Vidya K et al. / Vol 4 / Issue6 / 2014 /369-381.

International Journal of Pharmaceutical Research & Analysis

e-ISSN: 2249 – 7781
Print ISSN: 2249 – 779X

www.ijpra.com

A REVIEW ON CURRENT TRENDS AND ADVANCEMENTS IN BIOANALYTICAL METHODS

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ABSTRACT

This paper reviews the recent developments in bio-analysis sample preparation techniques and chromatographic techniques give an update on basic principles and a comparative discussion on the benefits and limitation of every technique. Any bio-analytical technique includes several steps, all of them being vital in order to attain reliable results. The first step is taking aliquots of samples for the analysis, followed by the extraction procedure and sample clean-up, chromatographic analysis and detection. Conventional sample preparation techniques including solid phase extraction (SPE), liquid-liquid extraction (LLE), protein precipitation (PP) and many modern approaches including molecularly imprinted polymers (MIP), solid phase micro extraction (SPME), liquid-liquid micro extraction (LLME), micro extraction by packed sorbent (MEPS) and plenty of others have additionally been featured as fundamental and significant step of bio-analytical methods. Current trends in fast liquid chromatographic separations involve monolith technology, high temperature liquid chromatography (HTLC) and ultra-high performance liquid chromatography (UHPLC). UHPLC has recently become a wide-spread analytical technique in many laboratories which focus on fast and sensitive bio-analytical assays. The key advantages of UHPLC are the increased speed of analysis, higher separation efficiency and resolution, higher sensitivity and much lower solvent consumption as compared to other analytical approaches. This is all enabled by specially designed instruments and sub-2-microne particle packed analytical columns.

Keywords: Bio-analytical, Solid phase extraction (SPE), Liquid liquid extraction (LLE), Protein precipitation (PP), Micro dialysis, Micro extraction, Molecularly imprinted polymer (MIP), Micro extraction in packed syringe (MEPS), Monolith, HILIC, UHPLC.

INTRODUCTION

Bio-analytical Methods are widely employed for the Quantitative analysis of the drugs and their metabolites in the biological matrix or media like saliva, urine, plasma, serum, etc also plays a significant role in the evaluation of bioavailability, bioequivalence, pharmacokinetics studies [1]. The purpose of sample preparation is to clean up the sample before analysis and/or to concentrate the sample. Material in biological samples which will interfere with analysis, the chromatographic column or the detector includes proteins, salts, endogenous macromolecules, small molecules and metabolic byproducts. A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system [2]. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase micro-extraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME). MEPS are a new technique and represent an attempt of SPE miniaturization using much reduced sorbent. Separation efficiency and speed of analysis may also be increased using superficially porous particles, often termed also fused core columns. The use of sub-2 micron particles in ultra high pressure systems presents another option in performing rapid analyses. HILIC is an alternative to conventional RP-HPLC (reversed phase HPLC) or NP-HPLC (normal phase

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HPLC). HILIC has proven quite convenient for the analysis of small polar molecule, weakly retained or eluted with dead volume in conventional RP-HPLC systems [3].

Sample collection and sample preparation

The biological media that contain the analyte are usually blood, plasma, urine, serum etc. Blood is usually collected from human subjects by vein puncture with a hypodermic syringe up to 5 to 7 ml (depending on the assay sensitivity and the total number of samples taken for a study being performed) into tubes with an anticoagulant, e.g. EDTA, heparin etc. Plasma is obtained by centrifugation at 4000 rpm for 15 min [2].

ADVANCES AND TRENDS IN SAMPLE PREPARATION TECHNIQUES

CONVENTIONAL SAMPLE PREPARATION TECHNIQUES

LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction is based on the principles of differential solubility and partitioning equilibrium of analyte molecules between aqueous (the original sample) and the organic phases [2].Extraction of analyte occurs from aqueous phase into organic phase. After extraction of analyte the organic layer is separated from aqueous phase and it is evaporated in presence of nitrogen gas to get the dry form of sample. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase micro-extraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME) [4]. Extraction efficiency can be improved by solvent choice, solvent volume, pH, and by using the salting-out effect.

The salting-out effect involves the addition of an inorganic salt used to enhance the activity coefficients of volatile components in aqueous solutions, thereby increasing the concentration in the organic layer. The salting-out effect is depends upon the analyte and type of salt applied. The higher is the number of carbon atoms in a compound the greater is that the salting-out effect. This results from the lower solubility of the high-molecular-weight compounds in water [5].

Advantages

- Clean extracts obtained.
- Greater sample capacity.
- The technique is simple, rapid and has relatively less cost per sample.

Limitations

- Sometimes pH control of the sample necessary for extraction.
- During evaporation, since the temperature is increased, the method cannot be used for thermo labile substances [4].

SOLID-PHASE EXTRACTION

SPE is a selective technique for sample preparation where the analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Due to many various choices of sorbents, SPE is a very powerful technique. SPE consists of 4 steps; conditioning, sample loading, washing and elution.

Conditioning

The column is activated with an organic solvent that acts as a wetting agent on the packing material and solvates the functional groups of the sorbent. Water or aqueous buffer is added to activate the column for proper adsorption mechanisms.

Sample loading

Once adjustment of pH, the sample is loaded on the column by gravity feed, pumping or aspirating by vacuum.

Washing

Interferences from the matrix are removed while retaining the analyte.

Elution

Disruption of analyte-sorbent interaction by appropriate solvent, removing as little of the remaining interferences as possible.

