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Overview of novel sample pretreatment methods and their pharmaceutical applications in bioanalysis

Jamshed Haneef*, Larzi Mohsin Bin Mohammed
Department of Pharmaceutical Chemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India.

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ABSTRACT

Sample pretreatment methods are a vital component in the pharmaceutical assays of analytes with the objectives of minimizing matrix effects and better recovery. The associated challenges involved are multistep procedures, time-consuming, labor-intensive, and a lack of selectivity. These limitations can be overcome by the advancement in novel extraction methods. These methods are preferred over traditional methods due to the ease of the clean-up procedure and the possibility of automation, miniaturization, and good recovery of target analytes. Therefore, the present review highlights the current progress in novel pretreatment methods and their applicability in the extraction of analytes from biological fluids. Various case studies have been covered about the pharmaceuticals and were summarized with critical parameters associated with method developments. In addition, considerable progress in novel methods for the extraction of biomarkers and diagnostics has been made. The integration of artificial intelligence and the adoption of green sampling approaches allow faster and reliable bioanalysis workflows. These developments have a significant impact in making bioanalysis more accessible, eco-conscious, and high accuracy in bioanalytical method developments.

1. INTRODUCTION

Sample pretreatment is an integral part of bioanalysis for the extraction of analytes from different matrices and their quantitation using analytical tools [1]. Biological matrices are often complicated due to the presence of endogenous (e.g., Proteins, lipids, or the target analyte's metabolites) or exogenous chemicals (additives used in analysis) [2]. Besides, matrix components that co-elute with target analytes during chromatographic separation affected the mass spectrometry (MS) detection of the analyte's response, either positively (ion enhancement impact) or negatively (ion suppression effect) [3].

Among the sample pretreatment methods available, protein precipitation is a straightforward and affordable method. The proteins in the sample are denatured by a high concentration of organic solvent (preferably acetonitrile) and eventually precipitate out of the sample by centrifugation [4]. Liquid–liquid extraction (LLE) is another commonly used pretreatment method, which involves the selection of two immiscible solvents for the extraction of the desired analyte. According to their partition coefficients, immiscible organic solvents separate the target analyte into the organic layer and can be extracted [5,6]. The advantage of this technique is that highly clean extracts with strong selectivity for the desired analyte can be produced with the careful selection of solvents, pH of the aqueous phase, polarity, and analyte solubility in the organic solvent. However, LLE suffers from certain limitations such as the use of large amounts of organic solvent, lack of automation, and time consumption [7]. Another important pretreatment method is solid phase extraction (SPE), which involves chemically separating the components of a sample by using chromatographic packing material, usually in cartridge-type devices. The selection of a suitable SPE extraction sorbent depends upon the interaction between the sorbent and the analyte of interest. SPE generally retains materials by van der Waals forces (nonpolar interactions) [8]. The extraction steps of

*Corresponding Author
Jamshed Haneef, Department of Pharmaceutical Chemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India. E-mail: Jamshedhaneef@jamiahamdard.ac.in

SPE include conditioning, equilibration, sample load, washing, and elution [9]. SPE is widely employed in the LC-MS/MS applications to quantify molecules in biofluids [10].

Overall, the traditional pretreatment methods are time-consuming, labor-intensive, and poorly suited for large as well as very small samples. Many conventional multistep procedures do not have adequate sensitivity, selectivity, and reliability. In addition, assays for metabolites or biomarker detection in clinical samples at very low concentrations face a similar set of challenges. In addition, improvements in sample preparation methods are increasingly focused on improving the clean-up procedure and exploring the possibility of automating, miniaturising, and improving their specificity [1]. Therefore, sample pretreatment has made significant progress in miniaturized and solvent-free extraction techniques, and faster separation methods designated as novel pretreatment methods [11]. A glimpse of the different available sample pretreatment methods is shown in Figure 1.

Recent comprehensive reviews on bioanalytical sample preparation techniques emphasize that matrix effects remain one of the most significant challenges in achieving accurate and reproducible analytical results [12]. Biological matrices such as blood, plasma, urine, and tissues contain various endogenous substances—proteins, lipids, and salts—that can interfere with the detection and quantification of target analytes. These interferences can suppress or enhance analytical signals, leading to compromised data quality. As a result, modern sample preparation methods increasingly focus on minimizing matrix effects through improved extraction efficiency, selectivity, and compatibility with advanced analytical platforms like LC-MS/MS.

This review focuses on the recent developments in novel pretreatment methods, covering their working principle, applications (mainly pharmaceutical uses), and recent updates. Much effort has been made to cover the maximum case studies that involve novel pretreatment methods for analyte extraction and hyphenated techniques for their detection. In addition, different matrices (blood, urine, saliva, and so on) treated with novel methods have been covered to understand their overall applicability. Besides, a comparative analysis of different novel methods has been performed to assess their overall performance based on critical parameters. Furthermore, a separate section dealing with recent trends in the bioanalytical pretreatment methods provides the innovative strategies being developed and explored in bioanalysis. The recent use of pretreatment methods for the separation of biomarkers and their diagnostic uses has also been covered.

2. NOVEL SAMPLE PRETREATMENT METHODS

Novel sample pretreatment methods are becoming more popular to overcome the limitations of traditional methods.

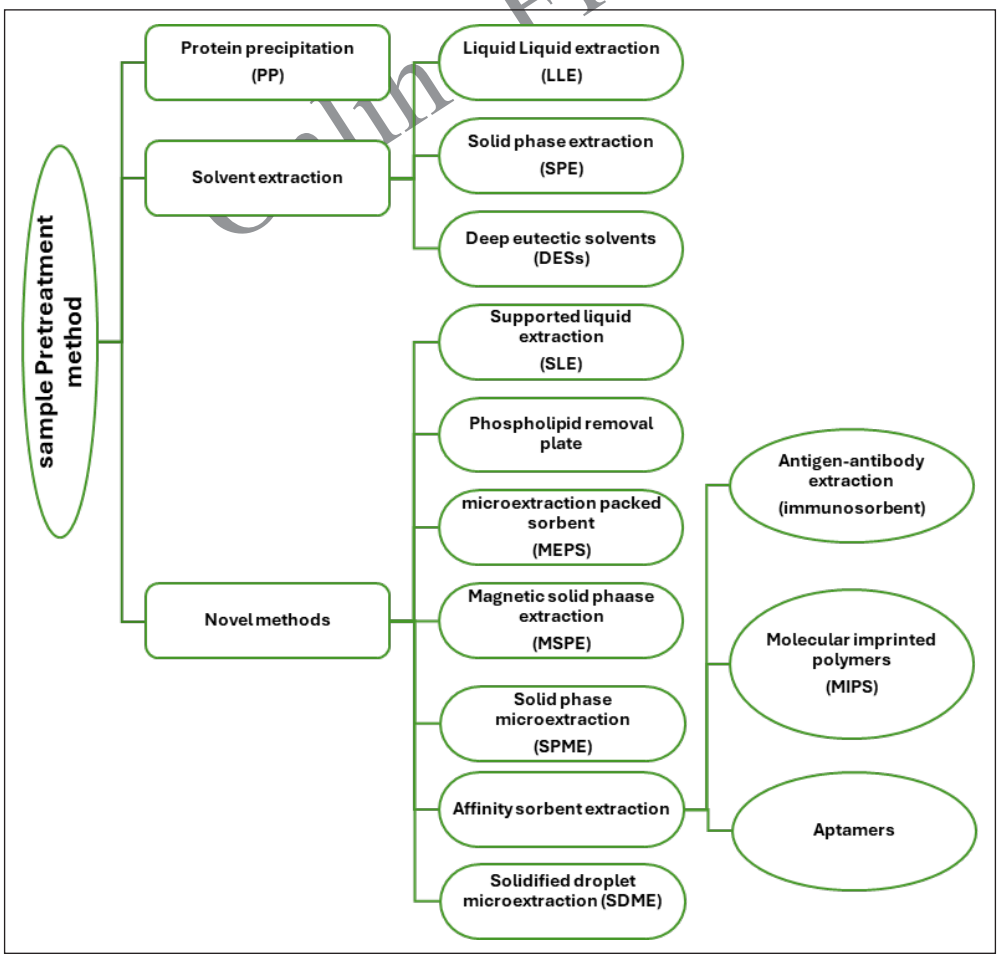

Figure 1. Overview of the traditional and novel sample pretreatment methods.

These methods are the modified versions of either SPE or LLE with the advantages of miniaturization, automation, and less time-consuming with good efficiency. ICH Q2(R^2) guideline emphasizes the integration of method validation within the broader analytical procedure lifecycle, aligning with ICH Q14. Pretreatment methods largely affect the limit of detection (LOD) and limit of quantitation (LOQ) of the analytical method and overall affect the sensitivity of the technology employed. A literature survey in the scientific database (PubMed) has been done for the last 10 years and showed a surge in the publications using these novel methods (Fig. 2). Among them, solid phase microextraction (SPME) showed the highest usage. Another survey in terms of the application of these novel methods in diverse applications. The trend showed that environment and food analysis are among the top, followed by pharmaceutical and clinical applications (Fig. 3). These surveys demonstrate the growing interest among the scientific community in novel methods to overcome the critical issues encountered in the traditional methods.

This section deals with different novel methods along with their working principle and is complemented by pharmaceutical applications. Prominent case studies under each novel method have been briefly described. Finally, all the case studies collectively are summarized in tabular form (Table 1) based on the target analyte, novel methods, matrix type, recovery, and hyphenated methods used, along with their detection limits.

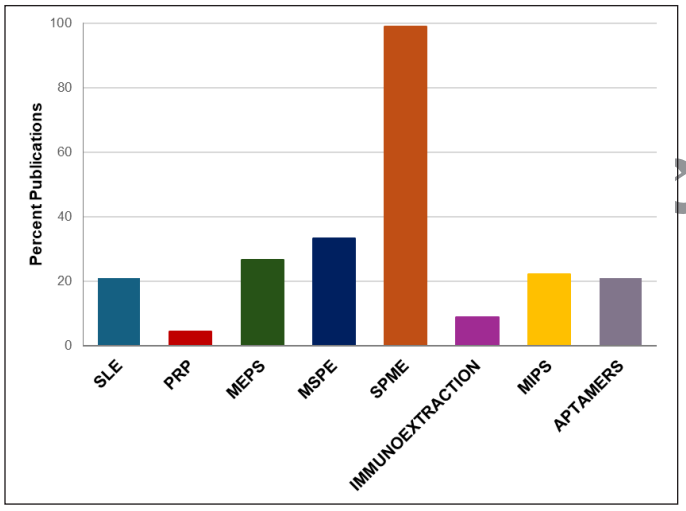

Figure 2. Trends of novel sample pretreatment methods in the scientific database (PubMed) in the last 10 years [till December 2024].

