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Review

New challenges in (bio)analytical sample treatment procedures for clinical applications

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Abstract: The primary cause of poor and ambiguous results obtained from bioanalytical process is the sample pre-treatment, especially in clinical analysis, e.g., because it involves dealing with complex sample matrices, such as whole blood, urine, saliva, serum and plasma. So, the aim of this review is to focus the attention on classical and new techniques of pre-treatment for biological samples used in the bioanalytical process. We discussed the methods generally used for these types of complex samples. Undoubtedly, it is a daunting task to deal with biological samples because the analyst may encounter substantial loss of the analytes of interest, or the overall analysis may be too much time consuming. Nowadays, we are inclined to use green solvents for the environment, but without sacrificing the analytical performance and selectivity. All the characteristics mentioned above should be added to the difficulty of withdrawal of samples like blood, because it can be an invasive practice. For these reasons, now we can also find in the literature, the use of saliva as an alternative biological sample and new techniques that don't require substantial sample pre-treatment such as fabric phase sorptive extraction (FPSE). The text has been divided into two distinct parts: in the first one, we described clinical applications under different subsections such as anticancer drugs, antibiotics, vitamins, antivirals, non-steroidal anti-inflammatory drugs, statin, imidazoles and triazoles. The second part is dedicated to sample preparation techniques for diagnostic purposes and is divided into different sample preparation techniques: solid phase microextraction (SPME), Microextraction by packed sorbent (MEPS), Dispersive liquid–liquid microextraction (DDLME), and Fabric Phase Sorptive Extraction (FPSE).

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1. Introduction

The analysis of biological samples requires an adequate sample preparation procedure that has an inevitable role in the analytical process. In fact, the sample preparation step may influence the accuracy of results, and it consumes most of the time needed for the overall analytical workflow.

The traditional methods applied in many fields including bioanalysis, such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE), have shown some limitations due to their use of toxic organic solvents and long processing time. Therefore, new methods based on microextraction techniques have been developed to achieve high recovery of target analytes from simple to complex sample matrices, and to comply with the concepts of the Green Chemistry (GC) and Green Analytical Chemistry (GAC). [1]

Biological samples include whole blood, plasma, serum, urine, feces, saliva, bile, hair, sweat, breast milk, cerebrospinal fluid (CSF), tissues, and bio analytes (DNA, defined proteins, drugs and specific metabolites or unknown molecules).

The stability of these samples is important, often they are rich in endogenous components such as carbohydrates, proteins, lipids, and salts that can interfere with the targets search due to matrix effects. Moreover, analytes are often present at very low concentration and for this reason the target analytes need to be preconcentrated prior to analysis that often enhance the levels of interfering molecules (e.g., drugs, salts and metabolites, nucleic acids, proteins, and peptides). [2]

After collection from patients and appropriate storage, the sample clean up and preparation step becomes crucial to avoid damage also caused by endogenous degrading properties (enzymes activities, cell death, etc.) and to perform an accurate and selective bioanalysis to obtain a real and correct "picture" of the studied system. [3]

These sample preparation techniques include liquid phase microextraction (LPME) with its different modifications such as single-drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME), hollow fiber liquid-phase microextraction (HF-LPME) and solidified floating organic drop microextraction (SFO-DME). In LPME, the extraction phase is based on the action of different solvents (e.g., DESs, ILs, and ferrofluids) that with its features and properties play an important role for the entire process (in terms of recovery, enrichment factor, selectivity).

Other techniques include solid phase microextraction (SPME), microextraction by packed sorbent (MEPS), and fabric phase sorptive extraction (FPSE). [4,5] These techniques manifest a rapid development in different fields, such as biological, environmental, food sciences, natural products, forensic medicine, and toxicology [1].

The clinical applications of (bio)analytical sample treatment procedures are very extensive in different fields, ranging from the search for biomarkers useful in the diagnosis of many diseases to those necessary for personalized therapeutic drug monitoring (TDM).

In this review, the attention is focused primarily on the clinical applications of these procedures, highlighting the major advantages and results that have been made in laboratory and clinical practices.

