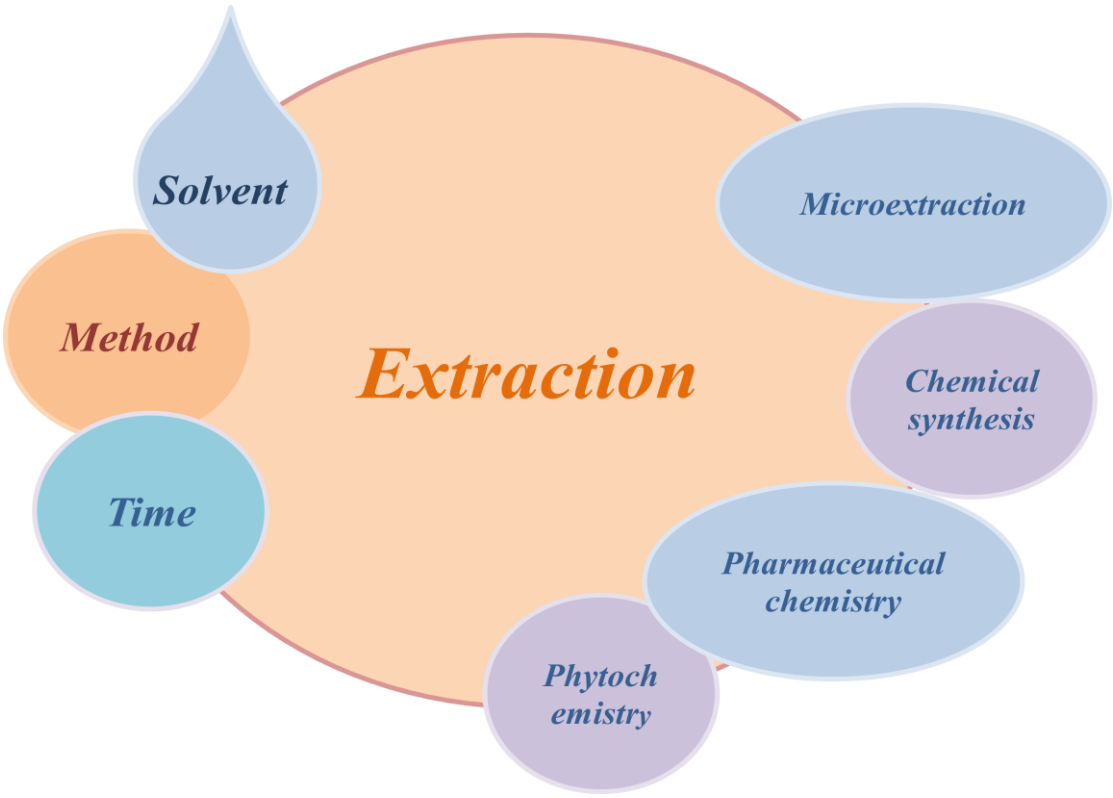
Chapter 1

INTRODUCTION



Chapter 1 Introduction

1. Introduction

The chapter discusses the history, developments, and role of extraction and chromatographic determinations over the years. The aspiration to achieve extraction with reduced time and efforts have led to these developments.

1.1 Extraction and its role

The term ***"Extraction"*** literally means ***"to draw out"***. Extraction has been used for drawing out desired chemicals or active molecules from natural as well as synthesized materials through various techniques. This transfer of one or more components from one phase to another, while both phases are in contact with each other and immiscible with each other, is based on the Nernst distribution law. Extraction has played a major role in pharmaceutical chemistry, phytochemistry and trace analysis and in commercial processes (figure 1). It forms the basis for development of microextraction techniques for trace level analyses.

Extraction was developed in the 1920's and 1930's in the petrochemical industry, with new developments in fundamental understanding, design procedures, applications and equipment evolving continuously from that period [1]. Extraction in the pharmaceutical industry has been used primarily in the isolation of antibiotics from fermenter broth, in the preparation of natural and synthetic vitamins, and in the preparation of drugs from naturally occurring materials [1]. Extraction from plants and herbs has been done for their exceptional medicinal properties and their selective action against specific disorders. This led to their application as medicine in Ayurveda, as churna or in combination with other ingredients or for external application. Over the years advances have been made to extract chemicals from these natural sources that have led to the development of novel drugs, preservatives and in development of various applications. Chemical analysis of plants commences with the extraction of plant material in water or alcohol or solvents of different polarity in order to extract bioactive constituents of the plant. Different extraction techniques such as Soxhlet extraction, solvent extraction, maceration or other such conventional techniques are used based on the conditions of source material. Once the extraction of major components in plants has been achieved, to segregate each chemical constituent or compound, chromatographic techniques such as planar chromatography, column chromatography, etc are used. After their separation, the chemical components are

identified by making use of spectroscopic techniques such as Mass Spectrometry (MS), Nuclear magnetic resonance (NMR), Infra red spectroscopy (IR), etc.

In natural products the concentration of the chemical constituents varies, and many exist at low concentrations. Also, the extraction of these constituents depends on extraction efficiency of the technique used. To enhance the extraction efficiency and the extraction yield of desired product, supercritical fluids, microwaves and ultrasound has been used successfully. Other advantages of these methods are lower extraction costs, use of minimal solvent making the processes green, reducing time consumption and lower extraction work up procedures.

Extraction is preferred over other separation techniques due to selectivity it provides by removing the non-essential matrix such as impurities, plant material, food, spices or a mixture of chemical reaction product and extracting the desired product from them. It has undergone modifications and development over the years, and in several cases it also serves as sample pre-treatment or pre-concentration step. Pre-concentration is thus an enhancement of the desired or target analyte concentration from a complex matrix, by removing the undesired compounds and narrowing down the complex matrix into simpler matrix. The remaining undesired products from the mixture can be separated by chromatographic techniques such as column chromatography, preparative thin layer chromatography. Pre-concentration is useful in trace analyses: determination of pollutants (organic or inorganic).

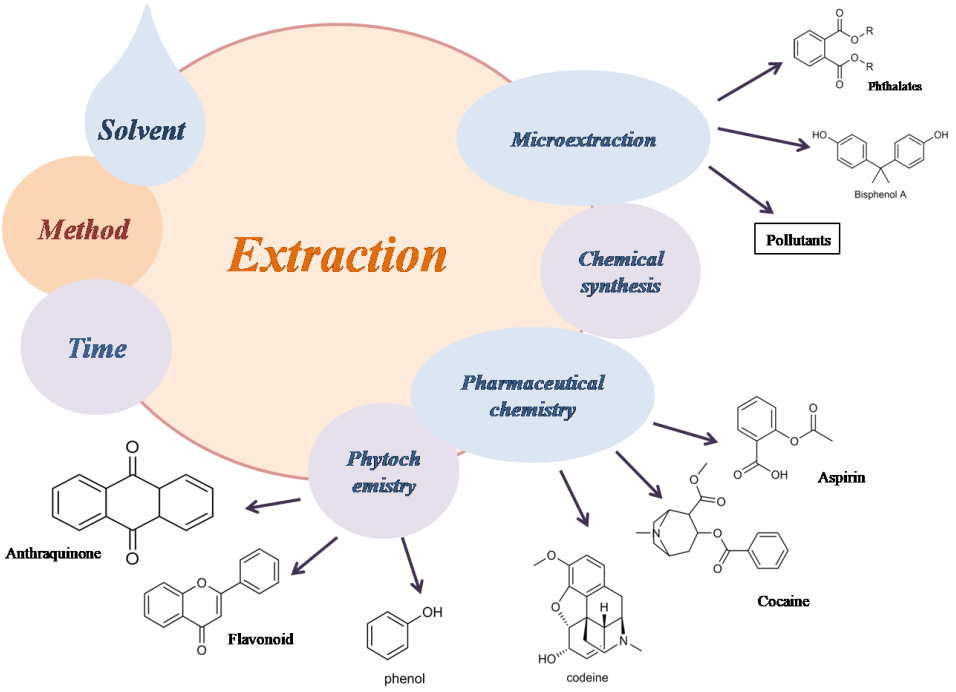


Figure 1 Extraction and its application

A technique (figure 2) in which the soluble solutes (bioactive components) from insoluble solid material (inert or inactive components) or other source material are separated for various purposes such as medicine, food, dye, essential oils, pigments, infusions, etc [2, 3, 4]. The material from the source is dissolved in an appropriate solvent to extract the target material and once the extraction is achieved, then the solvent is removed either through evaporation or distillation.