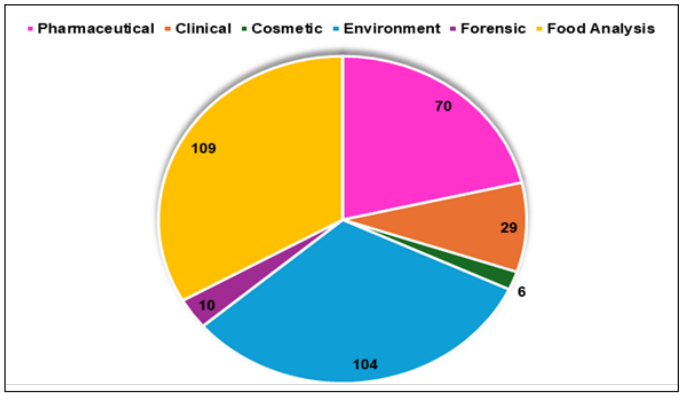

Figure 3. Applications of novel sample pretreatment methods in diverse areas based on the scientific database (PubMed).

2.1. Supported liquid extraction

SLE is a modified version of LLE in which the extraction is accomplished using a stationary phase, preferably inert diatomaceous earth [1], which gives solid support to divide the incompatible solvents in the SLE technique. In this method, samples are applied onto the plate with a brief suction pulse and then left to fully absorb the sorbent for a period. The extracting solvent is passed through the plate before being dried down, and the interfering matrix gets adsorbed to the solid substrate, and the solvent extracts the analyte. SLE offers several benefits as compared to conventional LLE, for instance, no emulsion formation, excellent extraction effectiveness, and automation compatibility [13]. In contrast to SPE, pre-deproteinization is not essential in SLE [14,15].

SLE is widely used in bioanalytical applications using different hyphenated tools such as liquid chromatography tandem MS (LC-MS/MS) and gas liquid chromatography tandem MS (GC-MS/MS), and is extensively used in drug metabolism, food, and environmental analysis. In addition, SLE is suitable for removing phospholipids, which frequently prevent the ionization of target components during MS analysis [16,17]. Zheng *et al.* [18] applied SLE in combination with UPLC-MS/MS for determining nine mental drugs in human plasma (100 µl). Analytes were loaded onto a 96-well SLE cartridge, and separation was done on Agilent Poroshell 120 EC-C_{18} columns. Recovery of the analyte was found to be in the range of 53.11%–132.98% and the LOD was 0.02–0.25 ng/ml. Similarly, Meunier *et al.* [19] have developed an LC-MS/MS-based detection of aldosterone in human plasma (450 µl). SLE was employed to extract the target analyte and was found to be faster and more convenient than the traditional methods (LLE and SPE) with a good LOD of 30–50 pmol/l. Another interesting study was conducted by Rositano *et al.* [15] for the simultaneous detection of psychostimulant drugs (methylamphetamine, methylenedioxymethamphetamine, and delta-9-tetrahydrocannabinol) in two biofluids (blood and oral fluid) of drivers using the LC-MS/MS technique. Target drugs were extracted using 96-well plates together with an internal standard, as well as elution solvent. The sample pretreatment method was found to be efficient with a recovery of 80% and a detection limit of 1–5 ng/ml. In addition, SLE has been exploited for diverse other analytes (benzodiazepine, eicosanoids and related metabolite, polycyclic hydrocarbon and its metabolites, alkaloids) in the biological matrices (plasma and urine) [20–24].

Overall, SLE offers more benefits than LLE and SPE due to the automation, low sample requirement, faster, minimal solvent waste, with good sensitivity. SLE is widely used in bioanalytical, food, and environmental testing due to its high recovery rates, reproducibility, and compatibility with automation platforms like 96-well plates. Technically, SLE simplifies workflows by eliminating the need for vigorous

Table 1. Extraction of different analytes using novel pretreatment methods from biological fluids.