Typically, sorbents used in SPE consists of 40μm diameter silica gel with approximately 60 Aº pore diameters. To the silica gel, functional groups are chemically bonded, for different modes of action. The most usually used format is a syringe barrel that contains a 20μm frit at the bottom of the syringe with the sorbent material and another frit on top, referred to as packed columns. Extraction disks are also placed in syringe barrels. These disks include 8–12μm particles of packing material imbedded into an inert matrix. Disks are conditioned and used in the same way as packed columns. The foremost advantage of disks compared to packed columns is that higher flow rates can be applied. Analytes can be classified into four categories; basic, acid, neutral and amphoteric compounds. Amphoteric analytes have each basic and acidic functional groups and can therefore function as cations, anions or zwitterions, depending on pH [2].

Advantages

- Low concentration of drug can be detected.
- Effective in selective removal of interferences.
- Different varieties of adsorbents can be used [4].
- SPE provides greater reproducibility as compared to other techniques.
- High recovery of the analyte.

- Sample process of about 40-50 matrix samples in a batch can be processed with an inexpensive vacuum manifold [6].

Limitations

- Extraction is troublesome for high-density materials.
- In extraction methodes a number of steps are to be carried out making it a time consuming process [4].

PROTEIN PRECIPITATION

Protein precipitation is commonly used in routine analysis to remove proteins. Precipitation may be induced by the addition of an organic modifier, a salt or by changing the pH that influence the solubility of the proteins. The samples are centrifuged and the supernatant can be injected into the LC system or be evaporated to dryness and thereafter dissolved in a suitable solvent. A concentration of the sample is then achieved. The samples typically contain protein residues and it's a non-selective sample cleanup technique, there is a risk that endogenous compounds or other drugs might interfere in the LC-system, but the protein precipitation technique is often combined with SPE to provide clean extract.

Methanol is most usually preferred solvent amongst the organic solvent because it can produce clear supernatant that is suitable for direct injection into LC-MS/MS. Salts are other alternatives to acid and organic solvent precipitation. This method is termed as salt-induced precipitation, because the salt concentration of a solution is increased, proteins aggregate and precipitate from the solution [2].

Advantages

- It's less time consuming.
- Smaller amounts of organic modifier or other solvents are use.
- This technique may be applied for extraction of each hydrophobic and hydrophilic substance.

Limitations

- Protein precipitation might clog the column [4].

MICRODIALYSIS

Micro dialysis is a minimally-invasive sampling technique that's used for continuous measuring of free, unbound analyte concentrations in the extracellular fluid of nearly any tissue. Analytes may include endogenous molecules (e.g. neurotransmitter, hormones, glucose, etc.) to assess their biochemical functions within the body, or exogenous compounds (e.g. pharmaceuticals) to determine their distribution inside the body. The micro dialysis technique needs the insertion of a small micro dialysis catheter (also referred to as micro dialysis probe) into the tissue of interest. The micro dialysis probe is meant to mimic a blood capillary and consists of a shaft with a semi permeable hollow fiber membrane at its tip that is connected to inlet and outlet tubing. The probe is continuously perfused with an aqueous solution (perfusate) that closely resembles the (ionic) composition of the surrounding tissue fluid at a low flow rate of approximately 0.1-5µL/min. Once inserted into the tissue or (body) fluid of interest, small solutes will cross the semi permeable membrane by passive diffusion. The direction of the analyte flow is decided by the respective concentration gradient and permits the usage of micro dialysis probes as sampling as well as delivery tools [7]. The solution leaving the probe (dialysate) is collected at certain time intervals for analysis.

Micro dialysis probes

There are a variety of probes with different membrane and shaft length combinations available. The molecular weight cutoff of commercially available micro dialysis probes covers a wide range of approximately 6-100kD, but also 1MD is available. While water soluble compounds generally diffuse freely across the micro dialysis membrane, the situation is not as clear for highly lipophilic analytes, where both successful (e.g. corticosteroids) and unsuccessful micro dialysis experiments (e.g. etsradiol, fusidic acid) are reported (Stahl M et al., 2002). However, the recovery of water soluble compounds usually decreases rapidly if the molecular weight of the analyte exceeds 25% of the membrane's molecular weight cutoff.

Advantages

1. To date, micro dialysis is that the only sampling technique that can continuously monitor drug or metabolite concentrations in the extracellular fluid of nearly any tissue. Depending on the exact application, analyte concentrations can be monitored over several hours, days, or even weeks. Combination of micro dialysis with trendy imaging techniques, such positron emission tomography, further allow for determination of intracellular concentrations.

2. Exchange of analyte across the semi permeable membrane and constant replacement of the sampling fluid with fresh perfusate prevents drainage of fluid from the sampling site that permits sampling without fluid loss. Micro dialysis will consequently be used without disturbing the tissue conditions by local fluid loss or pressure artifacts, which can occur when using other techniques, like microinjection or push-pull perfusion.

3. The semi permeable membrane prevents cells, cellular debris, and proteins from entering into the dialysate. Due to the lack of protein in the dialysate, a sample clean-up prior to analysis is not required and enzymatic degradation is not a concern.

Limitations

1) Despite scientific advances in making micro dialysis probes smaller and more efficient, the invasive nature of this method still poses some practical and moral limitations. For example, it has been shown that implantation of a micro dialysis probe will alter tissue morphology leading to disturbed microcirculation, rate of metabolism or integrity of physiological barriers, like the blood–brain barrier. Whereas acute reactions to probe insertion, like implantation traumas, needs sufficient recovery time, additional factors, such as necrosis, inflammatory responses, or wound healing processes need to be taken into consideration for long-term sampling as they may influence the experimental outcome. From a practical perspective, it's been suggested to perform micro dialysis experiments within an optimum time window, usually 24–48 hours after probe insertion [8-9].

