot; intoxication; the &quot;pleasantness&quot; of the states produced by both classes probably depends partially on expectancies; no approved therapeutic uses, but cannabinoids are being increasingly used for their antinausea, anxiolytic, and appetite-stimulating effects in severely ill patients (e.g., AIDS)</td>
<td>Includes two subclasses: hallucinogenics (e.g., lysergic acid diethylamine [LSD], mescaline, psilocybin) and cannabinoids (e.g., marijuana, hashish).</td>
</tr>
<tr>
<td>Stimulatory</td>
<td>Produce a mixture of psychomotor stimulant and hallucinogenic effects, depending on dose and other factors; no therapeutic uses, except phencyclidine as a veterinary anesthetic</td>
<td>MDMA (ecstasy), phencyclidine (PCP), ketamine</td>
</tr>
</tbody>
</table>

**Abbreviations:** *ADHD*, attention deficit hyperactivity disorder; *AIDS*, acquired immune deficiency syndrome; *CNS*, central nervous system; *OTC*, over-the-counter (nonprescription) medicines.
1.2.3. Potential for Abuse Classification (DEA Drug Schedules)

The U.S. Drug Enforcement Administration classifies drugs by their potential for abuse in five categories called schedules. These schedules govern the legal distribution and use of most substances with a significant abuse liability. The Drug Enforcement Administration is the primary federal agency charged with enforcing these regulations and with coordinating national and international efforts to reduce illicit drug supply. Hence, this classification scheme is often referred to as the DEA Schedules.

- **Schedule I** substances have a high abuse liability and no approved medical use.
- **Schedule II-IV** substances have decreasing abuse liabilities (II is the highest) and approved medical uses.
- **Schedule V** substances have a recognized abuse liability (and approved medical uses) but are generally not regulated (e.g., they are available without prescription).

1.2.4. Drugs Classification based on the Therapeutic effect

1. **Drugs affecting Central Nervous System**

   **Anesthetics:** Methoxyflurane, Halothane, Enflurane, Isoflurane etc.

   **Sedative-Hypnotics:** Benzodiazepines, barbiturates, etc.

   **Antiseizure Agents:** Carbamazepine, Diazepam, Phenobarbital, Primidone, Oxcarbazepine, etc.

   **Antidepressants:** Desipramine, Citalopram, Amitriptyline, Clomipramine, Mirtazepine, Trozodone, Bupropion, etc.

   **Antipsychotic and Anxiolytic Agents:** Risperidone, Clozapine, Aripiprazole, Oxazepam, Loxapine, etc.

   **Hallucinogens, Stimulant and Relative Drugs of Abuse:** Cannabinoids, Amphetamine, Fenfluramine, etc.
Opoid Analgesic: Morphine, Codeine, Mepridine, Tramadol, Pentazocine, Naloxone, etc.

Antiparkinsonian and Spasmolytic Agents: Levodopa, Carbidopa, Rasagiline, etc.

2. Drugs affecting the Cardiovascular System

Diuretics: Acetazolamide, Metolazone, Furosemide, Spironolactone, etc.

Cardiac Agents: Cardiac Glycosides, Antiaginal and Antiarrhythmic Drugs: Digoxin, Dobutaine, Phenytoin, Quinidine, etc.

Angiotensin-Converting enzyme Inhibitors, Antagonists, and Calcium Blockers: Enalapril, Fosinopril, Verapamil, etc.

Central and peripheral Sympatholytics and vasodilators: Acebutolol, Methyldopa, Reserpin, Pindolol, etc.

Antihyperlipoproteinemics and inhibitors of Cholesterol Biosynthesis: Atorvastatin, Gemfibrozil, Pravastatin, etc.

Antithrombotics, Thrombolytics, Coagulants, and Plasma Extenders: Dalteparin, Desirudin, Aspirin Reteplase Protamine, etc.

3. Drugs affecting the Hormonal System

Insulin and Antidiabetics: Lispro, Metformin, Glipizide, glimepiride, etc.

Adrenocorticoids: Betamethasone, Dexamethasone, Flunisolide, Aldosterone, etc.

Thyroid Drugs: Levothyroxine, Liotyronine, Iodide, Perchlorate, etc.

4. Drugs affecting the Immune System

Nonsteroidal Anti-inflammatory Drugs: Ibuprofen, Ketoprofen, Fenoprofen, etc.

Antihistamines and Related Antiallergic and Antiulcer Agents: Cetirizine, Lansoprazole, Ranitidine, etc.
5. Chemotherapeutic Agents

**Antibiotics and antimicrobial Agents:** Methenamine, Amoxicillin, Cephalosporins, Macrolides, Tetracyclines, etc.

**Antiparasitic Agents:** Metronidazole, Quinine, Thiabendazole, etc.

**Antifungal Agents:** Natamycin, Ketoconazole, Caspofungin, etc.

**Antimycobacterial Agents:** Streptomycin, Azithromycin, Dapsone, etc.

**Cancer and chemotherapy:** Busulfan, Cisplatin, Bleomycin, Vinblastin, etc.

**Antiviral agents:** Cidofovir, Ribavirin, Stavudine, etc.

1.3. Drugs as Environmental Pollutants

Pharmaceuticals are “emerging pollutants”, which, in most cases, correspond to unregulated contaminants that may be candidates for future regulation [1]. These are the compounds that are not currently covered by existing water-quality regulations, have not been studied before, and are thought to be potential threats to environmental ecosystems and human health and safety [4, 5]. They encompass a diverse group of compounds, including pharmaceuticals, drugs of abuse, personal care products (PCPs), steroids and hormones, surfactants, perfluorinated compounds, flame retardants, industrial additives and agents, and gasoline additives, as well as their transformation products [4, 6-8]. The way that organic compounds enter the environment depends on their pattern of usage and mode of application (e.g. disposal of municipal, industrial and agricultural wastes, excretion of pharmaceuticals and accidental spills).

Once in the environment, they can be widely distributed at any time between the moment of their production through to use and disposal. Because most emerging pollutants
are from human use, their emissions are an issue for some wastewater processes, so the study of the fate of the emerging pollutants in wastewater treatment plants is of most importance. Once released into the environment, emerging pollutants are subject to processes (e.g. biodegradation, chemical and photochemical degradation) that contribute to their elimination. Depending on the compartment in which synthetic chemicals are present in the environment (e.g. groundwater, surface water and sediment) or in the technosphere (e.g. WWTPs and drinking-water facilities), different transformations can take place, sometimes forming products that can differ in their environmental behaviour and ecotoxicological profile [9].