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Methylamphetamine, Methylenedio-xymethamphetamineand Delta-9-tetrahydrocannabi-nol	SLE	Oral fluid and blood	100 µl	Silica diatomaceous earth (96-well plates)	8% concentrated ammonia solution(v/v), methyl tert-butyl ether, methanol	65%–80%	1–5 ng/ml (LC-MS/MS)	- Analysis of hundreds of samples within hours - Faster analysis	[15]
Aldosterone	SLE	Blood plasma	450 µl	Silica diatomaceous earth	Methyl tert-butyl ether, methanol	> 97.7%	30 pmol/l (LC-MS/MS)	- Faster and more convenient method than LLE or SPE - Small sample size	[19]
Benzodiazepines (Alprazolam, chlordiazepoxide, clonazepam, diazepam, lorazepam)	SLE	Urine sample	100 µl	Silica diatomaceous earth	Methanol, ammonium acetate, ammonium acetate buffer, pH 5 & 1% formic acid	_	5.3–11,000 ng/ml (SERS)	- fast and clear extraction - Minimal solvent waste - Reduce total analysis time (under 20 min)	[20]
Eicosanoids and related metabolite	SLE	Blood plasma	375 µl	Silica diatomaceous earth	0.1% formic acid-water, ammonia	20%–70%	_ (LC-MRM-MS)	- Both protein removal and lipid extraction achieved simultaneously - Simple and automated method used for extraction of free fatty acid derived lipid mediators and platelet activating factors	[21]
Monohydroxylated polycyclic aromatic hydrocarbons	SLE	Urine sample (human)	100 µl	Silica diatomaceous earth minicartridge.	Acetate buffer, Dichloromethane /hexane (3:7 v/v), methanol	88.5%120.9%	0.06 – 0.30 µg/ml (LC-MS/MS)	- Compared with SPE, it showed high recovery, reproducibility as well as weak biological matrix interference - low emulsification.	[22]
Polycyclic aromatic hydrocarbon metabolites	SLE	Urine sample	1 ml	Silica Diatomaceous earth	Sodium acetate buffer pH 5.5, Pentane/chloroform (70/30 v/v), dodecane, toluene	25%–85%	2 – 13.8 pg/ml (GC-MS/MS)	- No preconditioning required - Higher throughput - Simplified extraction - No formation of emulsion. - Less time consuming and less expensive	[23]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Alkaloids (Colchicine, anisodamine, atropine, aconitine and Yun aconitine)	SLE	Blood plasma	0.5 ml	Cartridge	Ethyl acetate, buffer solution, pH 11	79.2 –95.8%	0.02 – 0.03 µg/ml (UPLC-MS/MS)	- Reduce time cost - Ease of operation and feasibility of automation - Application in clinical and forensic area	[24]
Budesonide (corticosteroid)	PRP	Blood plasma	350 µl	Ostro 96 well plate combined with C18 column of SPE	Acetonitrile, 2% formic acid, methanol	67%	24 pg/ml (LC-MS/MS)	- Lipid interferences successfully eliminate - Reduce maximum matrix effect	[28]
(S)-metoprolol and (S)-α-hydroxymetoprolol	PRP	Blood plasma	50 µl	Microelution-SPE (Oasis PRiME MCX micro elution 96 -well cartridge)	Water : 4% H_3PO_4 (1:1 v/v) Acetonitrile, ammonium formate, 2% formic acid	69.4%–78.7%	0.5 & 1.25 ng/ml (LC-MS/MS)	- Ease of use - Reduce matrix effect - Robustness. - Lower sample requirement. - Low solvent use	[29]
Anticoagulant (warfarin, pindone, definacoum)	PRP	Blood plasma	100 µl	Silica particle coated with zirconia Phree™ (Phenomenex)	Acetonitrile, 0.1% formic acid	80.6%–113.1%	0.02 – 0.3 ng/ml (UPLC-MS/MS)	- significantly reduce phospholipid content in blood - eluate can be directly injected - high cleanup efficiency - rapid sample pretreatment - Good analyte recovery	[30]
Avobenzone and oxybenzone (sunscreen)	PRP	Blood plasma	150 µl	Phree™ (Phenomenex)	Acetonitrile containing 1% formic acid	85.3%–105%	0.02 – 0.44 ng/ml (UHPLC-MS/MS)	- Reproducible and qauntitative recovery of analyte - Negligible matrix effect	[31]
Amitraz, chlordimeform, formetanate and metabolite (formamidine group of pesticide)	PRP	Blood plasma	100 µl	Phree™ (Phenomenex)	Acetonitrile containing 0.1% formic acid	88 %–103.5%	0.05 – 12 ng/ml (UPLC-MS/MS)	- Direct injection of eluate in UPLC-MS/MS - Excellent cleanup efficiency for LC long column life - Easy and fast method - Require less time	[32]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Ethyl glucuronide and ethyl sulfate in postmortem and antemortem	PRP	Blood plasma	100 µl	Phree™ (Phenomenex)	First protein precipitation then 96-well phospholipid removal plate using ice cold acetonitrile containing 0.1% formic acid, water,	61%–77%	0.0025 – 0.0089 ng/ml (UHPLC-MS/MS)	- Better efficiency of analyte compared with the protein precipitation - Good chromatographic performance of analyte - Negligible matrix effect	[33]
Haloperidol (Typical antipsychotics drug)	PRP	Blood plasma	100 µl	Ostro™ 96-well plate	acetonitrile with 1% formic acid	98.5%–100%	0.05 ng/ml (LC-MS/MS)	- Consistent recovery - Low matrix effect - Lowest time require for complition - Reduce possibilities of error - High throughput sample pretreatment	[34]
Direct oral anticoagulants (apixaban, dabigatran, edoxaban & rivaroxaban)	PRP	Blood Plasma	100 µl	Ostro™ 96-well plate	1% formic acid in acetonitrile	40%–50%	3, 12, 400 and 800 ng/ml (LC-MS/MS)	- High throughput - Washing, conditioning and elution steps not require - Diminish maximum matrix effect - Easy of handling	[35]
Non steroidal antiinflametory drugs (naproxen , diclofenac, & ibuprofen)	Magnetic beads	Urine	2.5 ml	2-aminobenzothiazol polymerized on Fe_3O_4 NPs, graphene oxide/Fe_3O_4 (GO/Fe_3O_4) and graphene/Fe_3O_4 (G/Fe_3O_4) nanocomposites	1:1 v/v ultrapure water	0.07 %–90.5%	0.07 – 0.3 µg/ml (HPLC)	- No filtration and centrifugation - Easier and faster method - Simple and economical method - Short duration and reduce sorbent/solvent utilization - Reutilization of sorbent (magnetic beads)	[42]
Puerarin	Magnetic beads	Blood plasma	200 µl	Fe_3O_4-Sio_2/C_{18}	Acetonitrile	85.2%–92.3%	0.05 µg/ml (HPLC)	- Simple, economic, and efficient method - Less toxic solvent expenditure	[43]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Cr(lll), Cu(ll), Pb(ll) and Zn(ll)	Magnetic beads	Hair sample	25 mg	Sio_2/H_2D_Z (Dithizone modified)	HNO_3	85%–104.5%	0.01 – 0.06 µg/ml (ICP-OES)	- Simple and high enrichment factor technique - Low consumption of solvent - No agglomeration after field removal - Suitable for trace element determination	[44]
Phthalate monoesters	Magnetic beads	Urine sample	2 ml	Magnetic nanoparticles onto Multiwall carbon nanotube	Ammonium acetate, Isopropanol	92.6%–98.8%	0.25 – 0.05 µg/ml (GC-MS)	- Sorbent easily isolated from matrix and can be reuse - Avoid time consuming, filtration steps - Rapid, low cost and simplified procedure	[45]
Fenitrothion	Magnetic beads	Plasma and urine	5 ml	Poly (St-co-DVB)	1-Propanol	98.5%–101.5%	0.5 ng/ml (UV-Spectrophotometry)	- More efficient method - Avoid multiple steps - Time saving - Low expenditure of solvent	[46]
Acetaminophen and metabolites	Magnetic beads	Blood serum	25 µl	Magnetized hyper-crosslinked polystyrene particle	Acetonitrile/water (70:30 v/v)	68%–95%	_ (LC-MS/MS)	- This method can be apply for wide range of polarity sample - Reduce time require for sample preparation - Less sample requirement - Protein and lipids are enriched in matrix	[47]
Aflatoxin	Magnetic beads	Blood serum	150 µl	Modified magnetic beads with gold nanoparticles (GNP)-labelled with anti-aflatoxin antibodies.	Disodium and monosodium phosphate, phosphate buffered saline (PBS)	92.8%–122%	12 ng/ml (Colorimetry)	- Noble metal having a large surface area thus diffuse freely in reaction mixture and easily separate - Fast and simple to handle	[48]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
1-hydroxypyrene (pyrene metabolite)	Magnetic beads	Urine	2 ml	n-octadecyl phosphonic acid modified magnetic mesoporous nanoparticles (OPA/ MMNPs)	Sodium acetate, β-Glucuronidase/ arylsulfatase, methanol	90%–115%	0.001 µg/ml (HPLC)	- More efficient, economic and convenient method - Direct analysis is possible because of sorbent - Minimum extraction time	[49]
Linezolid and ciprofloxacin	MEPS	Blood plasma	50 µl	C_{18} cartridge	methanol	92.4%–97.4%	0.007 & 0.004 µg/ml (UHPLC-PDA)	- Simplicity - Small sample volume - Short run time - Minimum solvent consumption	[56]
Metoprolol enantiomers	MEPS	Blood plasma & saliva	100 µl	C_8 cartridge sorbent	isopropanol, 5% methanol	93%–97.80%	0.5 ng/ml (HPLC-MS/MS)	- Small sample volume requirement - On-line connection with analytical technique - Automated method - Extraction of metabolite	[57]
Opiates	MEPS	Blood plasma	250 µl	(80% C_8 and 20% SCX)	Acetonitrile, formic acid, ammonium hydroxide	6%–23%	5 ng/ml (GC-MS/MS)	- Fast, simple, and robust technique - Can be applied for more complex matrix - Low solvent requirement - Sorbent can be reused	[58]
Amphetamine	MEPS	Urine sample	200 µl	C_{18} cartridge	methanol, acetonitrile, ammonium hydroxide	18.9%–52.39%	3-50 ng/ml (GC-MS)	- Simple and rapid method - Extract wide range of analytes - Low sample volume requirement	[59]
Lamotrigine	MEPS	Blood plasma & saliva	100 µl	C_{18} cartridge	Acetonitrile, 0.3% triethylamine water solution, methanol	64%–72%	0.1 µg/ml (HPLC-DAD)	- Qualitative and quantitively analysis of drugs and metabolite	[60]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
New psychoactive substances	MEPS	Oral fluid	300 µL	C_8/SCX Sorbent	Methanol, phosphate buffer, ammonium hydroxide	75%–125%	_ (UPLC-MS/MS)	- Short time extraction - Reduce sample and solvent consumption - Easy operation - 90 times sorbent reuse of oral fluid	[61]
Cannabinoids	MEPS	Oral fluid	125 µl	C_{18} cartridge	Ammonium hydroxide and methanol	50%–105%	0.008 – 0.12 ng/ml (LC-MS/MS)	- Rapid and effective cleanup - Satisfactory recovery and negligible matrix effect	[62]
Benznidazole	MEPS	Blood plasma	500 µl	C_{18} cartridge	Acetonitrile, methanol	25%	(HPLC)	- Minimize use of hazardous solvent and sample volume - Simplicity and robust technique	[63]
Rocuronium bromide and tranexamic acid	SPME	Blood plasma	0.8 ml	WCX TF-SPME (Weak cations exchange thin film)	methanol, acetonitrile, ammonium format buffer	18%–21%	0.04 – 0.1 µg/ml (LC-MS/MS)	- Combination of sampling, sample preparation and extraction in single steps - Effective clean up - Biocompatibility in clinical practice	[69]
Cannabinoids	SPME	Mice Brain	0.5 g	SPMEM (Solid phase microextraction by the membrane of polyamide and amid)	NaCl, desorption solvent (methanol, ethanol, acetone).	100%	_ (LC-MS)	- Easier, simpler, and more convenient method than other conventional methods - Solventless - Extraction of explosive residue possible	[70]
Non-steroidal anti-inflammatory drugs	SPME	Urine, serum, & plasma	0.15 ml (urine) 5ml (plasma)	$Fe_30_4/CU_3(BTC)_2$ metal organic framework coating	Acetonitrile: water (60:40 v/v)	94%–102%	0.03 – 0.05 µg/ml (HPLC)	- Use of magnetic sorbent in SPME having significance long span, reusability, stable against temperature, chemical and mechanical variations	[71]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
N-nitrosamines impurities	SPME	Blood sample	200 µl –	NH_2-MIL-101(Fe)	Acetonitrile, methanol	82.4%–116.8%	0.005 – 0.025 µg/ml (LC-MS/MS)	- Simple pretreatment method - Less consumption of sample volume, and solvent - High surface area of sorbent high efficiency and selectivity	[72]
Tacrolimus and sirolimus	SPME	Blood plasma	200 µl	Hydrophilic lipophilic balance particles coating on fiber	Zinc sulphate, acetonitrile, water	–	0.2 – 0.3 ng/ml (MOI-MS/MS)	- Low cost - Simplicity - Mechanical and chemical lysing of matrix (ensuring all analyte release from matrix)	[73]
Caffeine and its metabolite	SPME	Serum, saliva, & urine	2.5 µl, 6.25 µl, 40 µl	ZB-FFAP (Nitro terephthalic modified PEG)	Methanol and water	84%–112%	0.1 – 0.5 µg/ml (Capillary LC-DAD)	- One step extraction, purification, and concentration - Sample volume and preparation time reduce - Cost effective and solventless method	[74]
Methamphetamines	SPME	Oral fluid	50 µl	TRB/ MWCNTs	Carbonate buffer, fluorenylmethyloxycarbonyl	–	0.5 – 0.8 µg/ml (Capillary LC-FLD)	- Coating with the CNTs increases the extraction efficiency because of high surface area	[75]
Phenyl ethanolamine	Immunosorbent	Urine	100 µl	PA hapten linked with carrier protein (diazotization)	Phosphate buffer, Anti-PA mAb, TMB-H_2O_2	82%–107.4%	0.13 ng/ml (LC-MS)	- Simple, rapid, low cost, no complex procedure, result achieved within 5 min	[87]
Ketamine	Immunosorbent	Oral fluid	3 µl	Ketamine antibody, ketamine-HRP from fitzgerald	PBS-T buffer, TMB solution (color change)	–	0.03 ng/ml (Colorimetry, µPAD)	- Minimum time requirement. - Improve non-specific binding - Sensitive, user-friendly method	[90]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Methadone	MIPs	Blood plasma	200 µl	Molecular imprinted sol-gel tablet (Methadone-d_9 as template and 3-(propyl methacrylate)-trimethoxy silane as precursor)	Water and methanol	≥ 80%	1.0 ng/ml (LC-MS/MS)	- Novel, robust, and chemical stable method - Reduce extraction time - High selectivity - Sample volume reduces 5 times - Limit of detection and quantification improved	[96]
Naproxen	MIPs	Urine	25 ml	Molecular imprinted polymer-coated magnetic multi-walled carbon nanotubes (amidoamine as monomer, naproxen as template)	Britton-Robinson buffer, 1:1 (v/v) methanol/sodium hydroxide aq. Solution	≥ 97.7%	2.0 ng/ml (Spectro fluorometry)	- Simple and rapid sample pretreatment method - High adsorption capacity and high chemical stability - Adsorbent can easily attracted using magnet and can be separate	[97]
Bisphenols A	MIPs	Urine	1 ml	Bisphenol A imprinted polymer microspheres by emulsion polymerization and used in SPE	Acetonitrile, methanol	81.3%–106%	2.2 ng/ml (HPLC-DAD)	- Simplicity, high yields of polymer and control of final particle size - Specific adsorption capacity for BPA - Good selectivity and clean up efficiency	[98]
Sulfasalazine	MIPs	Blood serum	0.6 ml	Sulfasalazine selective MIP and non-imprinted polymer incorporate in carbon paste electrode.	Britton-Robinson buffer (pH 4.5), Water	94.5%–96.3%	4.6×10^{-9} mol/l (DPV)	- Easy fabrication, facile renewable surface, biocompatibility - Stable for different solvents - Relatively low-background characteristics	[99]
Prednisolone	MIPs	Urine	100 µl	Pipette-tip based on nano-sized dummy molecularly imprinted polymer (PT-DMIP) Betamethasone as template and 3-aminopropyltrimethoxysilane as a monomer	Hexane, methanol	89%–96.1%	0.085 µg/l (HPLC)	- Unique method in terms of facility and consumption of toxic solvents - Effect of template bleeding absolutely expiring	[100]