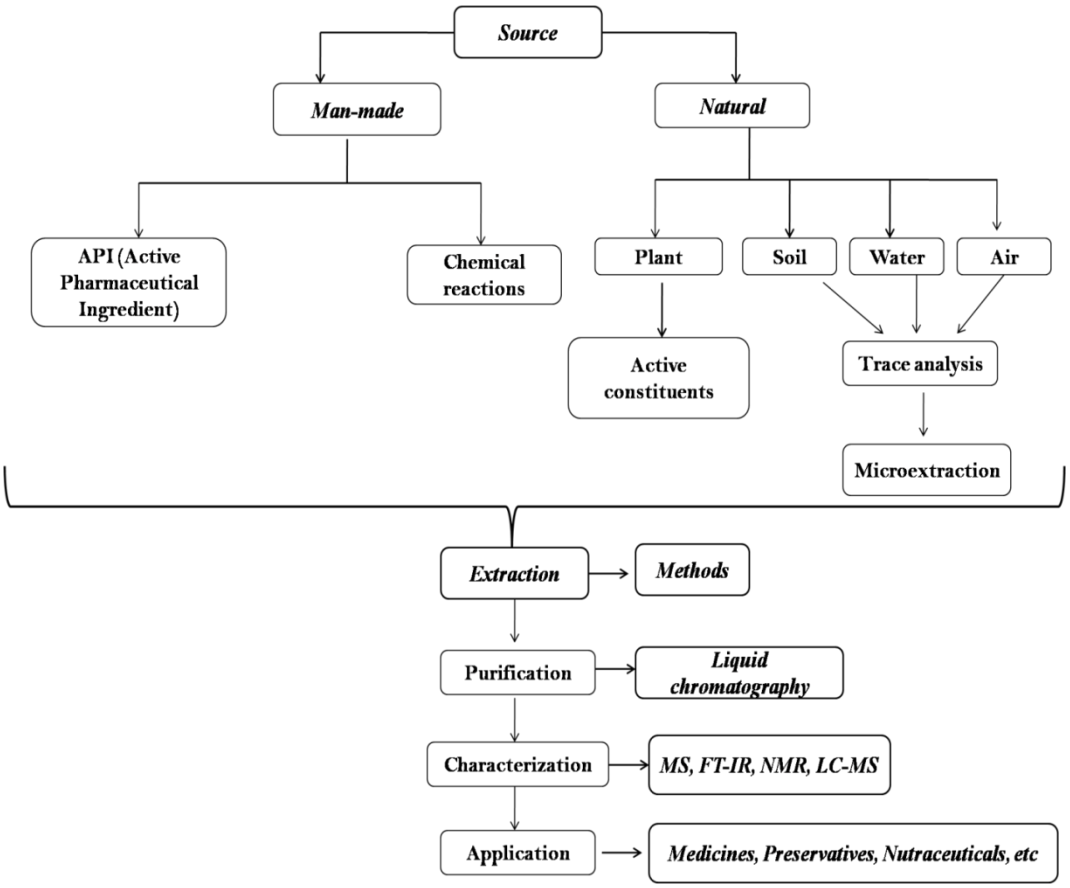


Figure 2 Methodology for extraction

Plants have been exploited over the years for different purposes such as wood, food, medicines, clothing, shelter, etc., as the development of mankind began. Extraction is a natural phenomenon that occurs in plants wherein they extract or take up the required nutrition (minerals uptake such as Na, K, N, P, etc) from the soil for their growth and development. So is the case in living beings as even here the extraction process occurs for acquiring the nutrients from the food they have consumed for their development and growth. Thus, extraction has application in biological system as well.

In case of extraction of desired chemicals from plants, the extract obtained is relatively crude material used directly as medicinal agents in the form of tinctures or

paste for external use. It contains a mixture of different metabolites [2, 3]. These consist of classes of preparations which are called infusions, fluidextracts, decoctions, tinctures, pilular (semisolid) extracts and powdered extracts [2]. Such types of preparations traditionally have been termed galenicals, named after a second century Greek physician; Galen [2].The procedure is mainly dependent on different parameters that affect it, which are as follows:

- Time for extraction
- Choice of solvent
- Temperature.
- Solvent to sample ratio

Plants possess complex mixtures of compounds which have specific role to play in the environment and for all living beings. The extracts prepared from plants are found to be a mixture of different class of compounds or secondary metabolites such as flavanoids, coumarin, phenols, terpenoids, anthraquinones, alkaloids, glycosides, insoluble forms such as lignin, cellulose, etc (figure 3).

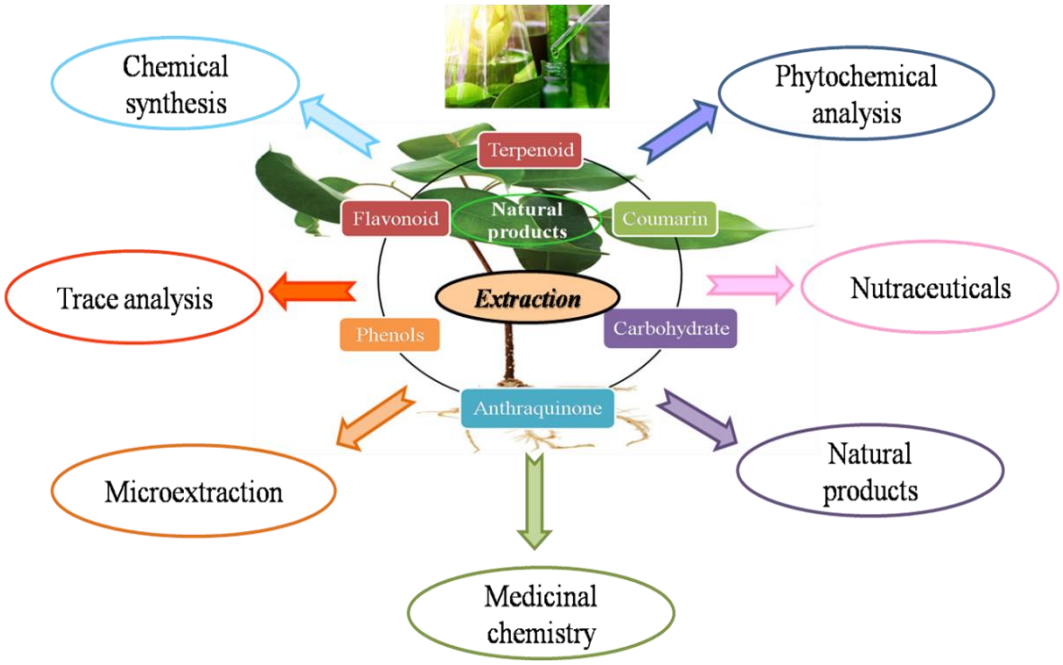


Figure 3 Extraction from plants

Extraction has found its application in not only biological ecosystems, natural products separation but also in chemical synthesis (figure 3), Medicinal chemistry, pharmaceutical chemistry, Microextraction, Nutraceuticals, etc. Chemical reactions occur between reactants to produce not just desired products but by-products as well

depending on the reaction mechanism. These side- or by-products may not be of desire always and thus can be treated as impurity.

To separate the desired product from the reaction mixture, extraction in an appropriate solvent is one of the separation techniques. Thus, the compound of interest is obtained, and the impurities are removed and the product can be used as per its application.

In a drug product, the active pharmaceutical ingredient (API) has specific action to perform at the target site in body for its therapeutic action. Purity of this specific active pharmaceutical ingredient (API) in a Pharma product is very important. The active ingredient could be obtained through extraction from a natural source such as plants and used as medicine. Therefore, the application and role of extraction becomes critical and has been developed over the years based on the requirement and short comings faced during the extraction performed. The basic and conventional extraction techniques used are Soxhlet extraction, liquid liquid extraction. For extraction from plants technique such as infusion, digestion, decoction, maceration, hot continuous extraction (Soxhlet), percolation, aqueous-alcoholic extraction by fermentation, supercritical fluid extraction, counter-current extraction, ultrasound extraction (sonication), microwave-assisted extraction, headspace trapping, solid phase microextraction, etc are used [2].

a. Extraction methods:

The general methods used for extraction from plants materials are as follows:

Maceration: The coarsely powdered material is mixed with a solvent and allowed to stand for a period of minimum 3 days with occasional stirring. The marc (damp solid material) is then pressed and filtered to remove the insoluble matrix (marc) [2].

Digestion: A form of maceration with application of gentle heat during extraction. It is applicable in cases when moderately elevated temperature is not an issue for the starting material, which in turn increases the extraction efficiency [2].