2) Micro dialysis has a relatively low temporal and spatial resolution compared to, for example, electro chemical biosensors. Whereas the temporal resolution is determined by the length of the sampling intervals (usually a few minutes), the spatial resolution is determined by the dimensions of the probe. The probe size will vary between totally different areas of application and covers a range of a few millimeters (intra cerebral application) up to a few centimeters (subcutaneous application) in length and a few hundred micrometers in diameter**.**

MODERN APPROACHES

MICRO EXTRACTION IN PACKED SYRINGE

MEPS are a new technique and represent an attempt of SPE miniaturization using much reduced sorbent. It's based on using a syringe with a special needle as an extraction device, sample preparation takes place on the packed bed which can be packed or coated to produce selective sampling. In MEPS the sorbent is inserted directly into the needle. The MEPS sorbent cartridge can be used for over 100 injections depending on the sample matrix. Another vital feature is that MEPS can handle sample volumes ranging from 10µL and up to 1000µL. MEPS can be used both manually and fully automated (the sampling, extraction and injection are on-line). The biological fluid is diluted before sample loading. For plasma the dilution factor is usually 5, whereas for whole blood it's typically 25. Thereafter, the sample is loaded by withdrawing and ejecting in the sample vial or ejecting to the waste. The loading step can be repeated many times to increase recovery of the analyte. Step two is the washing of the sorbent, to remove proteins and other unwanted material. The third step is the elution with organic solvent directly to the LC or GC injector, step four and step five is washing A and B and takes place after elution to eliminate the carry-over and conditioning the material for further use. Wash A is usually the elution solution and wash B is the washing solution used after sample loading, for a schematic presentation of the MEPS procedure.

Application of MEPS

This technique has been successfully used to extract wide range of drugs and their metabolites from different biological matrices like plasma, urine and blood. Other applications are investigated like polycyclic aromatic hydrocarbons in water, wine and hair. MEPS have been connected on-line to LC and GC mass spectrometry. The technique will replace most existing SPE methods by scaling the reagents and sample volume.

Advantages

- Reduce the time to prepare and inject samples from hours to minutes, eliminate all further steps between sample preparation and sample injection.
- Reduce buffer and solvent volume from Milliliters to Micro liters, reduce the sample volume required to as little as 3.6 Ml.

Disadvantages

- Recovery from MEPS can be affected by various factors, primarily the used sorbent and the influence of number of extraction cycles.
- Carry-over is an expected problem when using MEPS [10].

SOLID PHASE MICRO EXTRACTION

SPME was the first successful modern micro extraction technique. It's now commonly used for the analysis of trace components in a wide variety of matrices. The SPME technique uses a small polymer-coated fiber that is placed in solution or in the headspace of a sample for a period of time. Analytes are diffused to the surface of the polymer coating till equilibrium is achieved. The types of compounds which can be analyzed by this technique range from non-polar to semi-polar materials due to the different fiber materials available. Most applications favor headspace over immersion sampling, therefore the analytes should be sufficiently volatile to partition into the headspace of the vial being sampled. This extraction technique has become a standard technique, with fully automated systems available. In a similar way to SPE, there are two basic types of sorption process for the fibers: adsorbent-type fibers or absorbent-type fibers.

Once withdrawn from the sample source, in the case of gas chromatography (GC), the fiber is placed into the hot injection port and sorbedanalytes are thermally desorbed into the GC column to be separated and detected. This is the most common approach. Alternatively, HPLC can be employed. In this case, the analytes need to be solvent-extracted (or stripped) from the polymer coating, which can prove to be a much slower process than thermal desorption. One of the main drawbacks of SPME is

attaining accuracy and reproducibility. The following factors can affect recovery: vial size and selection, headspace versus direct sampling, salt addition, pH adjustment, stirring or agitation, fiber selection, extraction time and desorption conditions.

However, automation of SPME fiber addition to the sample and other steps can help with sample throughput, precision and accuracy [5].

Advantages

- No use of solvents.
- Easy handling and tiny equipment necessary.
- Fast method and easy automation.
- Good linearity and high sensitivity.

Disadvantages

- Extraction is very slow in contrast to LLE and SPE with bed columns.
- The recovery was very low, and the occurrence of a huge number of interferences in the chromatogram which come from endogenous trace substances in biological fluids prevents the analysis of target analyte at low concentrations [10].

MOLECULARLY IMPRINTED POLYMER

Molecular Imprinting Technology (MIT) is now a days a viable synthetic approach to design robust molecular recognition materials able to mimic natural recognition entities, like antibodies and biological receptors.

MIT is considered a versatile and promising technique which is able to recognize both biological and chemical molecules including amino acids and proteins, nucleotide derivatives, pollutants, drugs and food. Further, application areas include: separation sciences and purification, chemical sensors, catalysis, drug delivery, biological antibodies and receptors system.

MIT is based on the formation of a complex between an analyte (template) and a functional monomer. In the presence of a large excess of a cross-linking agent, a three-dimensional polymer network is formed. After polymerization process, the template is removed from the polymer leaving specific recognition sites complementary in shape, size and chemical functionality to the template molecule. Usually, intermolecular interactions like hydrogen bonds, dipole–dipole and ionic interactions between the template molecule and functional groups present in the polymer matrix drive the molecular recognition phenomena. Thus, the resultant polymer recognizes and binds selectively only the template molecules [11].