Continued

Analytes	Extraction technique	Matrix	Volume	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Lamotrigine	MIPs	Blood plasma	10 ml	Magnetic molecular imprinted polymers via lamotrigine as template molecule and methacrylic acid as functional monomers.	Deionized water, methanol/ TFA/HOAC (96:2:2 v/v/v)	96.6%–97%	0.5 µg/ml (HPLC-UV)	- High adsorption capacity and selectivity - Effectively avoid risk of template leakage - Ensure the reliability - MMIPs easily collected using an external magnetic field, avoiding traditional SPE column packing	[102]
Ephedrine	MIPs	Blood serum	5 ml	Monolithic molecular imprinted polymer with ephedrine as template and methacrylic acid as monomer	Ethanol, NaOH, NaCl, ammonia buffer, toluene	91%–10%	0.2 µg/ml (Capillary electrophoresis)	- Preparation of MIPs is reproducible and easy - No use of hazardous materials. - Low cost - Giving high Recovery of analyte	[103]
Tetracycline	Aptamer	Blood plasma	_	Aptamers conjugate with the dry CN-Br activated Sepharose and Packed in SPE	Methanol-water	86.5 %	0.019 µg/ml (ESI-IMS)	- Excellent selectivity and recovery of analyte - Much cheaper than the antibodies - Simple, fast and derivatization free method	[115]

SLE, Supported Liquid Extraction; PRP, Phospholipid Removal Plate; MEPs, Microextraction by Packed Sorbents; MIPs, Molecular Imprinted Polymers; SPME, Solid Phase Microextraction; SERS, Surface Enhance Raman Spectroscopy; LC-MS/MS, Liquid-Chromatography Tandem Mass Spectrometry; GC-MS/MS, Gas Chromatography Tandem Mass Spectrometry; UPLC-MS/MS, Ultra-High Performance Liquid Chromatography-Tandem Mass Spectroscopy; ICP-OES, Inductively Coupled Plasma-Optical Emission Spectroscopy; HPLC-DAD, High Performance Liquid Chromatography-Diode Array Detector; MOI-MS/MS, Microfluidic Open Interface- Mass Spectroscopy; ESI-IMS, Electron Spray Ionization- Ion Mobility Spectrometry.

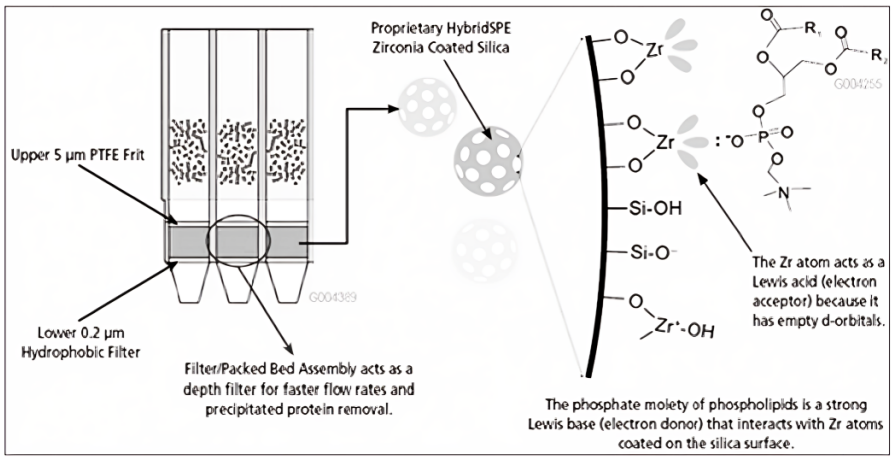

Figure 4. Mechanism of phospholipid removal plate-based extraction showing zirconia-coated silica sorbent with integrated filters enabling efficient flow that binds through Lewis acid-base interactions. (Adapted from Ahmad *et al.* [27].

mixing or phase separation, which are common in LLE. The process involves loading a pretreated aqueous sample onto the sorbent, allowing it to adsorb, and then eluting analytes with an organic solvent. This results in cleaner extracts with reduced matrix effects, making it ideal for LC-MS/MS applications. Nevertheless, SLE is less effective for highly polar or permanently ionized compounds, which may not partition well into the organic phase. Furthermore, the technique requires careful selection of sorbent type and solvent system based on analyte properties, and it may not be suitable for all matrices or analyte classes.

2.2. Phospholipid removal plate

A phospholipid removal plate is a specialized SPE device designed to selectively eliminate phospholipids from biological samples such as plasma and serum before analytical procedures like LC-MS. The plate contains a sorbent bed made of zirconia-coated silica particles, positioned between two filtration layers: an upper 5 µm frit and a lower 0.2 µm hydrophobic filter, which act as depth filters to maintain optimal flow rates and prevent clogging (Fig. 4).

The zirconium atoms on the silica surface act as Lewis acids due to their empty d orbitals, forming strong electron donor-acceptor complexes with the phosphate groups in phospholipids, which serve as Lewis bases. As the biofluid sample passes through the plate, phospholipids are selectively retained by the zirconia surface, while other components, such as proteins and metabolites, flow through unretained. This selective extraction significantly reduces matrix effects, enhances analyte recovery, and improves sensitivity and reproducibility in downstream analytical workflows [25]. Phospholipids are a major cause of ion suppression in blood and plasma analysis. To address this, Hybrid SPE precipitation plates have been developed wherein the plasma or serum samples are first treated with acetonitrile for protein precipitation, then passed through the Hybrid SPE precipitation plates packed bed for phospholipid removal. Structurally, phospholipids consist of a zwitterionic phosphonate moiety as the polar head group and two hydrophobic fatty acyl chains as tails. The phosphate group in all phospholipids interacts strongly with the zirconium atoms functionalized on the surface of the particles, enabling selective retention [26,27].

Phospholipid removal plates have been found to have potential applications in the plasma extraction of several drugs that are used in various indications, such as asthma, allergic rhinitis, and autoimmune hepatitis. Nilsson *et al.* [28] have implemented phospholipid removal plates in the bioanalysis of budesonide and quantified using UPLC/MS/MS and achieved a good LOD of 24 pg/ml. The developed method was applied to compute various pharmacokinetic parameters. Another interesting study reported the selective extraction of (S)-enantiomers of metoprolol (β blocker) in blood samples using LC-MS/MS [29]. Samples were extracted from the biological matrix using phospholipid removal micro elution-SPE plates, and eluates were reconstituted in acetonitrile with methyl benzyl isocyanate for chiral derivatization. The method offers maximum recovery (69.4%–78.7%) and a good LOQ of 0.5 ng/ml along with minimal matrix effect. In addition, this pretreatment method has been widely used in various areas, viz. cosmetic products, pesticides, as well as forensic sciences [30–35].

Technically, these plates streamline workflows by combining protein precipitation and phospholipid filtration in a single step, making them compatible with 96-well formats and automated systems. However, their selectivity can vary depending on analyte chemistry, and some compounds may be unintentionally retained or co-eluted. Despite its outperforming traditional methods in phospholipid removal; however, all matrix effects in complex samples may not be fully eliminated. Besides, relatively higher cost and assay compatibility can limit its routine adoption in analytical laboratories.

2.3. Magnetic solid phase extraction (MSPE)

The complexities associated with traditional SPE methods, such as sorbent selection, isolation, and enrichment of the analyte, can be overcome by using magnetic nanoparticles (MNPs). The core of the nanoparticles comprises iron, nickel, cobalt, and their oxides, while the inorganic materials (such as

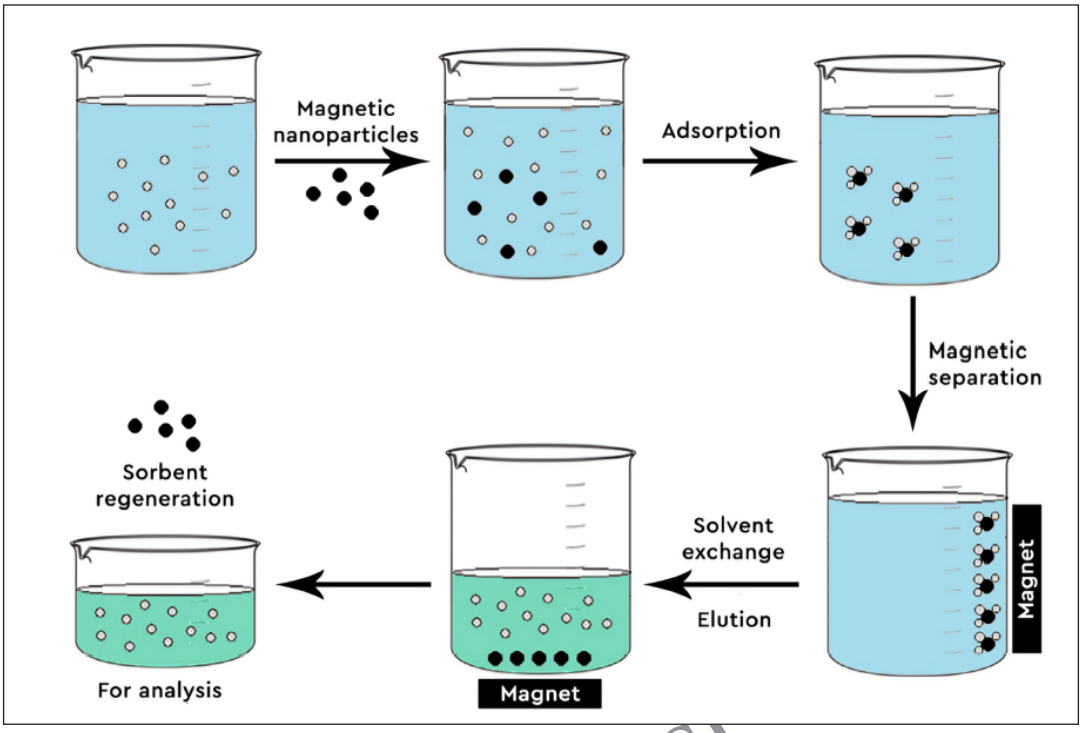

Figure 5. Illustration of the magnetic solid phase extraction process involving functionalized magnetic nanoparticles selectively capturing target analyte through adsorption, followed by magnetic separation, and elution. (Adapted from Plastiras *et al.* [39].