Many drugs from a wide array of therapeutic classes have been established as ever present environment pollutants in the surface and ground waters [10-14] generally occurring at concentration (e.g. ng/L-µg/L) far below the human therapeutic levels. Although drugs, by contrast with most conventional pollutants, are usually non-volatile, they can also end up on the land by disposal of sewage biosolids. Also drugs have longer environmental half lives than the regular pollutants, so the continual environmental introduction of drugs by sewage effluents makes them “pseudopersistent” pollutants with ramifications for aquatic organisms. It has been observed that the pollutant concentration in the interstitial sediment water and sediments is more than 10 times higher than that present in the overlying water column. So there is a need to detect and determine the trace levels of drugs from the waste and drinking water. However, for most emerging contaminants, occurrence, risk assessment and ecotoxicological data are not available, and it is difficult to predict their fate in the aquatic environment. Partly, the reason for this is a lack of analytical methods for their determination at trace concentrations.
Analysis of emerging contaminants is a real analytical challenge, not only because of the diversity of chemical properties of these compounds, but also because of generally low concentrations (usually at part per billion (ppb) or part per trillion (ppt) levels) and the complexity of matrices. Essentially, in most of the cases of interest, substantial analyte enrichment is necessary to isolate the target compounds from the matrix and to achieve the limits of detection (LOD) required.

1.4. Need of Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) is the measurement of specific drugs at timed intervals in order to maintain a relatively constant concentration of the medication in the bloodstream. TDM is required to optimise therapy of critical dose drugs with a narrow therapeutic range where there is a good chance of either overdosage or underdosage. Monitoring the drug concentration can guide the drug dosage to optimise therapeutic effectiveness whilst minimising the side effects. TDM has been performed for many years using immunoassay but it is recognised that immunoassay methods can suffer with non-specific interference from related compounds, metabolite interference or matrix effects.

The requirement to analyze drugs in biological samples and pharmaceutical products is becoming more and more frequent with the development of more selective and more effective drugs and with our need to understand more about their therapeutic and toxic effects. Knowledge of drug levels in body fluids, such as serum and urine, allows pharmacotherapy to be optimized and provides the basis for studies on patient compliance, bioavailability, pharmacokinetics and genetics, organ function and the influences of co-medication. [15].
There are numerous variables that influence the interpretation of drug concentration data e.g., route and dose of drug given, time of blood sampling, handling and storage conditions, precision and accuracy of the analytical method, validity of pharmacokinetic models and assumptions, co-medications and clinical status of the patient (i.e. disease, renal/hepatic status, biologic tolerance to drug therapy, etc.). In the clinical field, TDM is known to be a valid tool to optimize pharmacotherapy as it enables the clinician to adjust the dosage of drugs according to the pharmacokinetic characteristics of the individual patient. Over time, patients may acquire other chronic conditions that also require lifetime medication and that may affect the processing of their monitored drugs. Examples of these conditions include cardiovascular disease, kidney disease, thyroid disease, liver disease, and HIV/AIDS. TDM follows these changes and accommodates them.

TDM plays an important part in the optimal use of a select few drugs, commonly known as critical dose drugs. With the individualisation of drug therapy through measured concentrations the aim is to achieve maximum therapeutic response with minimal adverse effects. With these points in mind, accurate measurement of the drug in question is essential [16].

1.5. Introduction to studied categories of drugs

In our research work, we have considered a few categories of drugs i.e. antiepileptics, antidepressants, quinolones and acyl homoserine lactones. A brief introduction of these drugs is given below:

1.5.1. Introduction to antiepileptics

The antiepileptic is a diverse group of pharmaceuticals used in the treatment of epileptic seizures. The goal of an antiepileptic is to suppress the rapid and excessive firing
of neurons that start a seizure. There is no doubt that epilepsy belongs to the most encountered neurological conditions since the disease affects approximately 1% of the population. Around 75-80% of epileptic patients may be provided with adequate seizure control with the help of conventional antiepileptic drugs. Since the introduction of potassium bromide and paraldehyde (1850-1880), several generations of antiepileptic agents have been introduced into the clinical usage, greatly improving the life quality of many people suffering from seizures [17]. Carbamazepine, ethosuximide, phenobarbital, phenytoin, and valproate are the most frequently used conventional antiepileptics. The therapeutic failure in 20-25% of patients has stimulated intensive research on novel antiepileptic drugs and so far nine of them have been developed and licensed mainly as add-on treatment in patients poorly responding to conventional therapy. These are felbamat, gabapentin, lamotrigine, levetiracetam, oxcarbazepine, tiagabine, topiramate, vigabatrin, and zonisamide [18].

Majority of antiepileptic drugs possesses more than one mechanism of action. Deckers et al. [19] have proposed a classification of antiepileptic drugs based upon these mechanisms. The first group consists of antiepileptics (e.g.: carbamazepine, gabapentin, lamotrigine, oxcarbazepine, phenobarbital, phenytoin, topiramate, valproate) which block sustained repetitive firing in individual neurons, this effect being mainly due to the blockade of voltage-dependent sodium or calcium channels. These drugs are effective against generalized tonic-clonic and partial seizures. The second group includes drugs enhancing inhibitory events mediated by g-aminobutyric acid (GABA): benzodiazepines, gabapentin, phenobarbital, tiagabine, topiramate, vigabatrin, and valproate. Some of these drugs may be used in all seizure types. The third group practically consists of one drug - ethosuximide which blocks T-type calcium channels and is active against absences. Recent
evidence suggests that also zonisamide may be a T-type calcium channel inhibitor [20]. A separate category of drugs may be also suggested - these antiepileptic drugs reduce events mediated by excitatory amino acids (e.g. glutamate) and at present three antiepileptics meet these criteria: felbamate, phenobarbital, and topiramate [19].

Pharmacological treatment of the different forms of epilepsy has led to a better quality of life for the great majority of patients. Although monotherapy is currently the preferred approach to epilepsy treatment, a large proportion of patients with refractory seizures still receive multiple antiepileptic drugs (AED), tentatively to achieve a better clinical control [21, 22]. This often leads to complex and unpredictable pharmacokinetic and pharmacodynamic interactions [23, 24], with possible clinical consequences in terms of adverse side effects. Furthermore, antiepileptic therapy is chronic and may last for the whole patient’s life [25]. Thus, it is necessary to simultaneously monitor AEDs, including their active metabolites, to develop therapies without side effects.

TDM is a well established procedure which helps to maximise the effectiveness of antiepileptic therapy, increasing clinical efficacy while minimizing adverse effects [26, 27]. For optimal drug treatment of epilepsy the monitoring of concentrations of these new antiepileptic drugs in serum can be useful. However, the increasing number of antiepileptic drugs makes it difficult to offer such a service in clinical routine on a regular and economic base. Therefore integration of as much as possible antiepileptic drugs within one analytical method, which can also be automatized, is an usable approach, especially in patients on polytherapy with antiepileptic drugs. The drugs cause intoxication due to accidental overdosage or intentional abuse, and are therefore very frequently encountered in forensic science practice.
1.5.2. Introduction to antidepressants

Depression is a chronic or recurrent mood disorder that affects both economic and social functions of about 121 million people worldwide. According to the World Health Organization, depression will be the second leading contributor to the global burden of disease, confronting the world by the year 2020 [28-30]. This common mental disorder presents a highly variable set of symptoms such as depressed mood, loss of interest or pleasure, feelings of guilt or low self-esteem, disturbed sleep or appetite, low energy, and poor concentration. These problems lead to substantial impairments in an individual's ability to take care of his or her everyday responsibilities. At its worst, depression can lead to suicide, a tragic fatality associated with the loss of about 850 thousand lives every year.