2. Clinical applications

2.1 Anticancer drugs

Zufía, Aldaz, and Giráldez (2004) reported the use of a mixture of ethyl acetate and acetonitrile (4:1, *v*/*v*) after sample acidification with orthophosphoric acid to simultaneously extract capecitabine, 5′-DFUR (5′-deoxy- 5-fluorouridine), 5-FU (5-fluorouracil), and 5-FUH2 (5,6-dihydro-5-fluorouracil) from plasma. Similar extraction method is also

reported using LLE with a mixture of ethyl acetate and acetonitrile as the extractant (8:3, *v*/*v*). [6]

Piórkowska et al. (2014) have reported a similar extraction method using a mixture of ethyl acetate and acetonitrile (4:1, *v*/*v*) but without sample acidification. Acidification was not deemed necessary because the assay was developed to determine only Capecitabine concentrations (no metabolites). [6]

Table 1. Pre-treatment of biological samples for Capecitabine and its metabolites

Analyte	Sample	Treatment	LOD/LOQ	Ref.
Capecitabine 5′-DFUR 5-FU 5-FUH2	Human plasma	LLE	LOQ: 0.025ug/mL LOD: 0.01ug/mL	[7]
Capecitabine	Human plasma	LLE	CC: 0.05/10 ug/mL	[8]
Capecitabine	Human plasma	LLE	LLQ: 1.00/10.00 ng/mL	[9]
5-FU FBAL		LLE LLE + SPE		
Capecitabine 5′-DFCR 5′-DFUR 5-FU	Human plasma	SPE	LOD: 0.007(Capecitabine) 0.08 (5-DFUR) 0.6 (5-DFCR) 0.08 (5-FU) ng/mL LOQ: 0.02 (Capecitabine) 0.3 (5-DFUR) 2 (5-DFCR) 0.3 (5-FU) ng/mL	[10]
Capecitabine	Human plasma	SPE	LOD: 39 ng LOQ: 156 ng	[11]
Capecitabine	Human plasma	SPE	LOQ: 156 ng/mL LOD: 78 ng/mL	[12]
Capecitabine 5′-DFCR 5′-DFUR	Human plasma	Online SPE	LOQ 1.4 ng/mL (Capecitabine) 17.6 ng/mL (5-DFCR) 8.4 ng/mL (5-DFUR)	[13]
Capecitabine 5'-DFCR 5'-DFUR 5-FU	Human plasma	PP	LOQ: 10.0 (Capecitabine) 10.0 (DFCR) 10.0 (DFUR) 2.0 (5-FU)	[14]
Capecitabine	Human serum	PP	LOQ: 1 ug/mL	[15]
Capecitabine 5'-DFCR 5'-DFUR 5-FU	Plasma Tumor tissue liver	PP	LOQ ng/mL Plasma: 4.0(Capecitabine) 1.4 (DFCR) 3.3 (DFUR) 45.8 (5-FU) Tumor tissue: 1.3 Capecitabine	[16]

			1.7 DFCR 0.5 DFUR 50.0 5-FU Liver: 13.0 Capecitabine 3.0 DFCR 92.0 5-FU No interference DFUR	
Capecitabine 5'-DFCR 5'-DFUR 5-FU 5-FUH2	Human plasma	PP	LOQ: 1 ug/mL	[17]
Capecitabine 5′-DFCR 5′-DFUR 5-FU 5-FUH2 FBAL	Human plasma	LLE	LOQ: 20.0 ng/mL	[18]
5′-DFCR 5′-DFUR 5-FU 5-FUH2 FUPA FBAL	Human plasma	PP	LOQ: 50.0 ng/mL	[19]

Cap: Capecitabina; 5′-DFUR: 5′-deoxy- 5-fluorouridine; 5-FU: 5-fluorouracil; 5-FUH2: 5,6-dihydro-5-fluorouracil; 5-DFCR: 5′-deoxy-5- fluorocytidine; FBAL: α-fluoro-β-alanine; FUPA: α-fluoro-β-ureidopropionic acid; LLE: liquid-liquid extraction; PP: protein precipitation; SPE: solid phase extraction

Zufía, Aldaz, and Giráldez [7] used LLE, specifically using a mixture of ethyl acetate and acetonitrile (4:1, *v*/*v*) after acidification of samples to extract Capecitabine, 5′-dFUR, 5-FU and 5-FUH2 from human plasma. Piórkowska et al. [8] reported a similar LLE method using a mixture of ethyl acetate and acetonitrile (4:1, *v*/*v*), but without sample acidification, because they wanted to find only Capecitabine and not its metabolites.