silica, alumina, manganese oxide (IV), or graphene) are used for the surface coating. MNPs are distinguished by a large surface area, high sorption capacity, and good selectivity for analytes because of their small particle size, together with a shorter duration in the extraction process [36–39]. MSPE operates through a sequence of well-defined steps that leverage the unique properties of MNPs. The process begins with the dispersion of MNPs into a sample containing target analytes. These nanoparticles are engineered with surface functional groups that facilitate selective binding. During the adsorption phase, analytes interact with the nanoparticle surfaces primarily through hydrophobic and van der Waals interactions, as well as hydrogen bonding, electrostatic forces, or specific chemical affinities (Fig. 5). Once adsorption is complete, an external magnetic field is applied to perform magnetic separation, efficiently isolating the analyte-bound nanoparticles from the rest of the sample without the need for filtration or centrifugation. In the elution step, a suitable solvent is introduced to desorb the analytes from the nanoparticles, transferring them into a clean solution for downstream analysis such as LC-MS or inductively coupled plasma MS (ICP-MS). Finally, the sorbent regeneration step allows the magnetic nanoparticles to be washed and reused, making MSPE a cost-effective, rapid, and sustainable method for sample preparation [40].

Recent advancements in MSPE have focused on the integration of metal-organic frameworks (MOFs) with MNPs to enhance selectivity and efficiency in pharmaceutical analysis. A notable development involves the use of NH_2-MIL-101(Cr)-based magnetic nanocomposites, which combine the high surface area and porosity of MOFs with the magnetic responsiveness of Fe_3O_4 nanoparticles. These hybrid materials exhibit excellent adsorption capacity and photocatalytic activity, making them effective for the removal and analysis of pharmaceutical contaminants such as acebutolol from aqueous environments. The amino-functionalized MIL-101(Cr) enhances light absorption and charge transfer, while the magnetic core facilitates rapid separation and reusability, supporting sustainable and high-throughput analytical workflows [41].

Asgharinezhad and Ebrahimzadeh [42] extracted nonsteroidal anti-inflammatory drugs, viz. naproxen, diclofenac, and ibuprofen from urine samples by the MSPE method. Good recovery of analytes was observed (85%–90%) with a LOD in the range of 0.007–2000 µg/ml, which indicates the high efficiency of the extraction method. A preclinical study conducted by Wang *et al.* [43] in which the bioactive compound puerarin was efficiently extracted with the MSPE method based on C18 magnetic silica nanoparticles in rat plasma, and its bioanalysis was performed by HPLC. Another interesting study on hair samples pretreatment by MSPE for simultaneous determination of elements, viz. Cr(III), Cu(II), Pb(II), and Zn(II) and quantified by inductively coupled plasma-optical emission spectrometry [44]. The method was found highly efficient with good recovery (85%–104.5%) and a detection limit of 0.01–0.06 µg/ml, and can be suitable for trace element determination. Besides, the MSPE method has been extensively employed for the extraction of secondary metabolites of aflatoxins in food products, drug metabolites and phthalate detection in industrials and cosmetic products [45–50].

Overall, MSPE is a modern sample pretreatment method that employs functionalized magnetic nanoparticles

to selectively capture and concentrate target analytes from complex matrices such as biological fluids, environmental samples, and food extracts. It offers notable analytical benefits, including high selectivity, rapid processing, and reduced solvent consumption, making it particularly effective for trace-level detection. From a technical standpoint, MSPE involves dispersing MNPs into the sample, allowing analytes to bind to their surfaces, and then using an external magnet to isolate the analyte-bound particles, streamlining the workflow and enabling easy automation. However, its performance can be hindered by nanoparticle aggregation, reduced efficiency in viscous or particulate-rich samples. In addition, there is a need for precise surface functionalization to maintain specificity and stability across diverse analyte types [51].

2.4. Microextraction by packed sorbent (MEPS)

The miniaturisation of SPE modified into MEPS, in which the sorbent is integrated into the syringe; therefore, a separate robotic accessory is not required. MEPS may link online to different chromatographic systems (capillary, gas chromatography, and liquid chromatography) and can be fully automated [52]. MEPS comprises of syringe and barrel insert and needle (BIN) to perform sample extraction, and cleanup (Fig. 6). Thermo-packed sorbent is filled in a little tube of BIN, and target analytes are eluted in sequential phases after the syringe feeds the material through this BIN, enabling sample purification or speciation [53]. In addition, various commercial sorbents, including new varieties of graphitic sorbent, polyamide, polyaniline nanowires, functionalized silica monoliths, and so on, have been successfully used in MEPS devices to extract various groups of analytes [54,55].

MEPS is extensively used for the extraction of several drugs in biological fluids (blood, saliva, and urine) and their quantification with different analytical tools. Ferrone *et al.* [56] employed MEPS in the clinical study for the bioanalysis of ciprofloxacin and linezolid among pneumonia patients using the UHPLC-PDA technique. Good recovery (92.4%–97.4%) of target analytes was achieved with a detection limit of 0.007 and 0.004 ng/ml, respectively. Compared with the conventional SPE method, MEPS was found to be simpler, low-cost, requires a small sample volume (10 µl), as well as reduced sampling time. In another reported study [57], simultaneous extraction of the enantiomeric form (-R) and (-S) of metoprolol in blood plasma and saliva samples was accomplished with the help of the MEPS method. The recovery of the study was in the range of 93.1%–97.8% and bioanalysis was performed using LC-MS/MS with high sensitivity. Besides, MEPS has been used for the detection of drugs of abuse such as opiates and cannabis as well as CNS-acting drugs in blood samples using hyphenated tools [58–63]. This pretreatment method offers rapid and effective clean up sample preparations, satisfactory recovery, and minimum matrix effect.

In a nutshell, MEPS is a miniaturized, syringe-based solid-phase extraction technique that integrates sample extraction, cleanup, and preconcentration into a single, reusable device. It offers significant analytical advantages such as reduced sample and solvent consumption, high-throughput capability, and compatibility with automation and direct

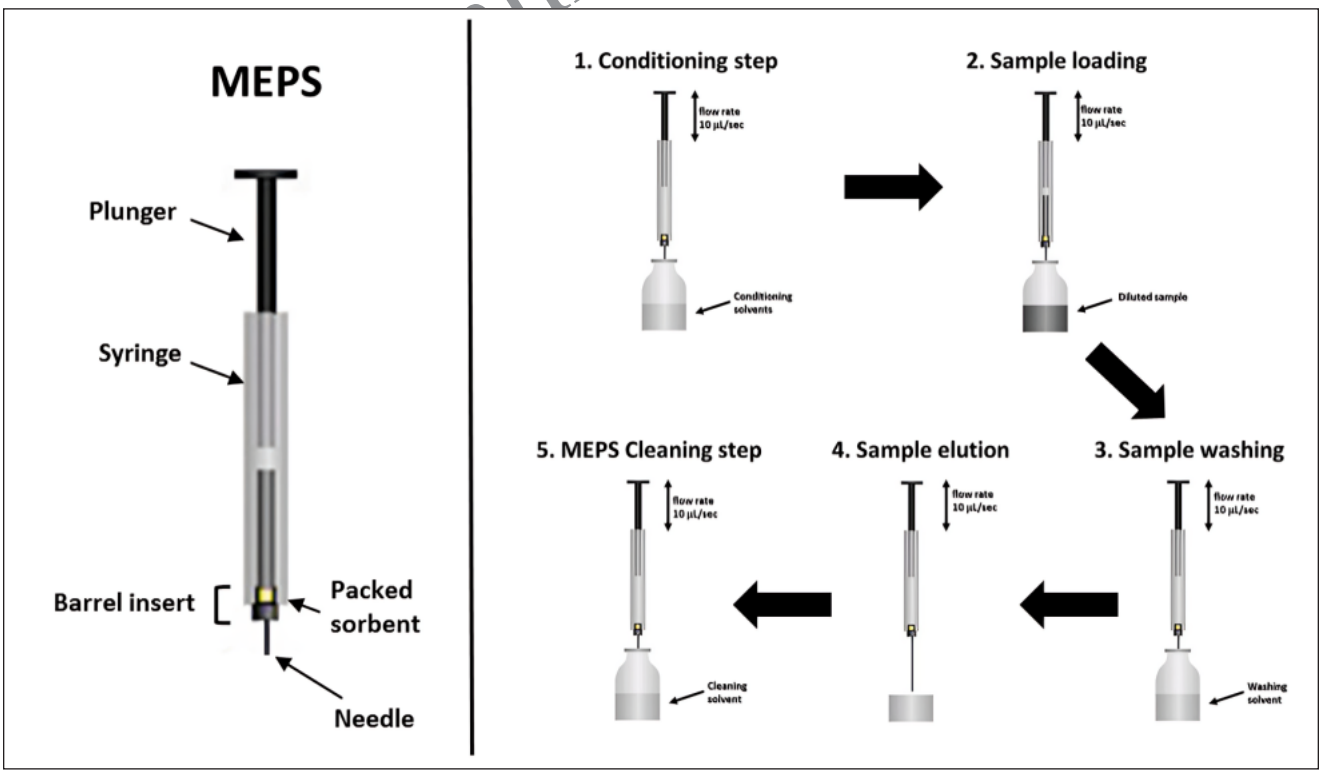

Figure 6. Schematic representation of the workflow of the microextraction by packed sorbent involving selective analyte retention, washing, elution, and cleaning steps. (Adapted from Kabir *et al.* [50].

coupling to LC-MS or GC-MS systems. Technically, MEPS uses a small amount of sorbent (1–4 mg) packed within a syringe or barrel insert, allowing for efficient analyte interaction through repeated aspiration cycles, followed by washing and elution steps all within microliter volumes. Recent advancements in sorbent materials for MEPS are the integration of molecularly imprinted polymers (MIPs), which act as synthetic receptors with high affinity for specific target molecules. These MIPs are tailored through imprinting techniques that create selective binding sites complementary in shape and functionality to the analyte, enabling precise extraction even in trace concentrations. Despite several stellar benefits of MEPS, its performance is highly dependent on sorbent selection, and manual operation in flow rate control can introduce variability due to user-dependent handling. In addition, the issue of sorbent degradation over multiple cycles can affect reproducibility and sensitivity [64].