Depression can be subdivided in bipolar disorder (manic-depression), dysthymia, and major depression (unipolar depression). An antidepressant is a psychiatric medication used to alleviate mood disorders, such as major depression and dysthymia and anxiety disorders such as social anxiety disorder.

Pharmacological treatment for depression has advanced greatly since the development of the first therapies in the 1950s, with the introduction of monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) [31]. However, their side effects, toxicity, and severe drug-drug interactions combined with an advanced understanding of the central nervous system have led to the introduction of several new ADs [32, 33]. Since the late 1980s, a whole new generation of chemically and neuropharmacologically unrelated agents have been introduced which appear to be safer and better tolerated [34]. Classes of these ADs are defined by their selectivity towards certain neurotransmitter transporters and receptors. These include: selective serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNaRIs),
noradrenergic and specific serotonergic antidepressants (NaSSAs) and noradrenaline reuptake inhibitors (NaRIs) [35].

Antidepressant drugs are becoming increasingly widely used for the treatment of depression and these drugs are frequently encountered in emergency toxicology screening, drug-abuse testing and forensic medical examinations [36]. TDM is underutilized in the field of psychiatry because the therapeutic ranges of antidepressants seem quite broad, leading to the generally accepted notion of low toxicity. On the other hand, the relationship between blood concentration and therapeutic effects is not always fully understood. TDM, though, could be of interest for monitoring patient compliance. In other situations, such as liver and kidney impairment, poor metabolism by CYP450 isoenzymes and comedication with inhibitors and inducers of those enzymes, and in the elderly population, TDM could provide valuable information for a cost-effective and more rational use of psychiatric drugs [35, 37-38]. The indications for using TDM are numerous, including treatment start-up, changes in dose, occurrence of unwanted side effects, lacking therapeutic effect, control for compliance, and pharmacokinetic interactions [39, 40]. This large number of indications combined with the marketing of new drugs and increased focus on drug use in psychiatry, augment the use of TDM.

These pharmaceutical drugs are used extensively in most developed countries and can enter the aquatic environment as a result of human excretion [41]. Occurrence of pharmaceutical drugs has been reported in sewage treatment effluents, surface water, and ground water [42-47]. Although the ecotoxicological effects are not quite known, it is important to develop methods for the analysis of these drugs in environmental samples for monitoring purposes.
1.5.3. Introduction to quinolones and fluoroquinolones

The quinolone class of antimicrobial agents has generated considerable interest since its discovery greater than 40 years ago. Substantial progress has been made in our understanding of the molecular mechanisms of the action of quinolones against pathogenic bacteria, the induction of resistance to quinolones in these organisms, and the potential of each quinolone compound to induce toxicity in treated patients. Unlike some of the first antibiotics discovered during the past century, the quinolone class of antimicrobial agents was not isolated from living organisms but, rather, was synthesized by chemists. The prolific development of the quinolones began in 1962, when Lesher et al. [48] made the accidental discovery of nalidixic acid as a by-product while synthesising the antimalarial compound chloroquine. This discovery led to the development of a library of quinolone compounds, especially the newer quinolones in clinical use at the present time [49]. Other discoveries followed, but only a few were of signal importance because they provided us with (1) a better understanding of the mechanisms of action of the quinolones; (2) the ability to modify the quinolone nucleus to improve potency and the spectrum of antibacterial activity; (3) the opportunity to prolong the elimination half-life and to improve the pharmacokinetic and pharmacodynamic properties of quinolones, resulting in effective once daily dosing; and (4) a clearer understanding of the importance of the structure-activity relationships (SARs) of the quinolones, with respect to their relative susceptibilities to the development of bacterial resistance and their potential for causing adverse events in treated patients [50, 51].

A new key finding in the evolution of quinolones was modification of the quinolones nucleus through the addition of different substituents at the N-1, C-6, C-7, and C-8 positions [51, 52]. These modifications altered the antimicrobial activity, pharmacokinetics, and metabolic properties of the quinolones and provided a better
understanding of the SARs in quinolone compounds. The addition of specifically selected substituents at these key positions on the quinolones nucleus made it possible to target specific groups of bacteria and to improve the pharmacokinetics of the earlier quinolone compounds [53-55].

![General structure of quinolone antibiotics](image)

**Fig. 1.5.1 General structure of quinolone antibiotics**

Fluoroquinolones (FQs) are antibacterial agents widely used for various infections because of characteristic of their broad activity spectrum and good oral absorption. Fluoroquinolones are applied as both human and veterinary medicine, and used at sub-therapeutic levels to promote growth for animals. Generally, FQs are prescribed 300-600 mg/day to the patient for therapeutic treatment. Due to quinolones and fluoroquinolones are only partially metabolised by patients, they are eliminated mainly as parent compounds, being consequently discharged into hospital sewage or municipal waste water. Unfortunately, sewage water treatment plants are not able to completely remove these compounds and thus important quantities of the active ingredient are transported to the environmental aquatic systems. Beside this, it has been reported that irrigation of crops with treated waste water can introduce antibiotics in surface waters through agricultural runoff [56, 57]. On the other hand, the use of both sewage sludge and livestock manure as fertilizers in agricultural crops in several countries is favouring the accumulation of antimicrobials in soils [58].

Benefits of the use of antibiotics in medicine are obvious, but the abuse made in last years has led to the accumulation of antibiotic residues in different environmental
compartments, thus increasing the risk of growing and promotion of antibiotic resistant bacteria [59-61].

The FQs administered to humans or animals are almost excreted as unchanged compounds in urine, and are mainly effluent from the wastewater treatment plants (WWTP) [62], but these compounds are not carried out monitoring on WWTP. Recently, several studies have indicated the presence of antibiotic residues at a pg/mL level in environmental water including municipal wastewater effluents and surface waters [63-70]. The literature for analysis of FQs in environmental waters, however, is very few. Several analytical methods by fluorescence or mass spectrometry detectors have reported to detect FQs in surface water, groundwater, and WWTP. Hospital wastewater is one of main source of contamination. In fact, some studies have demonstrated that bacterial resistance against FQs in the hospital wastewater is higher than that in the sewage treatment plant wastewater [71]. These antibiotics are rather resistant to microbial degradation [72, 73], and these compounds may be persisting within environmental waters because of their strong sorption properties. On the other hand, degradation of antibiotics, including photolysis [74-78] and chemical oxidation [79-81] may be significant on their environmental ecosystem. Effluence of FQs into the environmental waters occurs mainly as the parent compounds and as a consequence of inadequate treatment of human and animal excreta. It is apprehensive that bacteria exposed with antibiotics in environmental waters may acquire resistances against antibiotics. Additionally, it is important to perform more efficient and reliable environmental monitoring in order to know the stability of the FQs in the water samples. Therefore, it is necessary to develop a simple and sensitive analytical method for enabling the determination of these antibiotics at naturally occurring levels.
1.5.4. Introduction to N-Acyl homoserine lactones

N-Acyl homoserine lactones (AHLs) are a class of signaling molecules involved in bacterial quorum sensing. Quorum sensing is a method of communication between bacteria that enables the coordination of group-based behavior based on population density. The signal changes in gene expression, such as switching between the flagella gene and the gene for pili for the development of a biofilm.