Percolation: A frequently used method to extract the active components in the production of tincture and fluid extracts. A closed container termed percolator (a cone shaped vessel) is used in which the material is moistened with solvent for 4 hours, after which the container is closed. Additional solvent is added to form a shallow layer in which the material keeps macerating for a period for 24 hours after which the opening of the percolator is opened through which the solvent keeps dripping and is

collected. The macerate is pressed to collect remaining solvent, which is then mixed with the main percolated solvent [2].

Infusion: This technique is similar to that of maceration, percolation. These are prepared by macerating the crude product with water (cold or boiling) for a short period of time, mostly applied for galenical preparations [2].

Decoction: Decoction is solution containing the active ingredients of material achieved after boiling the powdered material in water. The sample (solute) to solvent ratio is generally maintained in the range of 1:4 or 1:16, which is reduced to $1/4^{th}$ of its volume during boiling. This method is generally used in Ayurveda to prepare extracts termed "kwath" [2].

Soxhlet extraction (Hot continuous extraction): The most commonly used extraction method in which the product is placed in a thimble. It is a cyclic process in which the solvent keeps on circulating and penetrating through the thimble and extracting the active constituents in the solvent, as shown in figure 4 [2]. The solvent is heated from the round bottom flask which evaporates and condenses to reach the thimble; the condensed solvent extracts the constituents by contact. The advantage of this method is that a large amount of product is subjected to extraction with much smaller quantity of solvent [2].

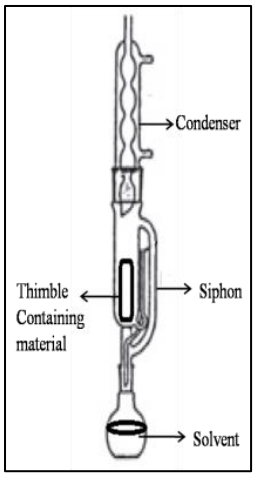


Figure 4 Soxhlet apparatus

This technique (leaching technique) has been used since 1879, developed by Franz Von Soxhlet, which has tremendous application in plant extraction. The solid material (plant material) is placed in a thimble which is brought repeatedly in contact with the solvent while heating such that continuous transfer of analytes from the material in the solvent occurs. The advantages of Soxhlet extraction are ease of operation

(unattended operation – once the experiment is set doesn't require continuous monitoring), it is not dependent on the matrix, total analyte present in the matrix gets extracted, also applicable at larger scale, better extraction efficiency and reproducibility is achieved. The major disadvantage of Soxhlet extraction lies in the longer extraction hours, also there remains the possibility of degradation of the analytes as extraction occurs at the boiling temperature of the solvent. Additional step of concentration of analytes by evaporation of the solvent, as large amount of solvent is required in the process. Also, to reduce the extraction time by providing continuous stirring in the Soxhlet apparatus is not possible [5].

Aqueous alcoholic extraction by fermentation: Specific preparations of Ayurveda such as asava and arista are produced by adopting the technique of fermentation for extracting the active constituents. The material is soaked in the form of a decoction (kasaya), for a specific time period, during which it undergoes fermentation to generate alcohol in situ. This facilitates the extraction of the active constituents from the plant material and the alcohol so generated also serves as preservative [2].

Counter current extraction: In this technique, using toothed disc disintegrators wet raw material is pulverized to produce fine slurry. The material moves in one direction within a cylindrical extractor where it comes in contact with the extraction solvent. As further the starting material moves in one direction, the richer the extract in concentration becomes. With optimized quantities of solvent and raw material and flow rate conditions, complete extraction is possible. The process is highly efficient, requires less time and is resistant from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end [2].

Ultrasound extraction: Ultrasound frequencies in the range of 20 to 2000 KHz are used to extract constituents from raw material. This increases the permeability through cell wall producing cavities which facilitates extraction. The process is useful in some cases such as extraction of rauwolfia roots. The application of this technique is limited due to higher costs and effect of ultrasound on active constituents of plants through formation of free radicals and undesirable changes in drug molecule [2].

Supercritical fluid extraction: This is an environmentally friendly extraction technique which involves use of CO_2 as an extraction solvent (CO_2 is pressurized at high temperature and pressure, above critical temperature and pressure, to attain super

critical fluid state) to extract the active constituents from plant, discussed elaborately in section 1.2.

Microwave assisted extraction: The technique uses microwave radiation to extract active constituents from the raw material. The use of microwave energy began in 1975 with the digestion of biological samples under pressurized conditions. Microwave energy was applied for the first time in extraction of organic compounds in 1986 [6]. It finds application in plant extraction and organic pollutants [7]. The sample absorbs electromagnetic energy and converts it into heat. Consequently, the heat produced facilitates movement of extraction solvent into the matrix of the raw material [8, 9 and 10]. When polar solvent is used, dipole rotation and migration of ions occur, increases solvent penetration and assists in extraction process. On the other hand, when nonpolar solvent is used, the microwave radiation released produces only small heat, which does not facilitate in achieving maximum extraction. Therefore, non polar solvents are not suitable for use during microwave assisted extraction [8].

1.1 Advances in extraction

Extraction technique has undergone tremendous changes and development over the years based on the demanding requirements to achieve maximum extraction efficiency with minimum efforts and the limitations and short comings the technique has faced. Most extraction techniques are based on the conventional technique: liquid liquid extraction (LLE) or solvent extraction. The basis of the technique lies in the distribution of the analytes in a mixture of a water immiscible solvent and the aqueous phase (a binary system). The mixture of analytes distributes between the binary system depending upon their polarity in either aqueous phase or the organic phase (figure 5).

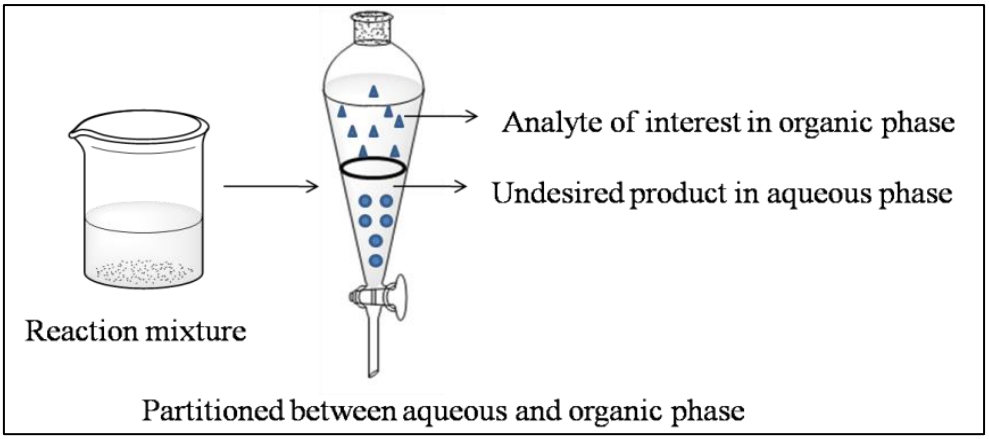


Figure 5 Pictorial representation of LLE

In an extraction technique choice of solvent, temperature and time are the major parameters that govern the selectivity and efficiency of extraction of a particular target analyte. Therefore, the solvent selection plays a major role in achieving the efficiency. The solvents are chosen based on its solubility in water, solvent evaporation rate during the concentration phase, polarity (intermolecular bonding such as H-bonding) that can increase the analyte recovery in the organic phase [11]. One of the limitations of this technique is formation of an emulsion. A milky white or cloudy solution that is formed during the extraction, which appears as one phase and no distinct boundary separation of the two phases can be observed, which makes the separation of either layer not possible is termed as emulsion. This emulsion can be broken by rapid centrifugation, by fast cooling or by addition of salt (such as NaCl) to the aqueous phase [11]. The other limitations include large solvent consumption, repetitive extraction with same volume of extraction solvent leading to accumulation of the repetitive batch and then concentration of the analytes is performed (leading to number of steps involvement) and use of several solvents that are not environment friendly. The technique and these observations have led to the development of microextraction techniques such as dispersive liquid microextraction (DLLME) [12], solid phase microextraction (SPME) [13, 14], single drop microextraction (SDME) [15] which has application in trace analysis. Continuous and discontinuous are the two types of approaches that are feasible with liquid liquid extraction. The discontinuous approach (non-continuous extraction) involves extraction of analytes between the two immiscible solvent mixtures in a separating funnel (figure 5) whereas continuous type approach is applicable in cases where the extraction process are slow, such that equilibrium of analyte between the organic phase and the aqueous phase is

poor. The organic solvent is boiled, condensed with the help of a water condenser attached over and again the solvent is mixed to percolate through the aqueous phase containing the target analytes and thus the organic solvent is enriched with the target analyte with repetitive cycles and thus the extraction takes several hours [3]. Therefore, over the period the limitations in the conventional techniques led to exploration of techniques by altering parameters such that it could enhance the extraction with minimal solvents, time, equipment, and thus led to advances in extraction. That's how the microextraction techniques were developed to determine analytes at trace levels.