2.5. Solid phase microextraction

Solid phase microextraction is a solvent-free technique used for extracting analytes from biofluids such as blood and plasma. It employs a sorbent-coated fibre or probe that is directly exposed to the biological matrix, allowing analytes to partition between the biofluid and the coating based on equilibrium dynamics. Biocompatible SPME devices typically use C18-bonded porous silica particles embedded in a nonswelling binder, which resists fouling and enables direct *in vivo* or *in vitro* sampling. This simplifies sample cleanup, stabilises labile analytes, and reduces preparation steps, making it ideal for pharmacokinetic and metabolomic studies [65,66]. A key feature of SPME is the use of fused silica fibres coated with stationary phases of varying thicknesses and polarities, offering high selectivity for different analytes. The efficiency of extraction depends on factors such as ionic strength, contact time, and temperature [67,68]. SPME can be performed in three distinct modes: (a) Direct mode, where the fiber is submerged in the aqueous sample and analytes transfer directly into the coating, often aided by agitation with a small stirring bar; (b) Headspace mode, where analytes are extracted from the gas phase above the sample to protect the fiber from matrix interferences like proteins; and (c) Membrane protection mode, where a selective membrane separates the sample from the fiber, allowing analytes to pass while blocking interfering substances. The membrane primarily serves to safeguard the fibre during extraction.

Gorynski *et al.* [69] have developed LC-MS/MS for the determination of rocuronium bromide and tranexamic acid in blood plasma (0.8 ml). Samples were extracted with a SPME containing a weak cation exchange thin film used as the sorbent and elution solvents for effective cleanup. Similarly, the determination and screening of cannabinoids in brain samples using polyamide-based SPME membrane coupled with LC-MS-APCI were conducted by Yang and Xie [70]. This method has resulted in the maximum recovery (100 %) of cannabinoids.

Another interesting study reported [71] using a novel SPME fibre coated with $Fe_30_4/CU_3(BTC)_2$ metal-organic framework as a sorbent. This unit of SPME connects online with the HPLC unit for the determination and screening of several anti-inflammatory drugs (ibuprofen, diclofenac, naproxen, and nalidixic acid) from human urine, serum, and plasma samples. Besides, SPME has been employed extensively for the extraction of different analytes [72–75].

SPME is a solvent-free, miniaturized sample preparation technique that integrates sampling, extraction, and preconcentration into a single step using a coated fiber or arrow device. It is widely used for volatile and semi-volatile compounds in environmental, food, and clinical analyses due to its simplicity, high sensitivity, and compatibility with GC and LC systems. Technically, SPME operates via adsorption or absorption of analytes onto a polymer-coated fiber, either through headspace or direct immersion modes, and is easily automated for high-throughput workflows. However, its limitations include lower mechanical stability of traditional fibres, potential phase saturation in complex matrices, and sensitivity to solvent swelling, which can affect reproducibility and fiber longevity. Nevertheless, the development of SPME arrow has addressed some of these issues by offering larger sorption phases and improved robustness [76].

2.6. Deep eutectic solvent (DESs)

Deep eutectic solvents are increasingly recognized as an innovative and sustainable alternative for sample pretreatment of biofluids. DESs offer customizable physicochemical properties, low toxicity, and high extraction efficiency. Their ability to disrupt complex biological matrices makes them ideal for isolating small molecules, metabolites, and biomarkers from biofluids such as urine, plasma, and saliva. DESs facilitate protein precipitation and phase separation, enabling cleaner extracts as well as preserving thermolabile compounds [77].

Compared to conventional organic solvent-based liquid–liquid extraction, the DES-based approach provided superior selectivity, reduced matrix effects, and minimized environmental impact. The DES facilitated efficient solubilization of hydrophobic analytes while maintaining sample integrity, making it a promising alternative for clinical and forensic bioanalysis. The advantages of DESs in biofluid pretreatment include high analyte recovery, low matrix interference, and eco-friendly profiles. For instance, DES, composed of choline chloride and phenol (1:2 molar ratio), has been explored for extracting steroid hormones from human urine. The developed method achieved good analyte recoveries (85%–98%) for testosterone, estradiol, and progesterone [78].

Finally, natural DESs derived from food-grade components have been explored for metabolomics and therapeutic drug monitoring. These developments facilitate DESs as key enablers of green chemistry in bioanalytical science, with strong potential for industrial-scale adoption. However, their practical use is not without challenges: some formulations may still involve toxic precursors, and their performance can be inconsistent due to variability in viscosity, solubility, and stability [79]. These factors necessitate careful formulation and validation, particularly when used in sensitive analytical or biological systems.

2.7. Solidified droplet microextraction (SDME)

Solidified droplet microextraction has emerged as a novel and efficient bioanalytical pretreatment method for biofluids over the past few years. This technique involves the use of a small volume of organic solvent suspended as a

droplet in the sample matrix, which solidifies under controlled conditions to facilitate analyte extraction. SDME is particularly advantageous for biofluid analysis due to its minimal solvent consumption, high enrichment factors, and compatibility with downstream analytical techniques such as GC-MS and LC-MS. The method is especially effective for isolating trace-level analytes from complex matrices such as urine, plasma, and saliva, offering a green and cost-effective alternative to traditional liquid–liquid extraction [80].

The integration of SDME with DESs represents a significant advancement in bioanalytical sample preparation. It combines the environmental benefits of green solvents with the operational simplicity of microextraction techniques. Recent developments in droplet microfluidics have further refined this method, enabling precise control over droplet formation, manipulation, and solidification [81]. These innovations have expanded the applicability of SDME to high-throughput clinical diagnostics, therapeutic drug monitoring, and metabolomics. A recent study [82] highlights the application of dispersive liquid–liquid microextraction based on the solidification of floating organic droplets for the extraction of different pharmaceutical residues (ibuprofen, naproxen, and diclofenac) from human urine. The researchers utilized a DES as the extraction medium, which was solidified post-extraction to isolate the analytes. The developed method showed enhanced extraction efficiency with good analyte recovery (88% to 95%). Besides, this approach also reduced matrix interference, leading to improved sensitivity and reproducibility in mass spectrometric analysis.

Overall, SDME is a minimalist, eco-conscious sample preparation technique that uses a single microdroplet of organic solvent either suspended directly in the sample or in its headspace to extract and preconcentrate analytes. Its analytical appeal lies in its simplicity, low cost, and minimal solvent usage, aligning well with green chemistry principles. Despite its advantages, SDME faces limitations such as droplet instability, slow extraction kinetics, and limited surface area, which can affect reproducibility and sensitivity. To address these issues, solidification of floating organic drop microextraction has been developed by stabilizing the droplet and improving recovery [82].

2.8. Affinity sorbent extraction

The most current developments in the field of sample preparation include frameworks modified with various biomolecules (such as proteins, nucleobases, amino acids, aptamers, and so on) as ligands to create affinity-based sorbents. These affinity materials' synthesis and inclusion methods, as well as their use as sorbents for the selective extraction of molecules and the cleanup of intricate samples. These sorbents have been used in sample treatment directly, or they have been specifically created to satisfy the needs of analytical applications [83]. Immunoextraction, molecular imprinted polymer, and aptamers are the methods that work on the principle of affinity sorbent extraction. The literature survey revealed that the application of the affinity sorbent extraction methods is primarily in the diagnosis of diseases by detecting the biomarkers in the blood samples. Representative case studies have been summarized in Table 2.

2.8.1. Immunosorbent

In this technique, antibodies have been immobilised on a solid phase, also known as immunoaffinity extraction sorbents. It offers good selectivity and high affinity due to the antigen–antibody interaction. The robust procedures allow one-step selective extraction and enrichment of specific compounds. Solid support is decorated with antibodies and packed in an SPE cartridge to create the immunosorbents. The formation of the antibody–antigen complex during this step is predominantly influenced by electrostatic forces. Desorption will only take place by significantly altering the experimental conditions due to the high energies of the biological interactions. However, matrix materials and sample additives can have an impact on analyte-antibody interactions, which can also reduce extraction recoveries [84]. Immunocapture methods are gaining renewed attention in pharmaceutical and biomedical analysis due to their high specificity and adaptability for complex biological matrices. These techniques utilise antibodies or antibody fragments to selectively bind and isolate target molecules such as therapeutic proteins, biomarkers, or drug metabolites. When coupled with advanced analytical platforms like LC-MS, immunocapture enables sensitive and selective quantification, particularly useful in pharmacokinetic and pharmacodynamic studies. Recent innovations have focused on hybrid immunocapture LC-MS workflows that combine ligand-binding specificity with mass spectrometric sensitivity, streamlining assay development and improving throughput. These methods are especially valuable in early-stage drug development, where rapid and reliable bioanalytical tools are essential [85].

Immunosorbent-based extraction methods largely find their application in the screening of biomarkers for disease diagnosis and their progression [86,87]. For instance, human chorionic gonadotropin, a biomarker, has been successfully extracted and analysed by Johannsen *et al.* [88] using the combination of paper-bound streptavidin as an anchor for biotinylated antibodies and quantified by LC-MS. Similarly, cytokeratin fragments are a high-potential biomarker in many epithelial cell cancers. A high-performance immunosorbent-based biosensor technique was employed for the extraction of this biomarker [89]. It involves the affinity of an antigen towards antibodies mechanism using Photographic paper/Ag ink/antibody as a capturing plate and $[Fe(CN)_6]^{3-/4-}$/KCl solution as a solvent. In addition, a rapid colourimetry sensing system using competitive enzyme-linked immunosorbent assay (ELISA) on a microfluidic paper-based analytical device has been developed for the detection and determination of ketamine in oral fluid (3 µl) using a coated ketamine antibody [90].