Many studies reported the use of protein precipitation like sample treatment for bioanalytical analysis. Deng et al. [14] and Thorat et al. [15] used methanol as the solvent to precipitate the proteins from human plasma and human serum. Furthermore, Vainchtein et al. [17] with the aim of not losing the drug, they added 10% *v*/*v* of trichloroacetic acid in water.

Another protocol used to extract anticancer drug, specifically Osimertinib, involved LLE. Fresnai et al. used tert-butyl methyl ether for simultaneous extraction and concentration of it. This method was developed to follow treatment for patients with non-small cell lung carcinoma with epidermal growth factor receptor activating mutation. [20]

Llopis et al. studied nine kinase inhibitors including cobimetinib, dasatinib, ibrutinib, imatinib, nilotinib, palbociclib, ruxolitinib, sorafenib and vemurafenib; two active metabolites of them: N-desmethyl imatinib, N-oxide sorafenib; and two Anti Androgen drugs: abiraterone and enzalutamide. They used a single step protein precipitation with 100 uL of aqueous Zinc Sulfate Monohydrate $ZnSO_4 \cdot H_2O$ (10% *w*/*v* pH 5.4), adding this to 50 uL of human plasma followed by vortexing and centrifugation. [21]

Locatelli et al. used whole blood, saliva, and urine samples. The difficulty, especially for whole blood, was to minimize the matrix interferents that can mess up the analysis. Usually, the first step is protein precipitation to convert whole blood into

plasma. In this case, the authors used directly whole blood without converting into plasma, thanks to the extraction protocol using Fabric Phase Sorptive Extraction (FPSE) to avoid the possible loss of analytical information related to the quantification of anastrozole, letrozole and exemestane used in treatment of metastatic breast cancer. [22]

2.2. Antibiotics

Ferrari et al. wanted to highlight the matter of a personal treatment of bacterial infections, because different people may respond to the drugs in different ways. So, they followed pharmacokinetics studies on Piperacillin, Meropenem, Linezolid and Teicoplanin in plasma samples from patients treated with the mentioned antibiotics. Pre-treatment involved a liquid-liquid extraction, vortexing for 10 seconds and addition of a precipitation buffer, and then vortexing followed by centrifugation. The supernatant was mixed with a dilution buffer and finally analyzed. [23]

Wongchang et al. performed their sample preparation through protein precipitation. Plasma samples were aliquoted, and they added acetonitrile with cefotaxime as internal standard. The samples were mixed and centrifuged. After this, the supernatant was loaded on Phree phospholipid removal plate and vacuum was applied. Finally, the samples were diluted, mixed and centrifuged. [24]

Krnac et al. used human plasma as the sample for their analysis. The aim of this study was to monitor tazobactam, piperacillin and meropenem in patients treated in intensive care unit. The authors used a mixture of methanol-acetonitrile-water (6:2:2, *v*/*v*/*v*), specifically 200 uL for 10 uL of human plasma plus 10 uL of internal standard, to precipitate proteins. After mixing, the samples were centrifuged, and the supernatant was used for the analyses. [25]

2.3 Vitamin

Rola et al. used LLE to find concentration of vitamin D and its metabolites in serum. In the beginning of the procedure, the authors applied LLE followed by PP, but due to low recovery of 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2$ D_3), they opted for only PP in two different steps: the first one for releasing the compound bound to the protein and the second step is to precipitate most proteins possible. In this case, the organic solvent was acetonitrile. [26]