Synthesis of MIP

MIPs can be synthesized following three different imprinting approaches as follows:

1. The non-covalent procedure is the most widely used because it is relatively simple experimentally and the complexation step during the synthesis is achieved by mixing the template with an appropriate functional monomer, or monomers, in a suitable porogen (solvent). After synthesis, the template is removed from the resultant polymer simply by washing it with a solvent or a mixture of solvents. Then, the rebinding step of the template by the MIP exploits non-covalent interactions.

2. The covalent protocol, which requires the formation of covalent bonds between the template and the functional monomer prior to polymerization. To remove the template from the polymer matrix after synthesis, it is necessary to cleave the covalent bonds. To this end, the polymer is then refluxed in a Soxhlet extraction or treated with reagents in solution.

3. The semi-covalent approach is a hybrid of the two previous methods. Thus, covalent bonds are established between the template and the functional monomers before polymerization, while, once the template has been removed from the polymer matrix, the subsequent re-binding of the analyte to the MIP exploits non-covalent interactions, as the non-covalent imprinting protocol [12].

Advantages

- Polymerization simplicity and universality, large scale possible.
- One-step, in-situ preparation, cost-efficient.

Limitations

- Tedious procedures of grinding, sieving, and column packing.
- Phase partitioning complicates system.
- Large amounts of template are needed, high dilution factor.
- Complicated system, time consuming.
- Extensive optimization required for each new template system [13].

LIQUID-PHASE MICROEXTRACTION

LPME is a solvent -minimized sample-pretreatment procedure of LLE, in which only several lL of solvent are required to concentrate analytes from various samples rather than hundreds of mL needed in traditional LLE. It is compatible with capillary gas chromatography (GC), capillary electrophoresis (CE) and HPLC.

In LPME, extraction normally takes place into a small amount of a water-immiscible solvent (acceptor phase) from an aqueous sample containing analytes (donor phase). It can be divided into three main categories:

(1) Single-drop micro extraction (SDME)
(2) Dispersive liquid–liquid micro extraction (DLLME)
(3) Hollow-fiber micro extraction (HF-LPME)

SINGLE-DROP MICRO EXTRACTION

SDME, using typically 1–3lL of an organic solvent at the tip of a micro syringe, has evolved from LPME. After extraction, the micro drop is retracted back into the syringe and transferred for further analysis.

In practice, two main approaches can be used to perform SDME

(1) Direct immersion (DI)-SDME

(2) Headspace (HS)-SDME

(1) In DI-SDME, a drop of a water-immiscible organic solvent is suspended directly from the tip of a micro syringe needle immersed in the aqueous sample.

Applications of DI-SDME are normally restricted to medium polarity, non-polar analytes and those whose polarities can be reduced before the extraction.

Advantages

- Fast agitation of the sample can be employed to enhance extraction efficiency, because agitation permits continuous exposure of the extraction surface to fresh aqueous sample and reduces the thickness of the static layer.

Disadvantage

- The DI-SDME is the instability of the droplet at high stirring speeds.

(2) In HS-SDME, a micro drop of appropriate solvent is placed in the headspace of the sample solution or in a flowing air sample stream to extract volatile or semi volatile analytes. In this mode the analytes are distributed among 3 phases (i.e., the water sample, the headspace and organic drop). HS-SDME has similar capabilities in terms of precision and speed of analysis to DI-SDME but has the advantage of a wider variety of solvents to choose from. Unlike in DI-SDME, water can be also used as the solvent in HSSDME to extract volatile and water-soluble analytes. This significantly enhances the range of extractable analytes as well as the range of analytical methods that can be coupled to SDME. In addition, HS-SDME is found to provide excellent clean up for samples with complicated matrices.

Advantages

- The selection of the extractant for HS-SDME is very flexible and its solubility in the sample solution need not be considered.

Disadvantages

- The solvent is that its vapor pressure must be low enough to avoid evaporation during sampling but, at the same time, it must be compatible with GC analysis; furthermore, when aqueous samples have to be analyzed, if the solvent is miscible with water, the drop size may increase, causing the drop to fall from the needle.

Dispersive Liquid–liquid Micro extraction

Recently Assadi and co-workers reported DLLME as a new LLE technique that uses lL volumes of extraction solvent along with a few mL of dispersive solvents. In this method, a cloudy solution is formed when an appropriate mixture of extraction and dispersive solvents is injected into an aqueous sample containing the analytes of interest. The hydrophobic solutes are enriched in the extraction solvent, which is dispersed into the bulk aqueous solution. After centrifugation, determination of the analytes in the settled phase can be performed by conventional analytical techniques.

In DLLME, the dispersive solvent plays a key role that helps extraction solvent form fine droplets in aqueous samples, representing about 97–99% of the total volume of the extraction mixture. Compared to other methods, abundant surface contact between fine droplets and the analyte in DLLME speeds up the mass-transfer processes of analytes from aquatic phase to organic phase, which not only greatly enhances extraction efficiency but also overcomes the problem of the time taken.For low toxicity and low cost, acetone, methanol, ethanol and acetonitrile have generally been used as dispersive solvents. However, they could apply some extraction solvents to form a constant, large volume of sediment when the dosage of extraction solvent is low. Though more costly and noxious than other dispersive solvents, tetra hydro furan (THF) could constitute larger settled volume, which could provide convenient operation and reduce the volume requirement of toxic, chlorinated extraction solvents. THF as dispersive solvent appears to have more advantages in DLLME.

Advantages

Simplicity of operation, rapidity, low cost, high recovery, high enrichment factor and very short extraction time (a few seconds).