2.8.2. Molecular imprinted polymers

MIP is a selective sorbent for SPE, which has gained popularity recently for selective extraction of analytes [91]. Functional polymers were grafted (Fig. 7) on the surface of silica particles and then cross-linked with the templates sustained by dipole interactions or hydrogen bonding [92]. MIPs are of tremendous interest because they can be produced

Table 2. Application of novel pretreatment methods for the extraction of biomarkers.

Biomarker	Extraction technique	Matrix	Volume (µl)	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Neopterin, tryptophan, creatinine, uric acid, kynurenine (Inflammatory biomarkers)	Immunosorbent	Saliva	200 µl	Microcon centrifugal filter device (Merck)	Sodium hydroxide	90%–114%	0.00036– 0.44 µmol/l (HPLC-FID/DAD)	− Simultaneous extraction of inflammatory biomarkers − Simple and time saving process − This method considered as gold standard for extraction	[86]
Human chorionic gonadotropin (Biomarker)	Immunosorbent	Serum	5 µl	Biotinylated antibodies Covalently linked to HEMA-VDM-functionalized paper.	PBS with Tween 20, ammonium bicarbonate buffer	33%	65 pg/ml (LC-MS)	− Convenient storage and transport − Analyte stability − Low sample amount − Minimal invasive sample collection	[88]
Cytokeratin fragment Cyfra 21.1 (cancer biomarker)	Immunosorbent	Saliva	5 µl	Photographic paper / Ag ink / antibody	$[Fe(CN)_6]^{3-/4-}$/ KCl solution	–	0.001–2.5 ng/ml (High performance biosensors)	− Practical, sensitive, fast, selective method − Portable and cost-effective	[89]
Hypoxia-inducible factor-1 (HIF-1α) (Myocardial infarction biomarker)	Aptamer	Blood serum	50 µl	AuNP-Coated Gold nanospheres functionalized with HIF-1α-binding aptamer	PBS buffer, gold seed growth solution, Na_2HPO_4, citric acid, H_2O_2, TMB	≥ 80%	0.2 ng/ml (colorimetry)	− Good stability − Not susceptible to environmental factors like pH temperature − Easy preparation − Low cost	[109]
Cortisol (stress biomarker)	Aptamer	Saliva	900 µl	Au-NP-duplex DNA	Ethanol	89%–114%	0.37 ng/ml (Lateral flow Assay Biosensor)	− Higher stability − Low cost − Potential improvement with the future SELEX procedure	[110]
Amyloid-β oligomers (Alzheimer's disease biomarker)	Aptamer	Blood serum	100 µl	AβO-specific aptamer immobilized on the surface of core-shell nanoparticles (silver and silica)	Phosphate buffer saline, pH 7.4	93%–107%	1.22 pg/ml (Transmission electron microscopy)	− Portable − Simplicity of sample preparation − Design versatility, facial modification − Low mol. wt, chemical stability − High binding affinity − High selectivity	[111]

Continued

Biomarker	Extraction technique	Matrix	Volume (µl)	Sorbent	Solvent	Recovery	Quantitation range (Technique used)	Significance	References
Exosome (Colorectal cancer biomarker)	Aptamer	Blood serum	5 µl	Graphene oxide-DNA aptamer (cy3 conjugated CD63-aptamer)	Tris HCl buffer, DNase-1 reaction buffer, EDTA	45%– 65%	2.4×10^4 particles/ µl (Fluorescence spectroscopy)	− Steps are simple and can be mixed with fluorescent dye-conjugated aptamers − No need of isolation step. − Time saving − Joint detection of two exosome biomarkers is possible − Improve the storage stability	[112]
Immunoglobin E (biomarker)	Aptamer	Blood serum	5 µl	Fabrication of zwitterionic peptide and IgE aptamer onto a microporous Au substrate.	Phosphate buffer saline, water	97%101%	42 pg/ml (Differential pulse voltammetry)	− Microporous Au greatly increases the surface area for sensitivity − Satisfying antifouling ability	[113]
Insulin (biomarker)	Aptamer	Blood plasma	5 µl	Poly-orthophenylene diamine ornate gold nanoparticles (GNPs) with single stranded DNA aptamer immobilized of pencil graphite electrode.	Trichloroacetic acid	88% –103%	1.0–1,000.0 nmol/l (Electrochemical impedance spectroscopy)	− Specific and sensitive tools for the extraction and detection of plasma insulin in presence of proteins − Mechanical and chemical stability − Modification at the terminal end of the aptamer sequence is possible − Reversibility under thermal denaturation	[114]
Thrombin (biomarker)	Aptamer	Blood serum	50 µl	Au-mNP functionalized core shell magnetic microspheres.	Tyrosine for direct digestion	≥ 99%	0.36 ng/ml (MALDI-TOF-MS)	− Simpler than other methods. − Combining easy immobilization − Fast separation − High throughput and high selectivity and sensitivity − Stability in harsh environment	[116]

SLE, Supported Liquid Extraction; PRP, Phospholipid Removal Plate; MEPs, Microextraction by Packed Sorbents; MIPs, Molecular Imprinted Polymers; SPME, Solid Phase Microextraction; MSPE, Magnetic Solid Phase Extraction.

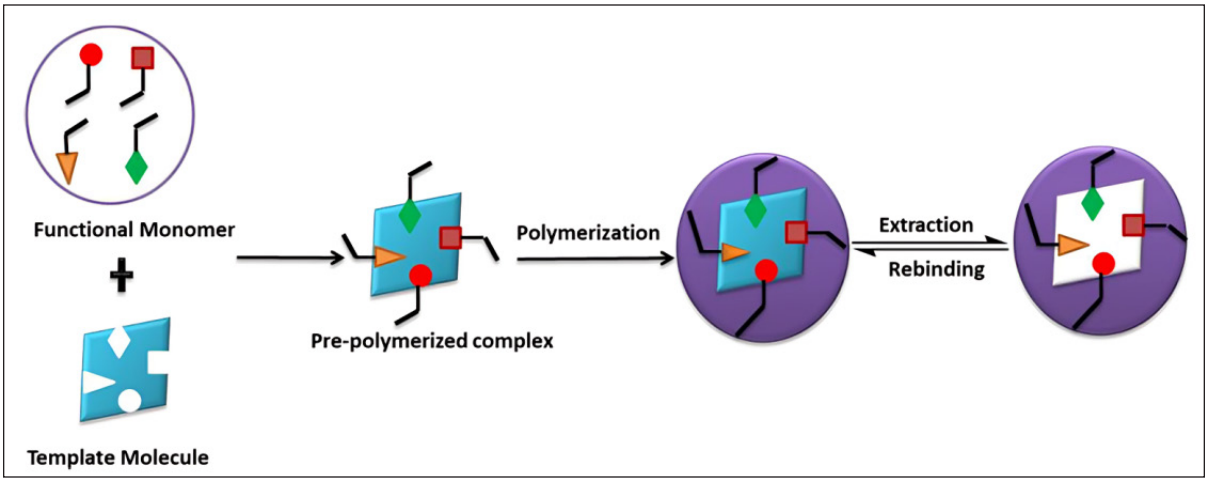

Figure 7. Mechanistic understanding of molecular imprinted polymer, where functional monomers are polymerized around a template molecule and subsequently removed to create selective binding sites complementary to the target analyte. (Adapted from Sajini and Mathew [95].

more cheaply, have greater chemical and physical stability, and can be reused. However, molecular imprinting takes a long time and can have issues like heterogeneous imprinting, whereas macromolecule imprinting is challenging [93–95].

Several studies have reported for the use of MIPs-based extraction procedures and be highly robust, chemically stable, have less extraction time, and have high selectivity [96]. Magnetic multiwalled carbon nanotubes have been developed by Madrakian *et al.* [97] via synthetic amidoamine as the monomer and naproxen as the template. This method has been used for the bioanalysis of naproxen in human urine and detected by spectrofluorometric methods. Yang *et al.* [98] have performed a test on a simple Pickering emulsion polymerization method used to prepare bisphenol A imprinted polymer microspheres and used for the extraction from the human urine sample. Similarly, several analytes have also been extracted by MIPs in different biological matrices with good [99–103].

MIPs are synthetic materials engineered to mimic natural molecular recognition systems, such as antibodies, by creating highly specific binding sites tailored to a target molecule. Their appeal lies in their robustness, chemical stability, and adaptability across various formats, making them valuable in applications such as sensors, separation, drug delivery, and environmental monitoring. Technically, MIPs are formed by polymerizing functional monomers around a template molecule, which is later removed to leave behind complementary cavities. This lock-and-key mechanism enables selective recognition even in complex matrices. However, a few challenges associated with MIPs are template removal, limited binding site accessibility, and reduced performance in aqueous environments due to disrupted noncovalent interactions. In addition, their commercial translation is hindered by difficulties in reproducibility, scalability, and integration into real-world devices. Finally, the innovative application of conducting polymer-based MIPs and stimuli-responsive designs is helping bridge the gap between laboratory research and practical deployment [104].

2.8.3. Aptamers

Aptamers are synthetic, single-stranded oligonucleotides that are short (up to 110 base pairs), fold into distinctive shapes with high specificity. Aptamers use conformational complementarity to identify and bind their target. Furthermore, adjustments in the buffering system (salt composition and ionic strength) cause the release of the bound target molecules. Aptamers also aid in reducing the utilization of research animals, unlike antibodies, which frequently need animals for both initial discovery and manufacturing. Finally, SELEX (Systematic Evolution of Ligands by Exponential Enrichment) can be used to create specific aptamers [105] for a given target analyte, as shown in (Fig. 8). Aptamer functionalized materials (AFMs) are created by immobilizing aptamers on a solid support using a linking agent (linker). Compared to free aptamers, AFMs have higher chemical, biological, and mechanical stability in addition to their intrinsic high selectivity and strong affinity [106]. The stellar benefits of aptamers, such as high thermal stability, tailored specificity, good affinity, and scalability of production, have resulted in large-scale use in rapid diagnostic applications [107]. Besides, they can be explored in various immunoassays such as ELISA, western blot, immunohistochemistry, and flow cytometry in biomedical research and clinical diagnostic applications [108].