These extraction techniques have major applications in plant extractions as the microwaves in Microwave assisted solvent extraction (MASE) and sound waves in ultrasound assisted extraction (UAE) results in cell disruption assisting in desorption of compounds from the matrix improving the extraction efficiency. MASE utilizes microwaves (electromagnetic radiation from 0.3 to 300 GHz) that operate at 2.45 GHz generally, which penetrates through the biomaterial to create heat by the interaction with the water molecule present in the sample [11]. In ultrasound assisted extraction (UAE), ultrasounds waves with frequencies higher than 20 KHz are used to extract the analytes of interest. Use of ultrasound assists in experimenting with the processing parameters such as decrease in temperature and pressure compared to that performed without ultrasound assistance [11].

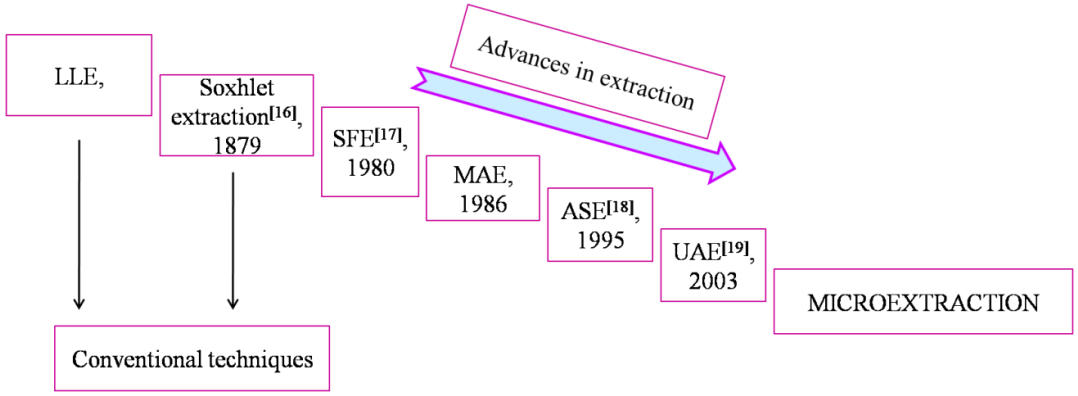


Figure 6 Advances in extraction

This could be achieved due to mechanical penetration of the sound waves into the matrix so that the analytes of interest are transferred from the matrix into the solvent. And thus, the advantages the technique bears are higher extraction yields and faster kinetics, with the application of this technique to thermally unstable molecules as the technique doesn't require higher temperature for extraction [11].

Accelerated solvent extraction (ASE) is a pressurized solvent extraction technique that is performed at elevated temperatures from 50 °C to 200 °C and pressure ranging from 10 and 15 MPa [10].

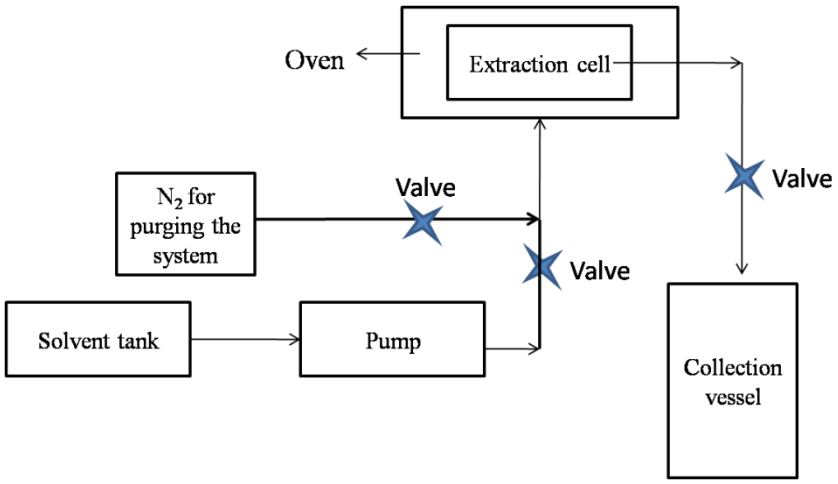


Figure 7 Schematic presentation of accelerated solvent extraction

In this technique, usually organic solvents are used for extraction, (figure 7) but pressurized hot water and sub-critical water has also been used and in that case it is termed as hot water extraction or subcritical water extraction [11]. Super critical fluid extraction is an extraction technique (figure 8) wherein CO_2 is pressurised at 7.3 MPa and a temperature of 31 °C K to achieve the supercritical state where the supercritical fluid has characteristics of both the gaseous and liquid state.

The advantage of this technique lies in the properties of the super critical fluid - higher diffusion coefficient, lower surface tension and viscosity in comparison with the usual organic liquid extraction solvents; this assists in rapid mass transfer and thus achieving higher extraction yields. CO_2 is used as solvent in this technique because of its non-toxicity, non-flammable properties. It is highly efficient in extraction of non-polar compounds.

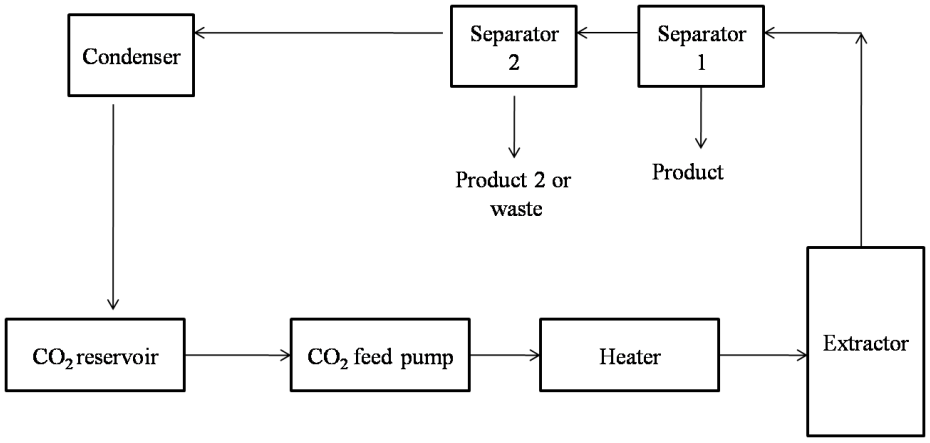


Figure 8 Schematic presentation for SFE

And thus, it limits the application of technique to only non-polar compounds and this limitation is overcome by addition of modifier (polar co-solvent) and the best suited solvent for this purpose is CH_3OH (methanol) and thus can be applicable in extraction of polar analytes. All these extraction techniques have application in plant (all type of plants) extraction.

All these techniques hold advantages and some limitations and based on these properties, their application to the extract active constituents of material is applied.

The application of extraction has been extended to determination of constituents at trace levels. And over the years development has been made to achieve higher extraction yields by optimizing the following parameters:

- Time for extraction
- Solvent consumption
- Quality of extracts
- Temperature at which extraction is performed
- Cost effective extraction technique
- User-friendly techniques

These advances have played a major role in the development of ***microextraction*** techniques. As the name suggests, these are small scale extractions with the use of minimal volumes of extraction solvents.

Extraction techniques	*Advantages*	*Limitations*
Liquid liquid extraction (LLE)	• Conventional extraction technique • Basic technique that led to extraction development	• Larger volume of solvent • Emulsion formation
Soxhlet extraction	• Large quantity of sample • Lower volume of solvent. • Applicable to heat resistant plant materials • Filtration not required • Application of high heat for extraction procedure	• Method not suitable for thermo labile materials
Microwave assisted extraction (MASE)	• Lower solvent consumption • Lower time of extraction • Increase in extraction performance • Method applicable for extraction of phenolic compounds and flavonoids only.	• Tannins and anthocyanins can degrade because of high temperature.
Ultrasound assisted extraction (UAE)	• Small sample size • Reduced extraction time • Reduced solvent volume • Higher extraction yield.	• Method reproducibility difficult. • High energy application can lead to degradation in case of phytochemical by producing free radical.
Accelerated solvent extraction (ASE)	• Reduced extraction time • Rapid and easy sample preparation • Reduced solvent volume • No solvent barrier (except strong acids, bases)	• High cost of equipment • Thus, not cost effective • Extraction not selective. • Extract volume not reproducible (say, in case 10 samples with same method used) [20].
Supercritical fluid extraction (SFE)	• Green technique • Allows the extraction of thermally labile compounds	• Expensive technique • High power consumption • Operation at elevated pressures.
Microextraction	• Lower solvent consumption • Quick, some techniques cost effective. • Effective at trace level analysis	• Adsorbents for techniques such as SPE, SPME costly.