Also, Hotta et al. used PP to understand pharmacokinetic profile of methylcobalamina, which is important in reaction of Vitamin B12. In this case, the authors used methanol as protein precipitation solvent, because the extraction of methylcobalamina was higher with methanol than with acetonitrile. [27]

2.4 Antiviral

Present HIV therapy appreciates the use of combination of different drugs, like dolutegravir, elvitegravir, reltegravir, nevirapine and etravirine. Bollen et al. studied the therapeutic drug monitoring of these antiretroviral drugs to analyze the pharmacokinetics trials. The authors used plasma samples that pretreat whit PP and used the supernatant as liquid to analyze. [28]

Elkady et al. analyzed the treatment of hepatitis C viral infection. Specifically, the combination therapy of sofosbuvir and velpatasvir was found success in the treatment. They used human plasma, which was pretreated with acetonitrile, then vortexed and finally centrifuged. [29]

2.5 Non-steroidal anti-inflammatory drugs (NSAIDs)

Raabova et al. used human serum taken from patients that were under treatment of intravenous infusion with 75 mg of sodium diclofenac. Blood samples were diluted 10 times with 20% aqueous acetonitrile, centrifuged, and the supernatant was analyzed. [30]

Kabir et al. in 2018 have focused attention on the analysis on inflammatory bowel disease (IBD). Among different treatment regimens used in IBD, therapeutic intervention by oral administration of cortisone, ciprofloxacin and/or sulfasalazine among others are common practices. As such, the authors used a combination of cortisone, ciprofloxacin, and sulfasalazine as an oral treatment regimen. For pharmacokinetic studies, the authors used physiological samples like plasma, serum, saliva, and urine. The whole blood was diluted with ultrapure water (1:5, *v*/*v*) and vortexed. Human plasma was directly vortexed after adding internal standard and the same protocol was used for urine. Finally, samples were ready to FPSE procedure to analyze the supernatant. [31]

Tartaglia et al. availed saliva samples from healthy volunteers in absence of NSAIDs, with the aim of analyzing furprofen, indoprofen, ketoprofen, fenbufen, flurbiprofen, and ibuprofen in the above samples. Thanks to FPSE, the authors did not need any pre-treatment, only the addition of the internal standard. [32]

D'angelo et al. developed an analytical method based on MEPS and HPLC to quantify simultaneously FANS and Fluoroquinolones. They collected plasma and urine samples from healthy volunteers after 5 h after oral administration of FANS and FLQ. The addiction of trichloroacetic acid aimed the denaturation of proteins, and after a centrifugation step, the supernatant was used for the MEPS extraction. [33]

2.6 Statin

Courlet et al. discussed the risk of cardiovascular events in HIV patients. Atorvastatin, rosuvastatin, and pravastatin are the most prescribed lipid-lowering agents in this class of patients. In their study, blood samples from different patients for pharmacokinetics studies were collected. Pre-treatment of these samples was protein precipitation. Following centrifugation, the supernatant was then evaporated until dryness and then reconstituted in methanol: H_2O with 0.1% formic acid, vortexed, sonicated, and centrifuged. Finally, the supernatant was ready to be analyzed. [34]