![General structure for AHLs](image)

Fig. 1.5.2 General structure for AHLs (where R = C₄ to C₁₈)

Signaling molecules are produced within the cell and are released into the environment. Members of the LuxI family of proteins synthesize these signals. The resulting concentration of signaling molecules in the environment is dependent upon population density. Once the population density has reached a particular threshold, gene expression can begin. This allows bacteria to coordinate group-based behavior. N-AHLs produced by different bacteria differ in the length of the R-group side-chain. Chain lengths vary from 4 to 18 carbon atoms. They also have either 3-oxo, 3-hydroxyl, or fully reduced methylene groups at the C-3 position or have an unsaturated bond [82, 83]. Although different bacterial species may produce AHL analogues, the communication systems share common regulatory features.

These signals function as “quorum sensing” molecules in that their concentration is an indicator of the relative bacterial population density. When the signal concentration reaches a threshold level, it interacts with a transcriptional activator protein which will then
induce specific genes. These cell to cell signals control a wide variety of bacterial functions such as symbiosis, virulence, competence, conjugation, antibiotic production, motility, cell differentiation, bioluminescence, growth, pigment production, sporulation, and biofilm formation [84]. The AHL signal systems are comprised of two genes, the first of which encodes a signal synthase protein that is a member of the LuxI-type family of proteins. The second gene encodes a member of the LuxR-type transcriptional activator protein family [85]. The activity of these transcriptional activators is dependent on their cognate AHL signal, which binds to the transcriptional activator protein and thereby leads to its activation. AHL signals share a common base structure of a homoserine lactone ring but they are highly variable with regard to the attached acyl side chain. When comparing signals from different systems, the acyl side chain varies in length, oxidation state, and saturation [83]. Such signal differences are what provides specificity for each LuxR-type transcriptional activator. The specificity of LuxR-type proteins is the limiting factor for detecting signals with the use of bioassays.

In selected foods AHLs contribute to product spoilage. In particular, many gram-negative plant pathogens produce AHLs and regulate their virulence by AHL mediated quorum sensing [86]. For instance, the Pectobacterium carotovorum subsp. carotovorum (Erwinia carotovora subsp. carotovora), which causes soft rot diseases in many plant species, induces the production of various exoenzymes and plant tissue maceration by AHLs [87]. A unique AHL detection system involves the pigmented bacterium Chromobacterium violaceum, which normally produces the purple compound violacein in response to the presence of N-hexanoyl homoserine lactone [88].

Environmental conditions may affect the AHL production, and it is possible that in the presence of quorum (dense population), environmental factors could affect the
accumulation of AHL [89]. Fuqua et al. explained the dynamics of AHLs accumulation. pH is an important factor that can affect the stability of AHLs. In alkaline conditions, most AHLs are unstable and a degradation of these molecules will occur [89]. Several bioassays have been designed for detecting AHLs [90-92]. While these can detect a variety of AHLs, they are limited in their ability to detect combinations of several important AHLs [93].

Quorum sensing attracts more and more interest in the scientific community in the field of environment and health. Moreover, these molecules are produced at very low concentrations, so they cannot usually be detected by conventional techniques. Taking into account the scientific relevance of AHLs and the fact that the bandwidth of analytic techniques is still limited, it is essential to develop simple and rapid methods for the determination and identification of AHLs.

1.6. Introduction to HPLC

High Performance Liquid chromatography (HPLC) is a chromatographic analytical technique used to separate particular analytes in solution. It consists of a mobile phase and a stationary phase, which adsorbs and desorbs the analyte of interest. In HPLC the mobile phase is a liquid (normally a mixture of aqueous buffer and organic solvent) and the stationary phase usually consists of surface modified silica or polymer particles. HPLC’s virtue lies in its versatility. It can be used to separate compounds of molecular weights from 54 to 450,000 Daltons. Amounts of material to be detected can vary from picograms and nanograms (analytical scale) to micrograms and milligrams (semi-preparative scale) to multigrams (preparative scale). There are no requirements for volatile compounds or derivatives. Aqueous samples can be run directly after a simple filtration. Compounds with a wide polarity range can be analyzed in a single run. Thermally labile compounds can be run.
1.6.1 Principle

In HPLC [94-97], the separation of the components is based on selective partitioning of the molecules of interest between two different phases. The mechanism of distribution includes surface adsorption, ion exchange, relative solubilities and steric effects. It is based on the general principles of liquid chromatography where the stationary phase may be a solid or liquid and the mobile phase is a liquid. A typical chromatographic unit consists of an injector to inject the sample, a pump to force the sample through the separation column (made up of tightly packed separation media particles), a detector for the detection of the separated ingredients as they migrate out of the separation column and a recorder to acquire, store and quantitate results. HPLC uses liquid as a mobile phase. Since liquids are viscous and exhibit more diffusion time than gases, the separation columns are operated at high (300 to 3,000 psi) pressures. Equilibrium rates can be improved by using the separation medium with small sized particles. HPLC is preferred over other types of chromatography especially for molecules which have high polarity, high molecular weight, a large number of ionic groups and thermal instability.

1.6.2 Types of HPLC [98-101]

1.6.2.1 Normal phase chromatography

Normal phase HPLC was the first kind of HPLC used, and it separates analytes based on polarity. This method uses a polar stationary phase and a nonpolar mobile phase, and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with the polar stationary phase. Adsorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time.
1.6.2.2 Reversed phase chromatography (RPC)

Reversed phase high performance liquid chromatography consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with $R(CH_3)_2SiCl$, where $R$ is a straight chain alkyl group such as $C_{18}H_{37}$ or $C_8H_{17}$. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. RPC is so commonly used that it is not uncommon for it to be incorrectly referred as “HPLC” without further specification.

RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent. This solvophobic effect is dominated by the force of water for “cavity-reduction” around the analyte and the $C_{18}$-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding less-polar solvent (MeOH) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically changing the polarity of the mobile phase during the course of the analysis.

1.6.2.3 Gel permeation chromatography

GPC is the type of chromatography in which the stationary phase is a molecular sieve. They are hydrophilic and therefore, capable of absorbing water leading to swelling and opening of this structure. Solvated molecules larger than largest pores of swollen gel
cannot penetrate the gel particles and therefore, will pass straight through the column through the spaces between the individual particles. Smaller molecules, however, will penetrate the open network in the particles to a varying degree, depending on their size and shape. They are, therefore, retarded to varying degree and will be eluted in order of decreasing molecular size. This is also known as gel filtration chromatography.

1.6.2.4 Ion-exchange chromatography

It is well suited for the separation of charged or highly polar sample ingredients such as salts, acids, etc. as it is based on the degree of charge. The sample is dissolved in an aqueous buffer and injected into the column. The column is selected in such a way that its charge is opposite to that of the sample ingredients of interest. The ingredients of injected sample get attached to the column by ion exchange and get separated by exchange of charges. The type of chromatography is frequently used for separating amino acids, organic acids, inorganic ions and proteins.

1.6.3 Instrumentation

HPLC consists of following parts as shown in Figure 1.6.1

1.6.3.1 Pump

The high pressure that must be applied to the liquid to force it through a column at a satisfactory rate can be obtained by a motor driven pump. The solvent reservoirs are filled with a range of miscible solvents of different polarities or different pH or different ionic strength and mixed in a predetermined manner.