1.2 Chromatographic determination

The determination of target analytes becomes a critical step, once they are extracted. Phytochemical screening is a complex work due to the presence of structurally similar compounds. Column chromatography is a technique that further helps in separating the components in the crude plant extracts and their identification can be performed by spectroscopic technique such as NMR, IR, MS [21]. Thin layer chromatography (TLC) is a very powerful technique both in one- and two- dimensional modes used in phytochemical detection. A huge number of saponins are determined in plant extracts by it [22]. Wang et al isolated anti-oxidative phenolic compounds from Sage (*Salvia Officinalis*) on a sephadex column and determined their spectral structures using NMR, IR and MS [23]. Lu et al identified two novel phenolic glycoside structures with ^{1}H and ^{13}C NMR and HPLC analysis [24]. Number of different separation techniques (TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC) are performed as typical practice in isolation of these phytochemicals to obtain pure compounds for the structure and biological activity determination [25]. The need of further separation arises due to the presence of mixture of structurally related forms in plants with very similar polarities, which remains a challenge. Glyphosphate (N-methyl phosphonomethyl glycine), a herbicide popular for its broad spectrum range, was determined using HPLC by the group of Nedelkoska and co-workers in both water and plant material after extraction and pre-column derivatisation with 9-fluorenylmethyl chloroformate [26]. In most of the studies for the determination of glyphosate in water Post-column derivatisation was used and was also recommended by the US Environmental Protection Agency (EPA) [26, 27].

Phenolics in plants have attracted great attention due to their anti-oxidative effect which promotes health. Five aromatic plants namely *Vitex agnus-castus (*Verbenaceae), *Origanum dictamnus* (Lamiaceae), *Teucriumpolium* (Lamiaceae), *Lavandula vera* (Lamiaceae) and *Lippia triphylla* (Verbenaceae), were examined by group of Proestos et al to determine their phenolic composition. The group quantified the phenolics in all these plants using RP-HPLC and identification of the phenolics was performed with GC-MS post silylation [28]. A group of Kiddle and co-workers have determined desulphoglucosinolates from *Brassica napus* L and related crops. Glucosinolates a class of compounds derived from amino acids. Intact glucosinolates and its associated hydrolytic products are known to be a part of general defence

mechanisms of crucifers against fungal infection and herbivores. The identities of these desulphoglucosinolates were validated by comparison of retention times with standards and by UV, ^{1}H- and ^{13}C-NMR and chemical ionisation MS analysis [29]. Luo et al investigated extraction and characterization by ^{1}H- NMR of sesquiterpene lactone isolated from *Vernonia amygdalina* leaves, a medicinal plant having anti-cancer activity [30]. Queiroz et al developed an on-line investigation of antifungal constituents from a plant used in the traditional medicine of Ivory Coast *Erythrina vogelii* by liquid chromatography with tandem MS, ultraviolet absorbance and NMR combined with liquid chromatographic micro-fractionantion [31].

Phytochemical analysis of the antioxidant ethanolic extract of *Alternanthera tenella Colla* led to the isolation of six flavonoids including vitexin, quercetin and kaempferol. Salvador and group established structures of all the Flavonoids by ESI-MS and NMR spectroscopic methods [32]. Zhang et al isolated and purified four flavone C-glycosides by ^{1}H- and ^{13}C- NMR, IR, UV MS from antioxidant of bamboo leaves by macroporous resin column chromatography and preparative high-performance liquid chromatography [33]. In the pursuit of discovery of new bioactive compounds from plant materials which could lead to new drugs, extracts have been subjected to chemical screening. Hyphenated techniques such as LC/UV, LC/MS and more recently LC/NMR are used to develop compound/structural profiles. These techniques provide plenty of structural information in a short time, leading to determination of the natural products of interest. Multiple hyphenation of several spectroscopic techniques in a single system was used for the identification of Ecdysteroids from crude extracts of Silene otites, Silene nutans and Silene frivaldiskiana investigated by a combination of spectrometers coupled with HPLC, which enables the on-flow collection of UV, ^{1}H NMR, IR, and MS spectra by group of Louden and co-workers [34, 35]. These hyphenated techniques have been used not only in determination of pollutants from a particular matrix but also in validating the chromatographic method used for the determination. For the first time Fiamegos et al reported Microwave-assisted phase-transfer catalysis (PTC) for the one-step extraction-derivatization pre-concentration and GC-MS determination of 20 phenols and 10 phenolic acids [36, 37]. For the determination of BPA and its chlorinated derivatives in complex sewage-sludge matrices Dorival-Garcia et al compared three extraction techniques (UAE, MAE and PLE) and evaluated their efficiency, detection of compounds and their quantification using LC-MS/MS [38]. Limam et al studied

biodegradability of phenol and bisphenol A using ^{13}C labeled contaminants during municipal waste anaerobic digestion [39].

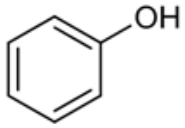
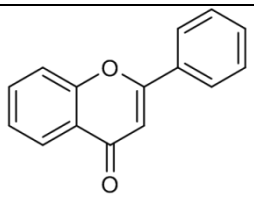
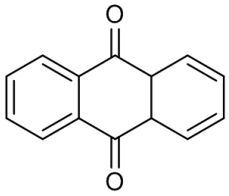
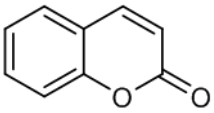
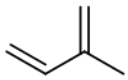
Therefore, it can be said that extraction and chromatographic determination are inter-related and go hand in hand, as identification of the analytes achieved after pre-treatment can be confirmed by these spectroscopic techniques.

1.3 Extraction in medicinal plants

Plants have been used since the evolution of mankind and were known to have beneficial, remedial and curative effects as a result of which they have application as medicine, in healing wounds or as tincture, decoction, etc. In short, plants that have medicinal properties are termed as medicinal plants. The efficacy of plants has been attributed to the biologically active ingredients present in them for healing effects. Such plants have been used since the primitive ages by humans to remediate themselves from many health issues. Turmeric (*Curcuma longa* L.), Tulsi (*Ocimum sanctum* L.), etc are some examples of plants that have been used as medicinal plants from the beginning of civilization and even now after so much development and advances made in medicinal and health industry. People are again heading back to the age old recipes and customs of using these plants and towards Ayurveda to find cure or remedies. With this aim, even in the early stages of development of mankind and now, extraction has been the source or tool for obtaining the active ingredients from the plants. Plants have a complex mixture of number of different classes of compounds. Extraction techniques help in the separation or enrichment of the medicinally active ingredient through the use of appropriate solvents. The extraction techniques used are based on the type of desired molecules that are extracted. This is based on the studies and advances made so far from the phytochemical studies. Phytochemical analyses, a branch that deals with the study of analysis of the chemicals or compounds that are extracted from plants through various extraction and chromatographic routes, their determination and then their characterization. Extraction techniques such as solvent extraction, Soxhlet extraction, MASE, UAE, etc extract or separate the soluble metabolites from the insoluble cellular matrix of plants to result in a mixture of compounds either in a paste (semi-solid) form, dry form or impure liquids after the removal of solvent [2]. It further needs to be subjected to chromatographic separation technique to purify and separate a particular analyte of interest.

A wide range of bioactive compounds like lipids, phytochemical, pharmaceutics, flavours, fragrances and pigments are present in plants, which are widely used in the food, pharmaceutical and cosmetics industries.

These secondary metabolites such as anthraquinones, flavonoids, phenols, coumarins, terpenes, saponins, contribute to providing beneficial properties to plants and ultimately serve in different applications. ***Secondary metabolites*** are substances produced as a result of metabolism (metabolic pathways) in organisms which do not have direct contribution in their growth and development but contribute by serving other requirements such as in defence mechanism. These compounds enable them to endure against the interspecies competition, defence mechanism and reproductive processes [40 and 41]. In addition to this, they also have biological properties that prove beneficial to organisms against diseases and serve as the basis of their application as medicines. The sources of secondary metabolites are mainly plants, fungi, bacteria, and marine organisms such as tunicates, corals, etc [40]. These secondary metabolites have been a source of research in drug discovery and are known to have biological properties such as antibacterial, anti-cancer, anti-parasitic, anti-biotic, anti-fungal and anti-viral and that is how the plants protect themselves against pathogens [42]. These compounds have specific roles and depending on the nature, characteristics; these compounds have applications such as medicines, ointments, dyes, pigments, fragrance, dietary supplements, nutrition, essential oils, skin and hair care products, etc. The primary natural source for these beneficial compounds is plants and the primary means of separation is the extraction technique.
Based on the chemical structure of these metabolites, they are categorized under different classes [42].