2.7 Imidazoles and triazoles

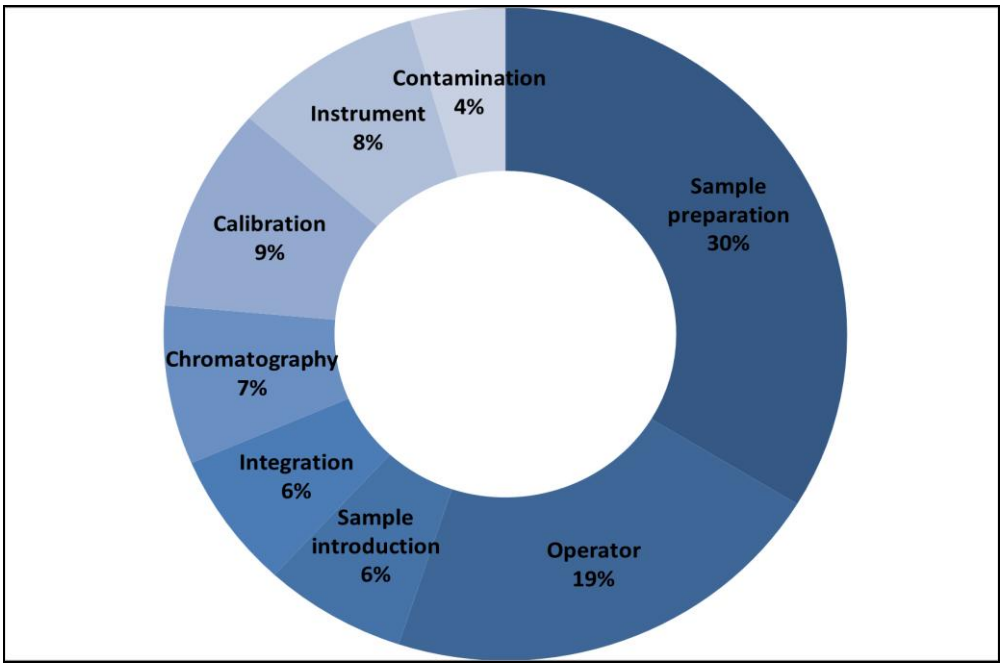
Campestre et al. studied imidazole as Ketoconazole, Bifonazole, Clotrimazole, Tioconazole, Econazole, Butoconazole and Miconazole and Triazole as Terconazole, Voriconazole, Posaconazole, Ravuconazole and Itraconazole. Specifically, the research group was interested about quantification of azole derivates in human samples collected from healthy volunteers (plasma and urine). The samples were mixed with the working analyte solution as well as internal standard and vortexed. With the aim of denaturing proteins and reducing sample density, the samples were pre-treated with trichloroacetic acid and centrifuged, then the supernatant was directly analyzed. [35]

The aim of this study was to simplify the clean-up procedures that precede the analysis in HPLC. These authors used plasma and urine samples from healthy volunteers, added analyte working solutions and internal standard to them and applied extraction procedure with FPSE membranes and then analyzed the supernatant in HPLC. [36]

3. Recent sample preparation techniques for clinical purposes

Particular attention is paid to novel extraction techniques, which significantly reduced the number of operations to be carried out during sample preparation. The aim was to develop extraction devices and technologies easily for operators to reduce time and minimize errors during operations. [37]

Fig.1 Sources of error generated during chromatographic analysis.



3.1 Solid Phase Microextraction (SPME)

Biological samples contain a variety of metabolites that can be used for clinical purposes in the diagnosis of various clinical conditions and many severe disorders.

Among sample preparation techniques, the most widely used procedure is solid phase microextraction (SPME) that includes sampling, extraction, and analyte pre–concentration into a single step. By contact with liquid bio-matrices, analytes are transferred to the adsorbent-coated solid surface thanks to the affinity of analytes to the coating material.

The direct immersion SPME (DI–SPME) and the headspace SPME (HS–SPME) are two modes of SPME operations. The first extraction technique is based on the sorption of analytes on the stationary phase immobilized on a fused silica glass rod that acts as the solid support. During the analyte's extraction, the coated fiber is directly immersed into the sample solution, while in the HS–SPME, the fiber is exposed in the vapor phase above the sample. At equilibrium, the quantity of analyte extracted by the fiber is proportional to its concentration in the sample. [38]

The main advantages of this methods include simplicity and speed of operations, high sensitivity, and the volume of solvent reduction, for these reasons it has been applied to the investigation of metabolites and neurotransmitters for diagnostic purposes. [39]

Many metabolites and neurotransmitters are useful for the diagnosis of neurological diseases. Neurotransmitters (NTs) are basic signaling molecules used for cells communications; they are involved in stress response mechanisms, in the regulation of motor coordination, in the control of psychomotor, gastrointestinal, and homeostatic function and neuronal communication.