HOLLOW-FIBER LIQUID-PHASE MICRO EXTRACTION

Pedersen-Bjergaard and Rasmussen introduced HFLPME. They used the basic principle of supported liquid membrane (SLM) for the first time utilizing polypropylene HFs as the membrane. The sample vial is filled with the aqueous sample. A short piece of a porous HF may be either a rod with a closed bottom or a u-shape where both ends are connected to guiding tubes. Prior to extraction, the HF is first dipped in the organic solvent for a few times to immobilize solvent in the pores, and excess solvent is removed. The solvent is immiscible with water to ensure that it remains within the pores during the extraction with no leakage to the aqueous sample. The organic solvent forms a thin layer within the wall of the HF. The extraction solvent must be compatible with the HF so that the pores in the wall of the HF can be filled completely [14].

TRENDS AND ADVANCES IN CHROMATOGRAPHIC APPROACHES MONOLITH COLUMNS

Use of sorbent instead of porous particles packed in columns has become popular in the field of bio-analytical applications in past few years. The efficiency and resolution of monolith sorbents are comparable to silica particles of 3µm in diameter. Monolith rods are made by sol-gel technology, which enables the formation of highly porous material, containing both macro pores and meso pores in its structure such an LC column consists of a single rod of silica or polymer-based material with two kinds of pores. The large pores (typically 2µm) enable low flow resistance and therefore allow the application of high eluent flow rates, while the small pores (about 12mm) ensure sufficient surface area in order to reach high separation efficiency. Because of this, much higher flow-rates can be used, while the resolution of the monolith rod column is much less affected in regards to particulate materials. Column back pressure remains low as well.

Advantages

- The short time needed for column equilibration when a mobile phase gradient is used.
- Monoliths can accept high flow-rates (up to 10mL min^{-1}) in conventional column lengths without generating high back-pressures.

Limitations

- Limited number of commercially available stationary phases (C_8, C18, plain silica only).
- Internal diameter of monolith columns (ie. 4.6 and 3.0 mm or 100µm i. d. are typical; however, 2.0 or 3.0 have not as yet been manufactured in all common column lengths).

Large internal column diameter, which are more readily available in all column lengths, are not fully compatible with MS and induce a high consumption of organic solvent, especially with flow-rates up to 10mLmin^{-1}. Finally, monoliths made of silica possess a limited chemical stability (pH range 2-8) [3].

FUSED CORE COLUMNS

Separation efficiency and speed of analysis may also be increased using superficially porous particles, often termed also fused core columns. The use of superficially porous particles was first reported in 1960s with the aim of reducing the diffusion distance of analytes to minimize mass transfer. The uses of fused cored silica particles have dramatically improved chromatographic peak efficiencies over fully porous particles in reverse phase as well as in HILIC separation mode, both gradient and isocratic elution. These properties might be explained using the HETP equation: by the combination of a 25% lower axial diffusion (due to the solid core of the particle) and a 20% lower eddy dispersion term (due to narrow particle size distributer). Specifically, the reduction in axial diffusion allows for higher flow-rates to be used without a detriment to chromatographic performance.

Advantages

- Greater efficiency in high speed analysis without the generation of high back-pressure, which is typical in UHPLC and required for special LC equipment.
- Various stationary phases commercially available.

Disadvantages

- Relatively higher solvent consumption comparing to UHPLC.
- Lower pH range (2-8) [3].

HIGH TEMPERATURE LIQUID CHROMATOGRAPHY

High temperature liquid chromatography (HTLC) ($T > 60^oC$) can also be used to perform rapid analysis using standard column lengths, since mobile phase viscosity and back-pressure are decreased. Efficiency, mass transfer, and optical velocity increases simultaneously with temperature permitting the application of high mobile phase velocity. The low viscosity and high diffusivity of a mobile phase at high temperatures produce much lower mass transfer resistance, which leads to flatter van Deemter curves. Therefore, HTLC can be faster and more efficient. The term used for HTLC, in which pure water is used as an eluent is pressurized hot water liquid chromatography (PHW-LC, water $>100^oC$). This is distinguished from conventional HPLC in that solvent strength is increased by temperature rather than by organic solvents. The absence of an organic solvent makes HTLC an environmentally friendly technique; therefore it is sometimes called green chromatography.

Advantages

- Low mobile phase viscosity and high mass transfer.
- Low back-pressure.
- Low concentration of organic modifier (green chromatography).
- Temperature as a variable to change method selectivity.

Disadvantages

- Limited availability of stable high temperature-resistant packing materials.
- A potential degradation of unstable compounds could occur.
- Difficulty with method transfer (selectivity) [3].

Ultra-High Performance Liquid Chromatography

The use of sub-2 micron particles in ultra high pressure systems presents another option in performing rapid analyses. Initially based on the theories of van

Deemter *et al*., then Giddings and finally Knox, the use of small particles is one of the best solutions in the quest to improve chromatographic performances. Optimal separations are also achieved at higher linear velocities and over a wider range of linear velocities because of the low mass transfer resistance of these supports. Thus, better resolution and shorter analysis time could be attained by reducing the particle size.

Advantages

- Substantial decrease in analysis time.
- Very high efficiency and resolution.
- Low solvent consumption.
- High mechanical and chemical stability of UHPLC stationary phases.
- Already, high variety of stationary phases available.

Disadvantages

- Dedicated instrumentation and stationary phases.

Lower sample loading capacity [3].