Class	*Basic Structural unit*	*Examples*
Phenolics		• Vanillin • Eugenol • Salicylic acid.
Flavonols		• Quercetin • Kaempferol • Hesperetin
Anthraquinone		• Alizarin • Purpurin • Rubiadin
Coumarin		• Aesculetin • Umbelliferone • Scopoletin
Terpenoid		• Linalool • Artemesinin
Alkaloids	N – containing class of compounds	• Morphine • Cocaine • Nicotine

Phenolics:

It is one of the major classes of secondary metabolites that is found in the highest concentrations in plants where they mainly contribute to aspects such as colour, taste and flavour of many herbs. They are found as simple phenolics with an aromatic ring or as long complex polymeric chain [42]. Phenolics such as quercetin are highly valued pharmacologically for anti-inflammatory property and anti-hepatotoxic property, for instance silybin. They also exhibit biological activities; act as free radicals scavengers and are effective antioxidants.

Anthraquinones:

Anthraquinones have anthracene as a core moiety. They are therefore referred to as 9, 10-dioxoanthracenes. Anthraquinones typically occur in their glycosidic forms. These have wide applications as dyes as they impart colour to plants and have been widely used as natural dyes [43]. The function is not just limited to colour but they also have anti-viral and anti-fungal effects and are used as laxative.

Flavonoids:

They are known to have biochemical and antioxidant effects associated with diseases such as Alzheimer's disease, cancer, atherosclerosis, etc [44 to 47].These are related with a wide spectrum of health-promoting effects and happen to be a requisite component in numerous applications of pharmaceutical, nutraceuticals, medicinal and cosmetic. This is mainly attributed to their anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme functions [47].

Alkaloids:

These are organic compounds with at least one nitrogen atom in the heterocyclic ring. The following fall under the basic types of alkaloids: acridones, aromatics, phenyl ethyl amines, imidazoles, indolizidines, indoles, bisindoles, oxindoles, quinolines, quinozolines, phenylisoquinolines, piperidines, purines, pyrrolidines, pyrrolizidines, pyrroloindoles, pyridines and simple tetrahydro isoquinolines [48]. Alkaloids exhibit a wide range of pharmacological activities including cardiac stimulation, analgesic, respiratory stimulation and relaxation, vasoconstriction, muscle relaxation and toxicity, as well as antineoplastic, hypertensive and hypotensive properties. These have toxic, cytotoxic activity, carcinogenic activity which has been reported in literature. As a defence mechanism they are also sufficiently toxic to animals to cause death if eaten [49, 50].

Terpenes:

The name"terpene" is derived from the word "turpentine," which is actually derived from the old Frenchter(e)bintb, which means "resin". They also fall under the largest group of plant secondary compounds. Structurally they have 5 -carbon isoprene units which assemble in different ways to produce different derivatives [50]. These are important plant metabolites as responsible for fragrance and thus serve as insect attractants [41]. Based on the number of isoprene units in the molecule, they are classified and a prefix in the name indicates the number of terpene units, which are as follows:

- Single isoprene unit – *Hemiterpenes*
- Double isoprene unit – *Monoterpenes*
- Three isoprene units – *Sesquiterpenes*
- Four isoprene units – *Diterpenes*

- Five isoprene units – *Sesterpenes*
- Six isoprene units – *Triterpenes*
- Eight isoprene units – *Carotenoids*

Coumarins:

These are derivatives of benzo-α-pyrone, the lactone of O-hydroxycinnamic acid [42]. They are reported to have biological activities such as anti-inflammatory, anticoagulant, anticancer and anti-Alzheimer's activity [51].

1.4. Microextraction

Various sample pre-treatment steps designed to extract the analytes of interest from complex matrices such as soil, sediment, water, beverages, food samples, cosmetics, industrial wastes etc. are based on microextraction. The basis of these extraction techniques are the conventional liquid liquid extraction (solvent extraction) and solid liquid extractions (figure 9). A major application of microextraction techniques is the determination of compounds that are found in trace amounts in biological matrices. These compounds contaminate the matrix and are found to be hazardous to the living system. These compounds are termed pollutants or contaminants. Pollutants or contaminants are a class of compounds that contaminate different biological matrices affecting the living systems by entering in the food chain or in the biological cycles. These can be organic or inorganic in nature.

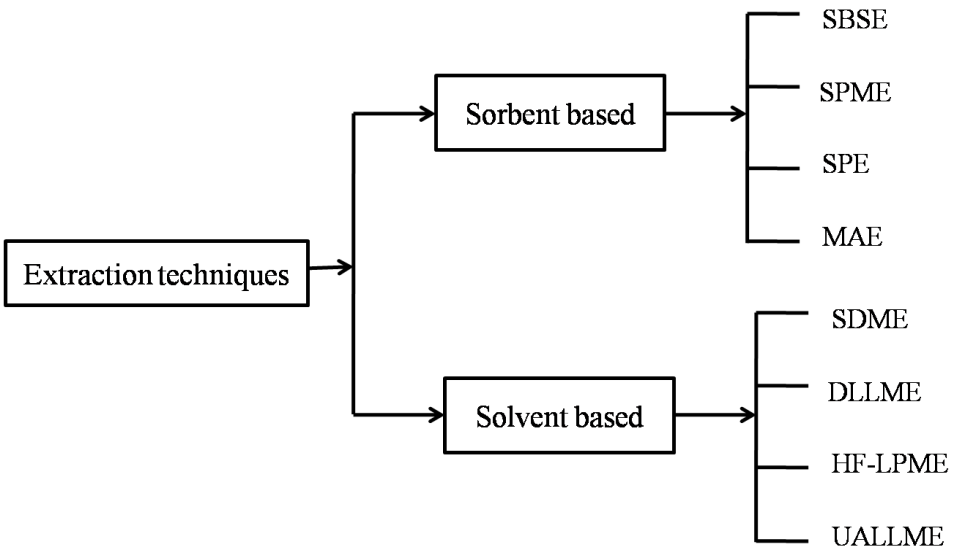


Figure 9 Extraction techniques

The methodology followed in trace analysis is to extract the analytes of interest from the real matrix by removing the interfering compounds by the use of various microextraction techniques (figure 10) and then perform their chromatographic analysis.

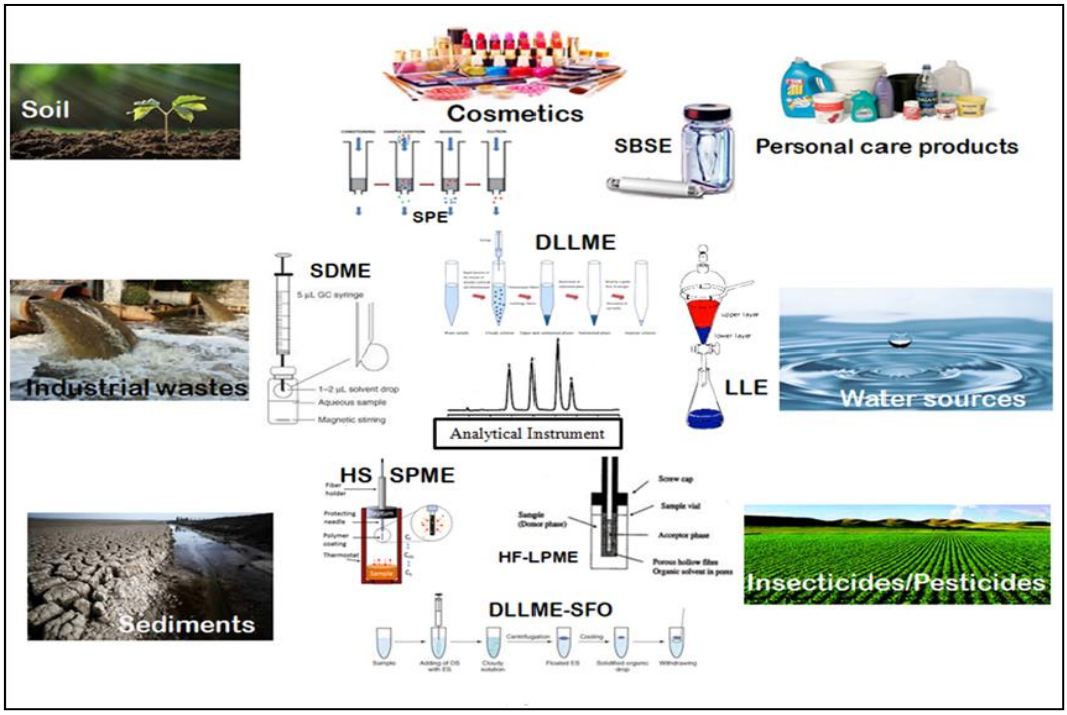


Figure 10 Methodology in trace analysis

Luke et al developed a method in 1975 where he used minimal clean up approach to extract the targets with acetone as extraction solvent [52]. Liquid liquid extraction (LLE) also called as partitioning and solvent extraction was derived from the Luke's method [52]. The method has been used as cleanup procedure for sample [52]. The method is applicable to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent [52]. The microextraction techniques DLLME, SDME, etc as shown in figure 10 have been developed based on the conventional technique of solvent extraction (liquid liquid extraction). These are categorized under the category of liquid phase microextraction (LPME). Liquid phase microextraction developed during the mid 1990's. Jeannot and Cantwell initiated experiments for using very low volumes of extraction solvent in the range of few micro litres [53, 54]. From here onwards, development has been made in LPME and thus evolved hollow fibre – liquid phase microextraction. These techniques use few micro litres of water – immiscible extraction solvent and achieve higher extraction recoveries with high enrichment factors.