1.6.3.2 Injector

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop (Fig. 1.6.2). The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via injection valve. A
rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. In modern HPLC systems, the sample injection is typically automated [102].
1.6.3.3 Columns

HPLC columns are typically 1-6 mm inside diameter, 30-100 cm in length and made of stainless steel or glass. The solid support for an immobile liquid phase can be diatomaceous earth which is porous or consist of particles with hard cores. A porous surface layer HPLC column is frequently operated at room temperature. The rate of diffusion and hence speed of separation can be increased somewhat by higher-temperature operation, so a heating jacket or oven is sometimes required. There are various columns that are secondary to the separating column or stationary phase. They are: Guard, Derivatizing, Capillary, Fast and Preparatory columns [103-104].

1.6.3.4 Mobile Phase

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase of an assay through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column [105].

1.6.3.5 Stationary phases

The stationary phase in HPLC [106] refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase.
### 1.6.3.6 Detectors

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. There are many types of detectors that can be used with HPLC. Some of the more common detectors include: Refractive index, Ultra-violet, Fluorescent, Radiochemical, Electrochemical, Near-infra red, Mass spectroscopy, Nuclear magnetic resonance, and Light scattering.

### 1.7. Introduction to GC-MS

GC-MS is a technique that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. In its initial stages of development it was applied to the analysis of gases and vapors from very volatile components. The work of Martin and Synge [107] and then James and Martin [108] in gas-liquid chromatography (GLC) opened the door for an analytical technique that has revolutionized chemical separations and analyses. As an analytical tool, GC can be used for the direct separation and analysis of gaseous samples, liquid solutions, and volatile solids. The GC portion of the system is typically a 30 meter capillary column that separates individual chemicals in a complex mixture. The separation of the component chemicals allows for both qualitative and quantitative analysis [109]. As the individual chemical components are released from the GC column, they enter the MS. In the MS, each chemical is ionized and passed through a magnetic mass analyzer that measures the amount of each ion present. The retention time of a chemical in the GC and the mass spectra provided by the MS allows for unambiguous identification of unknown chemicals. Therefore, it was not surprising that the combination of the two techniques was suggested
shortly after the development of GC in the mid-1950s. Gas chromatography and mass spectrometry are, in many ways, highly compatible techniques. In both techniques, the sample is in the vapour phase, and both techniques deal with about the same amount of sample (typically less than 1 ng).

1.7.1. Principle

GC involves the partitioning of gaseous solutes between an inert gas mobile phase and a stationary liquid or solid phase. The samples to be analyzed can be in solid, liquid or gas phase. Solid samples can be dissolved or extracted into liquid solutions that are injected into GC for analysis. Gaseous samples may be introduced by manual injection using a gas-tight syringe. Figure 1.7.1 is a simplified diagram of the major component parts of a gas chromatograph. These parts can be identified as the gas, the injection port, the column, the detector and the data acquisition system, consisting of an electrometer and recorder/integrating device.

Fig. 1.7.1 Schematic diagram of a Gas chromatograph
1.7.2. Instrumentation

1.7.2.1. Mobile phase

The first major component of a GC is the carrier gas. A carrier gas supply is always present and usually consists of helium, nitrogen, hydrogen, or a mixture of argon and methane. The function of the carrier gas is to carry the sample through the system. The gas chosen depends on the specific application and type of detector used. However, helium is one of the most commonly used gases.

1.7.2.2 Injection ports and sample introduction

The next major component of a GC is the injection port or GC inlet. The purpose of the inlet is to introduce the sample into the carrier gas stream. The most common sample introduction technique is the injection of 1 to 3 µL of a liquid sample into a heated inlet. A split injection port is available to allow only a fraction of the injected volume of the sample into the capillary column. Capillary columns have limited sample capacity, so some of the sample injected is allowed into the column and the remaining sample is vented or split away.

1.7.2.3 Columns

The third major component of the GC is the column, which is responsible for the separation of the components in the sample mixture. The first GC columns were packed columns developed in 1951 and 1952 [110-112]. The first open tubular or capillary columns was introduced in 1958 [113] and because of its significant ability for high quality separations, the capillary column has become the column of the choice.

When describing a column, one states the length in meters, diameter in millimetres, stationary phase film thickness in µm, and type of the stationary phase. The inner walls of
the capillary tube are coated with either a solid porous material or a viscous liquid material. The capillary column is made of fused silica quartz coated in the outside with polyimide to give it durability. The inner diameter commonly ranges from 0.005 to 0.53 mm, the length from 10 to 150 m, and the film thickness of the stationary phase from 0.05 to 3 µm.

1.7.2.4 Detectors

The fourth major component of the GC is the detector. It is the device that senses the presence of different components from the carrier gas and converts that information to an electrical signal. The characteristics influencing one’s choice of detector include selectivity and sensitivity. Selectivity is the ability of the detector to recognise and respond to the components of the interest. Not all detectors respond to all the components. Sensitivity is the concentration level detected and normally is defined as the change in the response with the change in detected quantity. One needs to match the selective properties of the detector to the components of interest in the sample mixture, as well as considering the lowest level of the detection of the detector and the concentration level of those components. The common detectors used in the GC analysis are thermal conductivity detector (TCD), flame ionisation detector (FID), electron capture detector (ECD), nitrogen-phosphorous detector (NPD), flame photometric detector (FPD), electrolytic conductivity detector (ELCD), photo ionisation detector (PID), infrared detector (IRD), atomic emission detector (AED), and mass selective detector (MSD).

1.7.2.5 GC and MS interface

There was a major incompatibility between the two techniques: The compound exiting the gas chromatograph is a trace component in the GC’s carrier gas at a pressure of about 760 torr, but the mass spectrometer operates at a vacuum of about $10^{-6}$ to $10^{-5}$ torr.
This is a difference in pressure of 8 to 9 orders of magnitude, a considerable problem. To maintain the high sensitivity of the two techniques, improved GC-MS interfaces were designed [114]. These interfaces reduced the pressure of the GC effluent from about 760 torr to \(10^{-6}\) to \(10^{-5}\) torr, but at the same time, they passed all (or most) of the analyte molecules from the GC into the mass spectrometer. These interfaces were no longer just GC carrier gas splitters, but carrier gas separators; that is, they separated the carrier gas from the organic analytes and actually increased the concentration of the organic compounds in the carrier gas stream. Prior to the introduction of capillary columns in GC, it was necessary to eliminate the larger volumes of carrier gas eluting from the chromatograph prior to introduction into the mass spectrometer. Various interfaces were developed for packed column GC. These included the jet, membrane and effusion separators. The purpose of all of these devices is to eliminate most of the carrier gas, thereby enriching the analyte concentration. The main requirement for these interfaces is that a constant temperature should be maintained across the entire length from oven to ion source with no “cold spots,” which may cause peak broadening or trapping of high boiling components.