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

HILIC is an alternative to conventional RP-HPLC (reversed phase HPLC) or NP-HPLC (normal phase HPLC). HILIC has proven quite convenient for the analysis of small polar molecule, weakly retained or eluted with dead volume in conventional RP-HPLC systems. This method is often substituted for normal phase chromatography because of the latter's bad reproducibility, low solubility of polar compounds in NP mobile phases as well as the great difficulties encountered when connection with MS detection is required. In HILIC, analyte retention is believed to be caused by the partitioning of the analyte between a water enriched layer of stagnant eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent, with the main components usually being 5-40% water in ACN. This is perhaps the most rational way to address compounds which are very hydrophilic and uncharged. The mobile phase is composed of a high percentage of organic solvent (typically ACN); this is complemented by a small percentage of water/volatile buffers. The water enriched liquid layer is established within the stationary phase, thus the partitioning of solutes from the mobile phase into the hydrophilic layer occurs. Under the HILIC conditions, the stationary phase is of polar character, usually containing hydroxyl-ethyl, diol and amino groups, or it could be, among other possibilities, a special kind of "zwitter ionic" stationary phase. The primary mechanism of separation is partitioning based on hydrogen bonding; the secondary mechanism, possibly influencing selectivity, is electrostatic interaction with charged stationary phases. Elution is enabled by increasing the polarity of the mobile phase, thus the content of water component. The application now encompass most categories of polar compounds, charged as well as uncharged, although HILIC is particularly well suited for solutes lacking chare, during which coulombic interactions cannot be used to mediate retention.

Advantages

- The increase need to analyze polar compounds in complicated mixtures.

Wide-spread use of MS coupled to HPLC, as HILIC mobile phases are well compatible and give high sensitivity [3].

Table 1. Summary of the mode of sorption of SPME fibers

Adsorbent- type fibers	Absorbent-type fibers
Physically traps or chemically reacts bonds with analytes: Porous material High surface area Chemical derivatizing agent	Analytes are extracted by partitioning the liquid phase: Retained by thickness of coating
Analytes may compete for sites.	Analytes do not compete for sites.
Fibers have limited capacity for extracted analtyes.	Fibers can have high capacity.

Table 2. Types of SPME fibers

Material	Type	Polarity
7-µm PDMS	Absorbent	Non polar
100-µm PDMS	Absorbent	Non polar
30-µm PDMS	Absorbent	Non polar
85-µm Poly acrylate	Absorbent	Polar
65-µm PDMS- divinyl benzene (DVB), StableFlex™	Adsorbent	Bipolar
65-µm CW-DVB, StableFlex	Adsorbent	Polar
85-µm carboxen-PDMS, StableFlex	Adsorbent	Bipolar
55-µm/30µm DVB/ Carboxen™-PDMS, StableFlex	Adsorbent	Bipolar

Table 3. Applications of monolith column in bio-analysis

Determined substances	Matrix Sample preparation	Stationary phase	Mobile phase	Detection	Validation data
Cyclosporin A [15]	Human plasma PP, On-line SPE	Chromolith performance RP 18e (10mm ×4.00mm)	ACN:ammonium acetate, 90:10 (v/v)	ESI-MS/MS	LOD-0.4ng mL^{-1} LOQ-0.015ng mL^{-1}
Montelukast [16]	Human plasma	Chromolith RP 18e(100mm×4.6 mm)	56%ACN and 50mM sodium dihydrogen phosphate and distilled water to 100%, adjusted to pH 7.0	FD	LOD- 5ng/ml^{-1}
Tramadol, O-desmethyl tramadol (M1) N-desmethyl tramadol (M2) and O,N-didesmethyl tramadol (M5) [17]	Human plasma, urine, saliva LLE	Chromolith performance RP 18e (100mm×4.6mm)	Methanol:water (95:5) v/v	FD	LOQ-2.5ng/ml
Retinol and α-tocopherol [18]	Human serum LLE	Chromolith Performance RP-18e (100 mm × 4.6 mm)	Acetonitrile:0.1% formic acid in water (40:60) v/v	Diode array detector	LOD- 0.02 mol l^{-1} and LOQ- 0.07 mol l^{-1} for retinol. LOD-0.1 mol l^{-1} and LOQ- 0.3 mol l^{-1} for alpha-tocopherol
Captopril [19]	Human plasma PP	Chromolith performance RP 18e (100mm ×4.6mm× 3 µm	40:60 (v/v) mixture of acetonitrile and 0.1% (v/v) formic acid in water.	ESI-MS/MS	r-0.993 LOQ-10ng mL^{-1} RSD-3.9%

Table 4. Applications of UHPLC in bio-analysis

Determined substances	Matrix Sample preparation	Stationary phase	Mobile phase	Detection	Validation data
5-hydroxy tryphopholglucuronide (GTOL) 5-hydroxy indoleacetic acid (HIAA) [20]	Urine SPE	ACQUITY HSS C18 (100mm×2.1mm, 1.8µm)	0.1% FAc:ACN (97:3) v/v	ESI-MS/MS	r-0.999 LOQ- 6.7 $nmolL^{-1}$ for GTOL r-0.975 LOQ- 0.02 $nmolL^{-1}$ for HIAA
Thyreostat [21]	Urine	Acquity UPLC HSS T3 column (1.8 m, 100 mm × 2.1 mm,	0.1% aqueous formic acid and 0.1% formic acid in methanol	MS/MS	Decision limits and Detection capabilities-1.1 and 5.5 g L−1 and 1.7 and 7.5 g L−1, respectively. RSD-lower than 15.5% r-0.982 and 0.999.