➢ ***Dispersive liquid liquid microextraction:*** A liquid phase microextraction technique developed by Rezaee et al in 2006 [55]. It is a ternary solvent mixture system wherein a mixture of an extraction solvent and disperser solvent is introduced rapidly in an aqueous sample containing the target analytes and then centrifuged to collect the extraction solvent, which is then introduced in the chromatographic system (GC, HPLC). Farajzadeh et al reported use of an elevated temperature DLLME for the determination of phthalate esters at trace levels from heated aqueous samples using DMSO as disperser solvent and 1, 2 – dibromoethane as extraction solvent achieving extraction recoveries up to 57 – 98 % [56]. The technique has been used to extract analytes such as organophosphorous pesticides [57], phthalate esters [58, 59], trihalomethanes [60], chlorobenzene [61], flame retardants [62], pyrethroid pesticides [63], chlorophenols [64], carbamate pesticides [65] and polychlorinated biphenyls [66]. Zeng et al determined chlorinated paraffins (CPs) a class of industrial chemicals produced by chlorination of n-alkane feedstock with a carbon chain of up to 10 to 30 carbons. These are mainly used as additives in cutting oils, secondary plasticizers, lubricants and flame retardants in polymeric materials for their low cost, electric insulation and fire resistance [67]. Zhou et al have determined chlorinated paraffin's from wine using DLLME coupled with high performance liquid chromatography [68]. The method has been applicable not only for the determination of organic pollutants [69, 70] but also for inorganic pollutants such as metals like lead [71], cadmium [72], palladium [73], copper and zinc [74]. The technique has been applied to determine pollutants from different matrices such as chloramphenicol in honey [75], volatile phenols in honey [76, 77] and phthalate esters in cow milk [78]. Phthalates heave been determined in water by using salt assisted DLLME [79], from beverages [80], in soybean milk [81], in wine using ultrasound-vortex- assisted DLLME [82], in bottled milk [83], in urine samples [84]. Phthalates are also determined in alcoholic beverages using ionic liquid based dispersive liquid liquid microextraction by Y Fan and co-workers [85].

➢ ***Single drop microextraction:*** In this technique, a single drop of extraction solvent is used, which is suspended through a micro syringe into the aqueous sample containing the target analytes. And after the extraction equilibrium is achieved, the extraction solvent is retracted in the syringe and introduced in the chromatographic system.

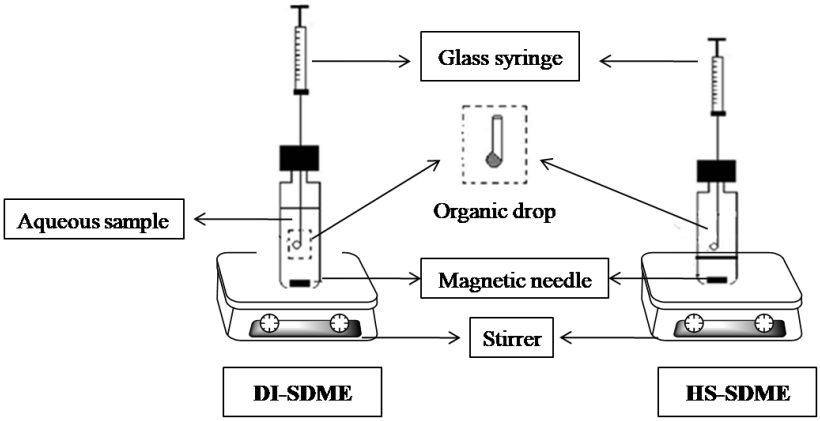


Figure 11 Experimental set up for Single drop microextraction

Dasgupta and co-workers in 1996 reported a drop-in-drop system to extract sodium dodecyl sulphate using a water-immiscible organic solvent micro drop of 1.3 µL immersed into a large flowing aqueous drop to achieve the extraction procedure [86]. During the same period, Jeannot and group introduced a procedure which they termed as solvent microextraction. In this process, they used 8 µL of 1-octanol as an extraction solvent held at the end of a Teflon rod and suspended in the aqueous sample for extraction [87]. After specific time, the Teflon rod was withdrawn from the aqueous solution; the organic phase was sampled with a micro syringe and injected into a GC system for analysis. But this served as a limitation for the process as the extraction and injection had to be performed one by one and using different apparatus. To overcome this limitation, Jeannot and co-workers developed the method using micro syringe instead of Teflon rod. Thus, a single micro syringe was used for both the extraction step and the injection step [88]. The microextraction technique has been used for the extraction of poly cyclic aromatic hydrocarbons from aqueous sample using 1-butanol as extraction solvent [89], phthalate esters [90, 91], organophosphorous pesticides from farm water using 0.9 µL of CCl_4 as extraction solvent [92], warfare agents [93], α and β – endosulfan in water [94]. Saraji and co-workers reported determination of phenols in water by single drop microextraction followed by in-syringe derivatization [95].

- ***Ultrasound assisted liquid liquid microextraction (UA-LLME):*** This technique involves the use of ultrasound for extraction of target analytes from an aqueous sample. Valproic acid (VPA), an anticonvulsant drug used for the treatment of epilepsy and bipolar disorders, was determined by this technique by the group of Rajeev Jain and co-workers [96]. Fluoroquinolines in pharmaceutical waste water [97], pyrethroids [98], benzodiazepines – class depressant drugs determined by Fernandez and co-workers using DLLME [99].
- ***Cloud point extraction (CPE):*** It is an inexpensive, fast, selective, accurate and precise procedure for pre-concentration. It offers many benefits over the traditional liquid-liquid extraction. An eco-friendly (green) extraction technique as it consumes no or minimum amount of toxic organic solvents. In 1976 the technique was first applied by Watanabe and co-workers, who extracted Ni^{2+} from aqueous solutions after complexation with 1-(2-Thiazolylazo)-2-naphthol (TAN) and Triton X-100 as a micelle-mediated extracting agent [100]. The CPE technique is based on the property of non-ionic surfactant which upon heating above definite temperature forms micelles in aqueous media (called as cloud point or cloud temperature) or by adding salt (salting-out phenomenon) [101].The separation is possible by centrifugation into two phases (an aqueous phase and a surfactant rich one). And the analyte is usually separated in the surfactant-rich phase. The technique has been used in pre-concentration of metal ions and other organic pollutants like persistent organic pollutants (POPs) [102], pesticides [103] fluoroquinolone antimicrobial agents [104].
- ***Hollow fibre liquid phase microextraction (HF-LPME)***: The technique was developed by Pedersen-Bjergaard and Rasmussen in 1999 [105] based on the use of a disposable and cost effective hollow fibre [106]. The extraction solvent is contained within the lumen of a porous hollow fibre and is not in direct contact with the sample solution. The analytes are extracted from the aqueous sample, through the organic phase in the pores of the hollow fibre, and further into an acceptor solution inside the lumen of the hollow fibre (figure 12).

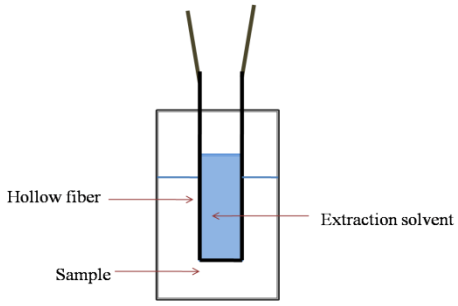


Figure 12 Experimental set up of HF-LPME

The technique has been utilized for the quantitative analysis of environmental and bio-logical samples from very complex matrices [107, 108] and also of organic pollutants [109] such as phthalate esters [110, 111], alkylphenols, chlorophenols [112] and bisphenol A [113]. Depending on the type of physicochemical characteristics of the desired analytes and levels of complexity of the sample (like its matrix), HF-LPME can be performed in a two- or three-phase mode. Different types of arrangements for HF-LPME have been reported in literature such as rod like, U - shaped, hollow-fiber solvent bar and knotted hollow-fiber [114].