The schematic diagram showing the major components of a typical capillary GCMS system is presented in Figure 1.7.2. The gaseous effluent from the chromatograph is directed through the transfer line into the ion source. The vaporized analytes are then ionized, producing molecular and/or fragment ions, which are then mass resolved utilizing a mass filter and detected. The resulting mass spectrum is displayed as a plot of the relative intensity of these ions versus their mass-to-charge ratio \((m/z)\). Since most ions produced are singly charged, their \(m/z\) values are indicative of their masses.
Gaseous analytes eluting from the chromatograph are directed into the spectrometer ion source where they are ionized. The ions produced are separated according to their $m/z$ values and detected.

### 1.8. Introduction to physical parameters

Chromatography in different forms today is the leading analytical method for the separation of the analytes in a mixture. Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible *stationary phase*. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary
phase. Techniques such as HPLC and GC use columns - narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called elution. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase.

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases;

\[ A_{\text{mobile}} \rightleftharpoons A_{\text{stationary}} \]

The equilibrium constant, \( K \), is termed as the partition coefficient; defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase.

The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the retention time (\( t_R \)). Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called \( t_M \).

![Fig. 1.8.1 Diagrammatic representation of retention time](image_url)
1.8.1. Retention factor ($k$)

The retention factor, as its name implies, is basically a measure of how long each compound stays on the column. It is often used to describe the migration rate of an analyte on a column and is the relative retention of each peak on the column. The retention factor for analyte A is defined as;

$$k_A = t_R - t_M / t_M$$

$t_R$ and $t_M$ are easily obtained from a chromatogram. When an analyte's retention factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between one and five.

1.8.2. Separation factor ($\alpha$)

The next factor, the separation factor ($\alpha$), represents the relative separation between any two peaks’ centers on a chromatogram. It is defined as the retention factor of the longer retaining peak ($k_B$) divided by the retention factor of the faster peak ($k_A$). Any pair of peaks in the chromatogram will have their own $\alpha$.

$$\alpha = k_B / k_A$$

When calculating the selectivity factor, species A elutes faster than species B. The selectivity factor is always greater than one.

1.8.3. Efficiency factor ($N$)

The next factor, the efficiency factor ($N$), measures the degree of sharpness of a given peak. It can be found by examining a chromatographic peak after elution;

$$N = \frac{5.55 t_R^2}{w_{1/2}^2}$$

where $w_{1/2}$ is the peak width at half-height.
### 1.8.4. Resolution equation \((R)\)

All of these three factors are combined in the resolution equation \((R)\), which predicts how each factor will affect the separation.

\[
R = \left( \frac{\sqrt{N}}{4} \right) \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{1 + k}{k} \right)
\]

Baseline resolution is achieved when \(R = 1.5\).

To obtain high resolution, the three terms must be maximised. An increase in \(N\), the efficiency factor, by lengthening the column leads to an increase in retention time and increased band broadening - which may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the size of the stationary phase particles. It is often found that by controlling the retention factor \((k)\), separations can be greatly improved. This can be achieved by changing the temperature (in Gas chromatography) or the composition of the mobile phase (in Liquid Chromatography).

### 1.8.5. Validation

When a method has been developed it is important to validate it to confirm that it is suitable for its intended purpose. The validation tells how good the methods are, specifically whether it is good enough for the intended application. The method validation is today an essential concern in the activity of analytical chemistry laboratories. The most common validation parameters will be briefly described below.

#### 1.8.5.1. Precision

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogenous volume of environmental or biological matrix [115]. The
precision is calculated as coefficient of variation i.e., relative standard deviation (RSD). The measured RSD can be subdivided into three categories: repeatedly (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision) [115-118].

1.8.5.2. Accuracy

Accuracy is the nearness of a measured value to the true or accepted value. It provides an indication of any systematic error or bias in the method. During the validation, accuracy is determined by measuring the recovery of the active component from an environmental matrix.

1.8.5.3. Limit of detection

The limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value [115]. The LOD is a parameter of limit tests (i.e., tests that only determine if the analyte concentration is above or below a specification limit). In analytical procedures such as HPLC or GC that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration (e.g., percentage, parts per billion) of analyte in the sample. There are several ways in which it can be determined, but it usually involves injecting samples, which generate an S/N of 3:1, and estimating the LOD.

1.8.5.4. Limit of quantitation

The quantitation limit (QL) or limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy [115]. The quantitation limit is a parameter of quantitative assays for low concentrations of compounds in sample
matrices and is used particularly for the determination of impurities and/or degradation products. It is usually expressed as the concentration (e.g., percentage, parts per million) of analyte in the sample. For analytical procedures such as HPLC or GC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviations (%RSDs) as well.

1.8.5.5. Linearity

Linearity evaluates the analytical procedure’s ability (within a give range) to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample [115]. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range [118]. Linearity is usually expressed as the confidence limit around the slope of the regression line. The line is calculated according to an established mathematical relationship from the test response obtained by the analysis of samples with varying concentrations of analyte. During validation, linearity may be established for all active substances, preservatives, and expected impurities. Evaluation is usually performed on standards.

1.8.5.6. Range

Range is defined as the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical method. During validation, range (sometimes
referred to as linearity of method) is evaluated using samples (usually spiked) and must encompass the specification range of the component assayed in the matrix [115, 118].

1.8.5.6. Recovery

The absolute recovery is the ratio of the response measured for spiked sample (in matrix) treated according to the whole analytical procedure to that of a non-matrix sample spiked (in aqueous solution) with the same quantity of the analyte substance and directly injected into the chromatographic system [118]. The relative recovery is the ratio of the responses between extracted spiked sample (in matrix) and extracted spiked pure sample (in aqueous solution). The relative recovery can be used, together with the absolute recovery, to reveal whether sample losses in the extraction are due to matrix effects or to bad extraction. High recovery of the analyte(s) from the matrix is a desirable outcome of sample preparation. And it is therefore an important characteristic of the extraction procedure.

1.9. Sample Preparation

An important step in the development of an analytical method is the extraction of the compounds of interest from the biological matrix as this will have implications on the overall sensitivity and selectivity of the method. Sample preparation will not only lead to highly concentrated extracts, but can remove potential interfering matrix compounds, resulting in enhanced selectivity and a more reproducible method independent of variations in the sample matrix.

Sample preparation is the process of extracting chemical residues from a sample and the subsequent purification of the extract to isolate the residues of interest and remove
any matrix interferents that may affect the detection system. Even with the advancement of
separation and detection techniques, sample preparation is a vital part of the analytical
process and effective sample preparation is essential for achieving reliable results and
maintaining instrument performance.

The quantitative and qualitative analysis of drugs and metabolites is extensively
applied in pharmacokinetic studies. Biological materials and pharmaceutical products are
complex and often contain proteins, salts, acids, bases and organic compounds with similar
properties to the analytes. In addition, the analytes often exist at low concentration in
samples. Depending on the origins of samples and analytical objectives, drug analyses
have been carried out using various analytical instruments in many circumstances such as
clinical control for diagnosis and treatment of diseases, doping control, forensic analysis
and toxicology. However, despite the advances in the development of highly efficient
analytical instrumentation for the endpoint determination of analytes in biological samples
and pharmaceutical products, sample pre-treatment is usually necessary in order to extract,
isolate and concentrate the analytes of interest from complex matrixes because most
analytical instruments cannot handle the matrix directly. In general, the analytical process
is divided into five steps: sampling; sample preparation; separation; detection; and, data
analysis. Over 80% of analysis time is spent on the sampling and sample-preparation steps.
Furthermore, the quality of these steps is a key factor in determining the success of
analysis from complex matrices, such as biological samples, so it is no exaggeration to say
that choice of an appropriate sample preparation method greatly influences the reliability
and accuracy of the analysis.
Sample preparation can be achieved by employing a wide range of techniques, but all methods have the same goal [119]:

- to remove potential interferences;
- to increase the concentration of an analyte;
- if necessary, to convert an analyte into a more suitable form; and,
- to provide a robust, reproducible method that is independent of variations in the sample matrix.