Vancomycin,Terbinafine, Spironolactone, Furosemide [22]	Human plasma SPE	Hypersil GOLD C18 e (50mm ×2.1mm ×1.7 µm)	Acetonitrile and 0.1% formic acid: Gradient eluent	UV Vancomycin-215nm Terbinafine-224nm Spironolactone-245nm Furosemide-280nm	r-0.994 LOD-0.01-0.07 microg ml^{-1}

Table 5. Applications of HILIC in bio-analysis

Determined substances	Matrix Sample preparation	Stationary phase	Mobile phase	Detection	Validation data
Glucosamine [23]	Dog plasma SPE	Zorbax SB-CN column (5µm×4.6mm i.d. ×250mm) ZIC-HILIC (20mm×2.1mm,3.5µm)	Methanol:5mM ammonium hydrogen carbonate buffer at pH 7.5 (95:5, v/v) 0.1% formic acid in water: ACN, 85:15 gradient	ESI-MS/MS	r-0.9981 LOQ- 1nM for GABA r-0.9940 LOQ-10nM for glutamate
GABA and glutamate [24]	Microdialysis	SeQuant AB Atlantis Hilic (3µm, 150mm×2.1mm)	ACN: water: ammonium acetate buffer (75:10:15, v/v/v)	ESI-MS/MS	r-0.996 RSD-1.3-7.8%
Miglustat [25]	Human plasma, cerebrospinal fluid PP	Poly hydroxyethyl A (35mm×2.1mm, 5µm)	ACN: 20mM ammonium formate with 0.1% formic acid, 97:3 gradient	MS/MS $(M+H)^+$	r-0.999 LOD-0.2 ngmL-1 LOQ-0.6 ngmL-1 for acetylcholine LOD-30ngmL-1 LOQ-80ngmL-1 for choline LOD-2.0ngmL-1 LOQ-15.0ngmL-1 for butyrobetaine r-0.994-0.997 r-0.994-0.997 RSD-2.01-11.05%
Acetylcholine, choline, butyrobetaine [26]	Human liver tissue	Supercosil LC-Si (4.6mm×250mm, 5µm)	0.01M ammonium acetate: ACN (40:66, v/v)	UV-235nm	r-0.998 RSD-1.8-7.7% LOQ-2.00ngmL-1
Metformin and its prodrugs [27]	Human blood, rat blood PP Human plsma LLE	Atlantis HILIC (50mm× 3.0mm×µ3m	Acetonitrile:100mM ammonium formate (85:15) v/v	UV-235nm	
Metoclopramide [28]		Atlantis HILIC silica column	acetonitrile–ammonium formate (100 mM, pH 6.5) (85:15, v/v)	ESI-MS/MS	*r*2 = 0.998