Hypoxia-inducible factor-1 (HIF-1α) is a transcription factor that has been proven to be widely involved in hypoxia metabolism and has developed into an essential regulator of myocardial injury. A highly sensitive colorimetry technique has been created to measure HIF-1α in a circulating rat serum exosome, which is an early indicator for myocardial infarction. Wang *et al.* [109] investigated the growth of gold nanospheres functionalized with HIF-1α binding aptamer using seed-mediated culture. Na_2HPO_4, citric acid, H_2O_2, and TMB (coloring agent) were used as solvents in the detection and extraction of HIF-1α biomarker in 50 µl of serum sample. This method was able to offer good recovery (≥ 80%) of biomarkers, and the lowest LOD was found to be 0.2 ng/l. Dalirirad *et al.* [110] reported the detection

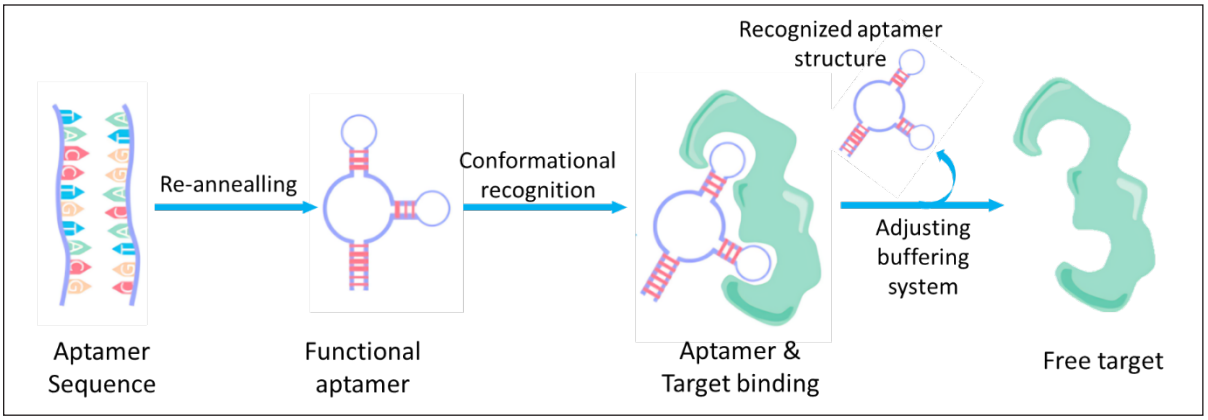

Figure 8. Representation of aptamer-based recognition mechanism, binding to the target, and subsequently target release. (Adapted from Mayol *et al.* [117].

of cortisol (stress biomarker) using duplex deoxyribonucleic acid (DNA) aptamer conjugated to the surface of gold nanoparticles by Au-S bonds as the sensor probe in a lateral flow assay. Similarly, the aptamer method of extraction is widely used in the diagnosis of biomarkers for diseases like cancer, diabetes, and diseases related to the central nervous system and analytical application in biological samples reported in studies [111–117].

Aptamers are short, single-stranded DNA or RNA molecules selected through the SELEX process for their ability to bind specific targets with high affinity and specificity, offering a promising alternative to antibodies in diagnostics, therapeutics, and biosensing. Their key analytical advantages include low immunogenicity, ease of chemical synthesis, and the ability to function under a wide range of conditions, making them ideal for applications in complex biological environments. Technically, aptamers can be engineered and modified post-SELEX to enhance stability, binding strength, and resistance to nucleases, with strategies such as truncation, multivalent integration, and chemical modification improving their performance. However, aptamers face notable limitations: they are prone to rapid degradation in biological fluids, often exhibit poor *in vivo* stability, and their small size leads to fast renal clearance. In addition, the SELEX process can be labor-intensive and may yield aptamers with suboptimal binding characteristics, requiring further optimization to ensure reproducibility and clinical viability [118].

In summary, all the novel sample pretreatment methods are found to offer stellar benefits of automation, good recovery, and faster cleanup (Table 3). However, they differ in terms of their versatile application and cost-effectiveness. Among all, immunosorbents and aptamers were found to be relatively costly due to frequent changes in the sorbent and have very specific applications in biomarker studies. On the other hand, other novel methods possess wide applications in drug analysis and are found to be less costly. Despite several advantages, there are some associated limitations, for instance, clogging of the sorbent, carryover effect, issues about hydrophobic analytes, template bleeding, and so on. However, these limitations have been overcome by applying innovative interventions in the pretreatment procedure to some extent.

3. RECENT TRENDS IN BIOANALYTICAL PRETREATMENT METHODS

Bioanalytical laboratories are increasingly prioritizing green sample preparation methods to align with sustainability goals and reduce environmental impact. In addition, bioanalytical pretreatment methods for biofluids have undergone a significant transformation, driven by the need for higher sensitivity, sustainability, and automation. For instance, solvent-free or solvent-reducing techniques such as microextraction and direct chromatographic methods that eliminate or minimize the need for hazardous solvents. The integration of biocompatible materials, miniaturized devices, and multitarget extraction systems—capable of isolating diverse analytes in a single step—further supports eco-friendly practices [119].

In drug analysis, green sample preparation is gaining traction through innovations that combine sustainability with high analytical performance, reflecting a broader commitment to environmentally responsible science. Techniques such as solidified droplet microextraction, DESs, and natural DESs are replacing traditional solvent-intensive methods. These approaches reduce environmental impact, minimize sample volume requirements, and improve analyte stability, aligning with sustainability goals and decentralized healthcare models. Similarly, the development of advanced microextraction strategies like dispersive liquid–liquid microextraction, hollow fiber liquid-phase microextraction, and electro-membrane extraction offers improved analyte recovery, reduced solvent usage, and compatibility with high-throughput workflows. Microsampling technologies such as volumetric absorptive microsampling, microneedle-based sampling, and dried blood spot methods are also gaining huge attraction among pharmaceutical scientists. These technologies enable decentralized and offer minimally invasive sample collection while maintaining analyte stability [50,120].

In recent years, a major innovation has been the integration of artificial intelligence (AI) and online automation into bioanalytical workflows [121]. AI is being used to optimize sample processing, predict analyte behavior, and enhance data interpretation, particularly in metabolomics

Table 3. Comparative analysis of different novel sample pretreatment methods.

Novel pretreatment method	Miniaturization	Automation	Cleanup speed	Recovery	Matrix effect	Cost	Application	Reusable	Limitation
SLE	Yes	Yes	Good	Not satisfactory	Yes	Medium cost	Versatile	One time use	Single use consumable requirement
PRP	Yes	Yes	Good	Satisfactory	No	Low cost	Versatile	**Reusable**	Limitations for Hydrophobic analyte
MSPE	Yes	Yes	Fast	Satisfactory	No	Low cost	Versatile	**Reusable**	-Set magnetic beads apart from other methods of sample isolation, which might have different protocols for different types of samples.
MEPS	Yes	Yes	Fast	Satisfactory	No	High cost	Versatile	**Reusable**	Clogging of Sorbent, large volume sample performing problem, & carry over effect
SPME	Yes	Yes	Fast	Satisfactory	No	Low cost	Versatile	**Reusable**	Low effectiveness of process due to small amount of PDMS coated on fiber
IMMUNOSORBENT	Yes	Yes	Time consuming	Satisfactory	No	High cost	Specific	One time use	Animal requirement, specific antigen require, time consuming, & risk of cross reactivity
MIPS	Yes	No	Fast	Satisfactory	No	Medium cost	Specific	**Reusable**	Possible template bleeding, sometimes tedious synthesis procedures, & problematic application to aqueous samples.
APTAMERS	Yes	No	Fast	Satisfactory	No	Medium cost	Specific	One time use	Susceptible to nuclease degradation, limited building block diversity, PCR bias in SELEX method

and personalized medicine. Automation technologies such as robotic liquid handlers and online SPE systems are streamlining sample preparation, improving reproducibility, and enabling high-throughput analysis [122]. These systems not only reduce human error but also significantly increase sample throughput, as compared to manual methods. Predictive analytics and real-time monitoring are also being incorporated to support adaptive trial designs and faster decision-making in clinical research.

Overall, the adoption of green sampling technologies together with the convergence of AI and automation not only reduces waste and energy consumption but also enhances analytical efficiency. These developments have a significant impact in making bioanalytical science more accessible, eco-conscious, and adaptable to modern healthcare needs.

4. CONCLUSION

The present review comprehensively explores the landscape of novel sample pretreatment methods and their transformative role in pharmaceutical bioanalysis, particularly in the extraction of drugs and biomarkers from complex biological matrices such as plasma, serum, and urine. These innovative techniques are designed to address the persistent challenges of matrix effects, low recovery, and labor-intensive workflows that are common in conventional sample preparation methods.

A central focus of the review is the working principles of various emerging techniques, including SPME, MSPE, and novel sorbent-based methods such as immunosorbents and aptamer-functionalized materials. These methods are evaluated not only for their analytical performance but also for their compatibility with miniaturization and automation, two critical factors for high-throughput pharmaceutical analysis. The review systematically compiles case studies that detail the analytical parameters of these methods, such as recovery rates, LOD, and the detection techniques employed (e.g., LC-MS/MS, GC-MS, and HPLC). Immunosorbents and aptamers have shown exceptional promise in clinical diagnostics due to their

high specificity and binding affinity, making them ideal for the selective extraction of biomarkers. Despite their advantages, the widespread adoption of these novel methods in routine pharmaceutical workflows is currently limited by higher initial costs and the need for specialized materials or instrumentation. However, ongoing research and technological advancements are steadily reducing these barriers, paving the way for broader implementation.

In a nutshell, the review underscores that while traditional methods still hold value, the integration of novel pretreatment techniques is essential for improving the efficiency, sensitivity, and reliability of pharmaceutical assays. These advancements are expected to significantly influence the future of bioanalytical workflows, especially as the demand for personalized medicine and rapid diagnostics continues to grow.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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