Although many traditional sample-preparation methods are still in use, there have been trends in recent years towards [119]:

- use of smaller initial sample sizes, small volumes or no organic solvents;
- greater specificity or greater selectivity in extraction; and,
- increased potential for automation.

However, previous sample-preparation techniques have involved various drawbacks, such as complicated, time-consuming procedures, large amounts of sample and organic solvent and difficulty in automation. For example, if a long time is required for sample preparation, this limits the number of samples, and multi-step procedures are prone to lose analytes. Furthermore, use of harmful chemicals and large amounts of solvent cause environmental pollution, health hazards to laboratory personnel and extra operational costs for waste treatment. Ideally, sample-preparation techniques should be fast, easy to use, inexpensive and compatible with a range of analytical instruments. Specialized sample preparations, such as self-assembly of analytes on nanoparticles for surface enhancement, have also evolved. In the present work, solid phase extraction (SPE) and microextraction in packed syringe (MEPS) are used as the sample preparation and preconcentration techniques.
1.9.1. Solid Phase Extraction (SPE)

1.9.1.1. Introduction

Despite the selectivity and sensitivity of analytical techniques, preconcentration of trace elements before their analysis due to their frequent low concentrations in numerous samples is necessary. Additionally, since high levels of non-toxic components usually accompany analytes, a clean-up step is often required. Liquid-liquid extraction is a classical method for preconcentrating and/or matrix removal. Solid phase extraction (SPE) is another approach that offers a number of important benefits. It reduces solvent usage and exposure, disposal costs and extraction time for sample preparation.

SPE is probably the most widely adopted technique for preparing samples in the analysis of pharmaceuticals and drugs of abuse in biological matrices. The large variety of sorbents commercially available makes this technique suitable for the determination of analytes with divergent chemical structures and polarities. As a result current research into sorbents focuses mainly in the development of novel sorbents allowing higher loading capacities, higher efficiency for retention of highly polar analytes from aqueous matrices, and in the synthesis of class-selective immuno-sorbents (ISPEs) and molecular imprinted polymers (MIPs) allowing an improved selectivity during the retention process and so contributing to the simplification of the subsequent clean-up and/or detection steps [120, 121].

SPE offers the following advantages over conventional liquid-liquid procedures:

- higher recovery;
- more effective concentration;
- less organic solvent usage;
• no foaming or emulsion problems;
• shorter sample-preparation time;
• easier operation;
• easier incorporation into an automated process.

1.9.1.2. Theory

The principle of SPE is similar to that of liquid-liquid extraction (LLE), involving a partitioning of solutes between two phases [122]. However, instead of two immiscible liquid phases, as in LLE, SPE involves partitioning between a liquid (sample matrix) and a solid (sorbent) phase [123] whereby the intermolecular forces between the phases influences retention and elution. Retention may involve non-polar, polar or ionic interactions. The wide range of SPE sorbents available provides a wide range of interactions. This sample treatment technique enables the concentration and purification of analytes from solution by sorption on a solid sorbent. The basic approach involves passing the liquid sample through a column, a cartridge, a tube or a disk containing an adsorbent that retains the analytes. After the entire sample has been passed through the sorbent, retained analytes are subsequently recovered upon elution with an appropriate solvent. The first experimental applications of SPE started fifty years ago. However, its growing development as an alternative approach to liquid-liquid extraction for sample preparation started only in the mid-1970s. It has been extensively used in the past fifteen years for the preconcentration of organic micropollutants, especially drugs, in water and biological samples. However, numerous studies have also shown the great potential of this technique for speciation studies.
MIPs are capable of molecular recognition and are stable enough for long-term storage, easy to prepare and inexpensive. Thus, they may be considered to be a new artificial affinity media. Different modes of MIP based SPE have been demonstrated, including various modes of offline and on-line SPE for pre-concentration or pre-treatment of analytes and for conventional SPE where the MIP is packed into columns or cartridges.

1.9.1.3. Working

An SPE [124] method always consists of three to four successive steps (Fig. 1.9.1). First, the solid sorbent should be conditioned using an appropriate solvent, followed by the same solvent as the sample solvent. This step is crucial, as it enables the wetting of the packing material and the solvation of the functional groups. In addition, it removes possible impurities initially contained in the sorbent or the packaging. Also, this step removes the air present in the column and fills the void volume with solvent. The nature of the conditioning solvent depends on the nature of the solid sorbent. Typically, for reversed phase sorbent (such as octadecyl-bonded silica), methanol is frequently used, followed by water or aqueous buffer whose pH and ionic strength are similar to that of the sample. Care must be taken not to allow the solid sorbent to dry between the conditioning and the sample treatment steps, otherwise the analytes will not be efficiently retained and poor recoveries will be obtained. If the sorbent dries for more than several minutes, it must be reconditioned.

The second step is the percolation of the sample through the solid sorbent. Depending on the system used, volumes can range from 1 mL to 1L. The sample may be applied to the column by gravity, pumping, aspirated by vacuum or by an automated system. The sample flow-rate through the sorbent should be low enough to enable efficient
retention of the analytes, and high enough to avoid excessive duration. During this step, the analytes are concentrated on the sorbent. Even though matrix components may also be retained by the solid sorbent, some of them pass through, thus enabling some purification (matrix separation) of the sample.

The third step (which is optional) may be the washing of the solid sorbent with an appropriate solvent, having low elution strength, to eliminate matrix components that have been retained by the solid sorbent, without displacing the analytes. A drying step may also be advisable, especially for aqueous matrices, to remove traces of water from the solid sorbent. This will eliminate the presence of water in the final extract, which, in some cases, may hinder the subsequent concentration of the extract and/or the analysis.

Fig. 1.9.1 Steps involved in the SPE process

The final step consists of elution of the analytes of interest by an appropriate solvent, without removing retained matrix components. The solvent volume should be
adjusted so that quantitative recovery of the analytes is achieved with subsequent low dilution. In addition, the flow-rate should be correctly adjusted to ensure efficient elution. It is often recommended that the solvent volume be fractionated into two aliquots, and before the elution let the solid sorbent soak the solvent.

The cartridges drip into the chamber below (Fig. 1.9.2), where tubes collect the effluent. A vacuum port with gauge is used to control the vacuum applied to the chamber.