Figure 1. General solid-phase extraction procedure

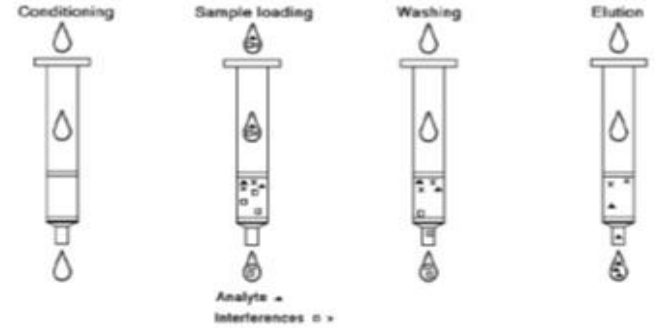


Figure 2. Micro dialysis extraction with a probe

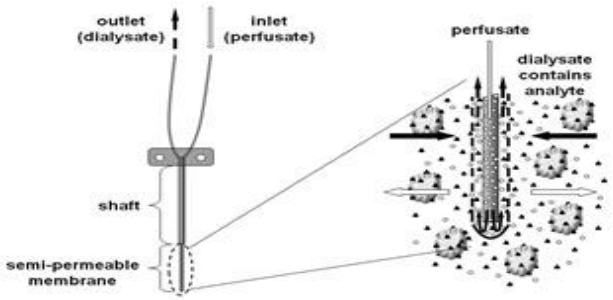


Figure 3.Schematic of the MEPS BIN in the syringe needle

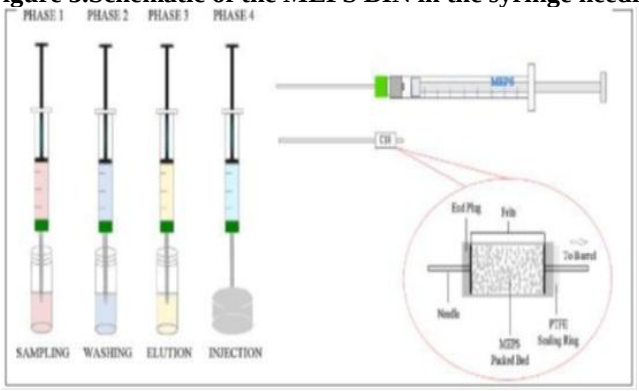


Figure 4. Schematic of molecular imprinting

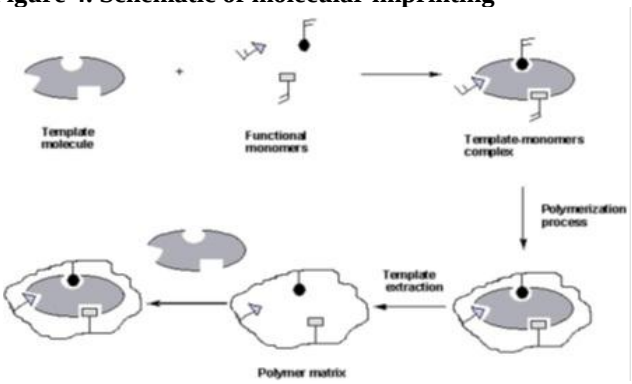


Figure 5. Direct-immersion single-drop micro extraction

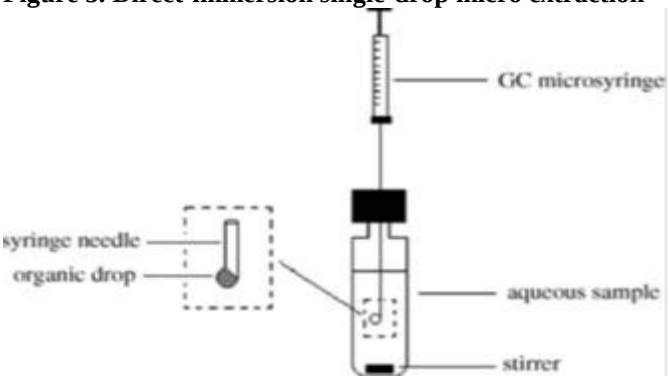


Figure 6. Headspace single-drop micro extraction

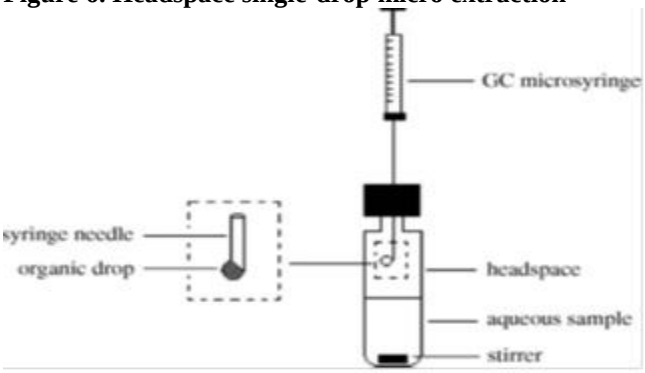


Figure 7. Different steps in dispersive liquid-liquid micro extraction

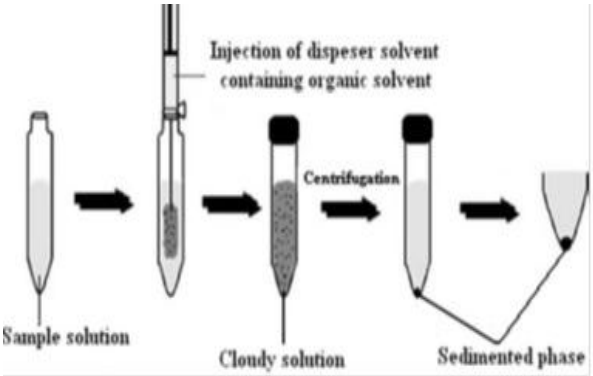
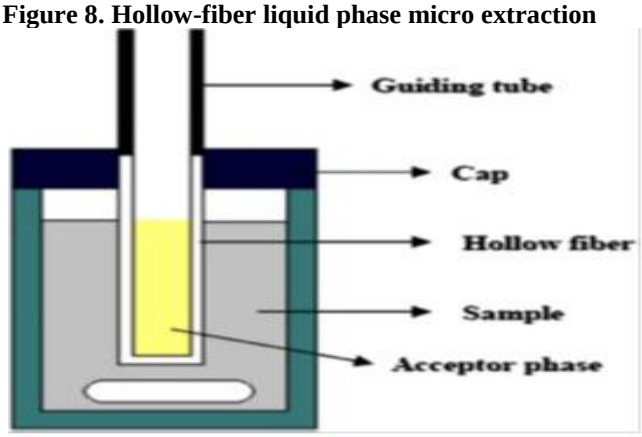


Figure 8. Hollow-fiber liquid phase micro extraction



CONCLUSION

The modern sample preparation techniques should be not only simple, reliable, cheap and take into account chemical laboratory waste problems, but also must be similar to common analytical techniques, in order to reduce errors. For these reasons, modern trends in analytical chemistry are towards the simplification and miniaturization of sample preparation, and the minimization of sample size and organic solvent used. The development of such procedures combined with modern chromatographic-mass spectrometric techniques will enable analysis at the low levels. Current sample preparation techniques employ small amounts of sample as well as simpler methods which are "just enough" prior to analysis, as more steps could introduce more errors. New developments have been attempted to enhance selectivity (MIP) as well as to reduce solvent consumption, thus making sample preparation environmentally friendly (micro extraciton approaches). Finally, such methods also feature high-throughput automated techniques. Different micro extraction techniques have found an important place in sample preparation because of their inherent advantages over the conventional procedures. Sample preparation techniques must be chosen and optimized with the regard to method purpose. As is stated in the ICH guidelines, the validated method must be appropriate for the intended purposes. An appropriate technique should be chosen with the regard to extraction time, selectivity, the number of steps, solvent consumption and the possibility of the use of on-line methods arrangement.

Monoliths seem to be more convenient for routine use in bio-analytical applications, however their application still remains limited due to the restricted availability of stationary phase chemistries and the low pH stability of silica ones. The high solvent consumption induced by the high flow-rates applied is one of the main drawbacks of monolith columns. As a result, fused core columns and UHPLC seem to be the most convenient approaches for modern, high-throughput, efficient, economic and ultra-fast analysis. UPHLC seems to be slightly more advantageous, because chemically more stable stationary phases are available. Commercially available fused core columns are manufactured from silica that is applicable within the conventionally restricted pH range of 2–8. UHPLC in its design needs special instrumental equipment.

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