- ***Solid phase microextraction (SPME):*** It is a simple, rapid extraction technique developed by Pawliszyn and co-worker in the year 1990, figure 13 [115, 116].

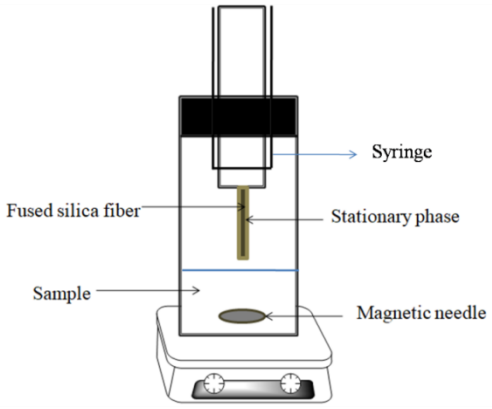


Figure 13 Schematic presentation of SPME

The method is a solvent free technique based on the equilibrium sorption of analytes onto a small microfiber, which is made of a fused-silica optical fibre, coated with a stationary phase (hydrophobic polymer). Analytes from either air (Headspace – SPME) or in a water sample (Direct immersion – SPME) gets adsorbed on the solid stationary phase based on its affinity [117].

- ***Solid Phase extraction (SPE):*** SPE was developed as an alternative approach to LLE in the mid-1970 [52]. The method was developed for separation, purification, pre-concentration and solvent exchange of solutes for solution [52, 118].The

technique is similar to that of column chromatography wherein a solid adsorbent (stationary phase) is packed in a column, which is activated and aqueous sample containing the target analytes is passed through the packed column, then dried under vacuum and eluted with an extraction solvent. The technique has been applied to organic compounds [119] such as polycyclic aromatic hydrocarbons [120, 121 and 122], organochlorine pesticides [123], trace elements [124, 125] and priority pesticides in ground and water samples [126, 127] in honey samples [128], from fruits and vegetables [129]. SPE was initially thought of as a replacement for the conventional liquid liquid extraction with benefits of shorter processing times, lower solvent consumption [130]. M D Carlo et al determined endocrine disrupting chemicals - phthalate esters in wine using SPE – GC-MS [131], to determine phthalate esters in packaged foods magnetic micro-solid phase was developed by Makkliang et al [132] and in aquatic environment by Fatoki et al [133].

- ***Stir bar sorptive extraction (SBSE):*** A stir bar coated with adsorbent is stirred in an aqueous sample. The analytes gets adsorbed on the surface of the stir bar which is then subjected for thermal desorption. The major features of the technique includes:

a. High inertness and thermal stability

b. High enrichment factors for polar and reactive analytes

c. Degradation products of PDMS (polydimethyl siloxane) are low in intensity and easily detectable from target analytes by mass spectrometry [134].

Organic pollutants such as phthalate esters have been determined in PVC bag by stir bar sorptive extraction [135]. Simultaneous determination of polycyclic aromatic hydrocarbon, phthalate esters, nonyl phenols in water was performed by group of Prieto and co-workers [136], organic pollutants in water samples [137], organophosphorous pesticides have been determined in potato and cucumber by a group of Wenmin Lu and co-workers [138].

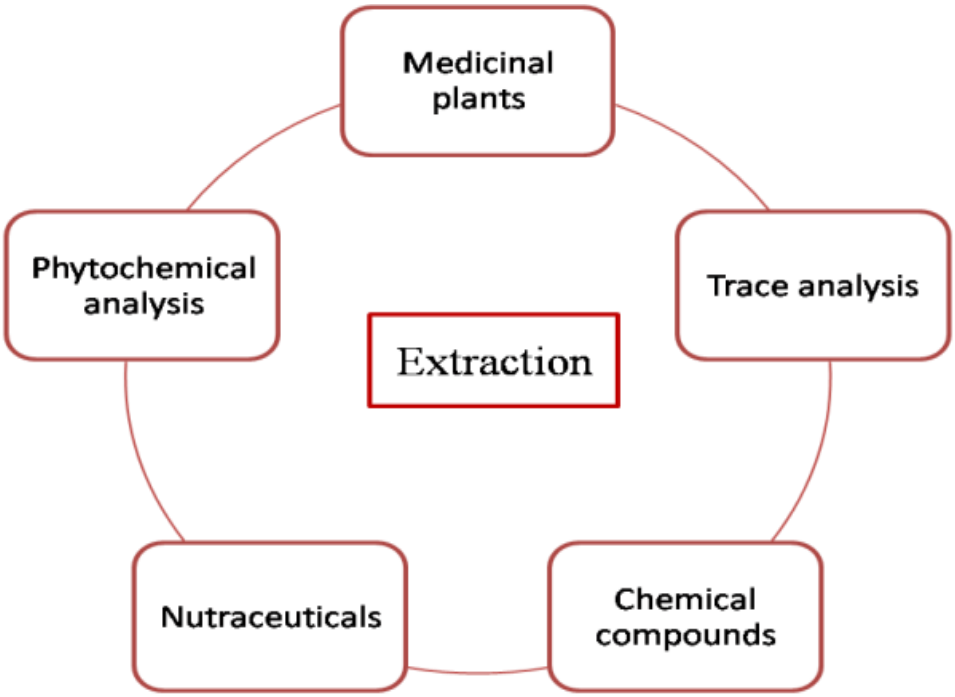
The selection of the technique depends on the type of analytes targeted and also the matrix from which the analytes are to be determined. These techniques are highly applicable as compared to the conventional extraction techniques as these require solvent in just few micro litres (lower extraction solvent volume), equilibrium is achieved faster, higher extraction recoveries and enrichment factors achieved user friendly experimental set ups, lower extraction time required. These techniques are useful in determining the analytes at trace levels, which is not always possible with the conventional techniques.

1.5 Organic pollutants

Organic compounds have application in our day to day life in areas from domestic or household supplies to personal care products, packaging, cosmetics, etc. With the development of human civilization, their application and demand has increased tremendously. These have thus found a way to enter the biological and life cycles as they leach out or disperse in the environment. Organic pollutants are a class of compounds that are a major concern to all the natural resources available on the earth and to the environment, polluting these bio-resources as these are resilient to environmental degradation through chemical, photolytic or biological processes. As it is said, anything in excess is always hazardous and so is the case with chemicals which have tremendous applications and have made lives easy but at the end of the day, contaminated the environment. The most common instance is that of plastic (polyethylene) bags, which do not decompose but pollute the environment extremely. Different classes of compounds fall under this category such as phthalate esters, chlorophenols, bisphenol A, etc. The discharge from industries in the form of effluents, wastes generated from households and small scale industries, etc are the cause and sources for pollutants to contaminate the environment and thus, enter the biological cycles [139]. Phthalate esters, phenols, substituted phenols, etc are considered as priority pollutants. These are of commercial importance and used extensively as insecticides, fungicides, pharmaceuticals, intermediate in chemical synthesis. These can form on degradation of chlorinated pesticides. These are toxic to humans at very lower concentrations [140].

1.6 Summary

It can be summarized that extraction plays a vital role in different areas of science, which has undergone number of modifications to extend its application.



2. Aim and outline of thesis

Medicinal plants are known to have beneficial effects and have been used in Ayurveda since ages. Organic pollutants pollute the environment and are found to be hazardous to human beings. Extraction techniques can be useful in separation of chemical constituents from medicinal plants and also in determination of organic pollutants at trace levels. Thus, we aim at developing chromatographic technique for the determination of the chemical constituents from medicinal plant – *Rubia cordifolia* L. and determining and comparing the microextraction technique for the determination of organic pollutant – phthalate ester in water. We have used microextraction for determination of pollutants, while preparative extraction only for extractive crude from plant material.

Chapter 1 discusses the significance and the developments made in extraction techniques over the years. **Chapter 2a** is focused on the development of mixed stationary phase for separation of chemicals from medicinal plants. **Chapter 2b** encompasses the determination of elemental composition and characterization in the medicinal plant with the help of a pre-concentration step – ashing. **Chapter 3** focuses on determining organic pollutants – phthalate esters and comparing the microextraction techniques.

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