Fig. 1.9.2 A typical solid phase extraction manifolds

1.9.2. Micro Extraction by Packed Sorbent (MEPS)

1.9.2.1. Introduction

Micro extraction by packed sorbent is a new development in the fields of sample preparation and sample handling [140-146]. MEPS is the miniaturization of conventional SPE packed bed devices from millilitre bed volumes to microlitre volumes. The commercially available presentation of MEPS uses the same sorbents as conventional SPE
columns. Unlike conventional SPE columns, the MEPS sorbent bed is integrated into a liquid handling syringe that allows for low void volume sample manipulations either manually or in combination with laboratory robotics. MEPS can combine sample processing, extraction and injection steps fully automated as an on-line/at-line sampling/injecting device to GC or LC.

In MEPS the sorbent, 1-4 mg is either inserted into the syringe (100-250 µL) barrel as a plug or between the needle and the barrel as a cartridge. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. Any sorbent material such silica based (C2, C8, C18), strong cation exchanger (SCX) using sulfonic acid bonded silica, restricted access material (RAM), carbon, polystyrene-divinylbenzene copolymer (PS-DVB) or molecular imprinted polymers (MIPs) can be used.

While the capacity for truly miniaturized extractions allows sparing use of sample and reagents, a more significant strength of MEPS is the ability to fully elute the sorbent with volumes of less than 10 µL through a syringe needle. Such capability allows MEPS to be adapted for on-line use with both GC and LC inlets.

MEPS performs the same functions as SPE - the removal of interfering matrix components and the selective isolation and concentration of analytes. MEPS increases the advantages of conventional SPE in the following ways:

- Significantly reduces the time needed to prepare and inject samples.
- Can be combined with LC or GC automation - the extraction step and injection step are performed on-line using the same syringe.
- Significantly reduces the volume of solvents needed.
- Ability to work with samples as small as some µL versus several hundred mL for SPE
1.9.2.2. Working of MEPS

In MEPS the sorbent material, about 1-4 mg, is either inserted into the barrel of a syringe as a plug with polyethylene filters on both sides, or between the syringe barrel and the injection needle as a cartridge (Figure 1.9.3). A scheme of MEPS working is shown in Figure 1.9.4.

![MEPS schematic](image)

**Fig. 1.9.3 MEPS needle and schematic of the MEPS BIN in the syringe needle**

i) Sampling

- Dilution of samples is required to reduce viscosity of blood or plasma samples to prevent coagulations and blockage in MEPS cartridge and to achieve low back-pressure. Dilution can be done with pure water or 0.1% formic acid in water (dilution, 1:5 for plasma and :25 for blood). Remove unnecessary macro particles by centrifugation (1-2 min at 3000 rpm).
- Adjust pH to reduce ionization of weak acids and bases for reversed-phase sampling.

- Constant ionic strength for samples and standards when using reversed-phase sampling conditions. Ionic strength is a critical parameter for ion-exchange extraction.

- Deproteination may be required for satisfactory recovery for reversed-phase sampling.

- The speed of the sample through the sorbent can be ranged from 10 to 20 μLs$^{-1}$ for better interaction between analyte and sorbent.

- Sample loading: extraction recovery can be increased by increasing the number of MEPS loading cycles (1-10 times, draw-eject).

Fig. 1.9.4 A scheme of MEPS working
ii) Washing

- Small volume (50-100 µL) of water with 5-10% organic solvent (methanol, isopropanol or acetonitrile) to elute matrix unwanted components and to keep analytes immobilized on the sorbent.

iii) Drying

- It is recommended but not necessary due to a small bed of sorbent.

iv) Eluting solvent

- Should be an organic solvent like methanol, isopropanol or acetonitrile, pure or mixed with acid or base solutions (0.1-3%) and should be able to displace all analytes from the sorbent in a small volume (20-50 µL).
- Normally should be volatile and miscible with the sample solvent or LC mobile phase.

v) Washing of sorbent after injection

- Two washing solutions are recommended. The first one (strong washing) should be an organic solvent such as methanol or acetonitrile and should include isopropanol (10-20%) with some acid or base depending on the nature of the analytes (0.2% formic acid/0.2% ammonium hydroxide, v/v). The second one (weak washing) can be pure water or 5% methanol in water. The both solution will be used for washing and re-conditioning of the sorbent.
- Eluting solution can be suitable as a strong washing solution followed by water (4 times for each step). This step was to reduce or eliminate carryover for reusing of MEPS sorbent.
• Carryover from the sorbent can be reduced further by adding additional conditioning rinses to the method. Carryover has been found to be less than 0.1% with 4-5 washes of.

A key factor in MEPS is that the volume of solvent used to elute the analytes from the extraction process is of a suitable order of magnitude to be injected directly into an LC or GC system. MEPS can be regarded as a short LC column in a syringe. The bed dimensions are scaled from the conventional SPE bed and so MEPS can be adapted for most existing SPE methods simply by scaling the reagents and sample volumes from the conventional device to the MEPS. The MEPS cartridge cannot be used for more than 100 extractions of plasma samples as plasma matrix can result in changing of sorbent surface chemistry and sorption properties of the solid phase. Many factors, such as volumes and composition of washing solution and elution solution, sorbent amount and sorbent type affect the performance of MEPS.

1.9.2.3. Advantages of MEPS over other extraction techniques

• MEPS and SPE/LLE: MEPS is a new sort of solid phase extraction that has been miniaturized to work with sample volumes as small as 10 µL. The MEPS differs from commercial solid phase extraction that the sorbent bed in MEPS is integrated into a syringe that allow for low void volume sample manipulations either manually or automatically in combination with autosampler. In addition MEPS allows sparing use of sample and reagents, a more significant strength of MEPS is the ability to fully elute the sorbent with volumes of less than 10 µL. By comparison with the SPE and LLE methods MEPS technique is simple, rapid, non-solvent consuming and can achieve the same sensitivity.
**MEPS and SPME:** SPME is suited for online use in combination with GC or LC methods; its ability to handle low concentrated analytes in small sample volumes is limited. In SPME, the sampling fibre is quite sensitive for the nature of sample matrix such as urine, plasma or blood. MEPS can be used for complex matrices such as plasma, blood and urine. SPME fibre has a very short life time and can perform 5-50 extractions while MEPS can perform 100-400 extractions depending upon the type of matrix. Table 1.1 shows a comparison between MEPS and other extraction techniques such as SPE and SPME.

Table 1.9.2 Comparison of MEPS, SPE and SPME

<table>
<thead>
<tr>
<th>MEPS</th>
<th>MEPS</th>
<th>SPE</th>
<th>SPME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbent amount</td>
<td>2-4 mg</td>
<td>50-2000 mg</td>
<td>thickness 150 μm</td>
</tr>
<tr>
<td>Handling time</td>
<td>1-2 min</td>
<td>15-20 min</td>
<td>20-40 min</td>
</tr>
<tr>
<td>Cartridge re-uses</td>
<td>100 extractions</td>
<td>Once</td>
<td>50 extractions</td>
</tr>
<tr>
<td>Recovery</td>
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<td>good</td>
<td>low</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>good</td>
<td>good</td>
<td>low</td>
</tr>
</tbody>
</table>

**MEPS and SBSE:** SBSE technique is known as more difficult to automate but semi-automated protocols have been described already. SBSE has a good recovery but has a long extraction time, 30 min or more, compared to 1-3 min using MEPS.
References