Many psychomotor, psychiatric, neurodegenerative, and oncological disorders are characterized by an abnormal production, release and/or metabolism of NTs, for this reason NTs can be used as a potential biomarker for diagnoses of Alzheimer's and Parkinson's diseases, neuroendocrine cancers and psychiatric illness such as schizophrenia.

The most common biological fluids in NT analysis are urine and blood, but other alternative matrices include CSF and cerebral tissue, with the related difficulty due to the presence of interfering molecules and low levels of the target analytes. Therefore, the development of validated methods of sample preparation and analytical techniques for the quantitative analysis of NT in biological samples is needed. [40]

Naccaro et al. developed a method for the determination of NTs such as dopamine (DOP), norepinephrine (NE) and serotonin (5-HT) in human urine by SPME coupled with Gas Chromatography-Triple Quadrupole Mass Spectrometry (GC-QqQ-MS).

The optimized SPME method provides a first step of extraction with polyacrylate fiber at room temperature for 45 min without addition of NaCl, the second step was desorption at 300 °C, followed by derivatization reaction carried out directly in urine sample and SPME extraction in immersion mode in the same vial (without organic solvent or further sample treatment).

The developed method has shown good linearity with correlation coefficient values of 0.9995 for DOP, 0.9999 for 5-HT and 0.9987 for NE; the accuracy values were between 92.8 % and 103.0 % and RSD% ranged between 0.67 % and 4.5 %. LOD values was 0.587 μg/L for DOP, 0.381 μg/L for 5-HT and 13.5 μg/L for NE and LOQ values was 0.81 μg/L for DOP, 0.74 μg/L for 5-HT and 21.3 μg/L for NE.

The obtained satisfactory results indicate that the proposed method can be adapted for analysis of urine samples, which contain much lower concentration of DOP, 5-HT and NE than those determined for healthy adults. Thanks to the rapidity and simplicity of this SPME method with the advantages of minimal sample handling and high sensitivity, this procedure might be considered as valuable tool for diagnosis of cancerous and neurological diseases correlated with urinary levels of analyzed amines. [41]

Among the markers proposed for the diagnosis of oncological diseases, the scientific literature proposes the analysis of volatile organic compounds (VOCs) as a valid alternative, due to their importance on identification and differentiation of cancer samples. In particular, the determination of urinary VOCs has been proposed as alternative cancer biomarkers for lung, bladder, and breast cancer.

Monteiro et al. validated a new HS-SPME method to obtain a urine volatile metabolomics profile useful for renal cell carcinoma (RCC). These compounds generally require a high efficiency of extraction that depends on the sample preparation method selected.

In this study, the authors validated a HS-SPME method after the optimization of different factors that may influence the results, such as pH of urine sample, fiber coating, time of incubation, and time and temperature of extraction. They tested different conditions (3 pH levels, time and temperatures of extraction, temperature of incubation) and fibers (5 types of SPME fibers), amongst these the best results were obtained with the exposition of 2 mL of urine sample with optimal pH 2.0.

The use of an acidic pH value confirmed a better HS-SPME extraction in terms of the total of compounds and total chromatographic peak area, but strong acidic conditions may determine some error due to chemical degradation of some pH-sensitive compounds.

Moreover, the fiber with highest and better performance for urinary volatile metabolites was divinylbenzene/polydimethylsiloxane (DVB/PDMS). The optimal analytical performance was obtained also with addition of 0.59 g of NaCl, 9 min of incubation and extraction at 68 °C in 24 min.

This method was simple, solvent-free, inexpensive, fast with combination of extraction, and preconcentration of sample with minimal pre-treatment. [42]

Deeva et al. validated an SPME method to obtain preconcentration of VOCs in urine sample of patients with prostate cancer already confirmed by prostatic puncture biopsy, for subsequent non-target GC-MS analysis.

In this procedure, 3 mL of urine with 0.3 g of sodium chloride was placed inside the headspace vial, the extraction equilibrium was obtained with stirring (250 rpm) on the

hotplate. 100-µm-thick polydimethylsiloxane fibers were used for SPME, these fibers were treated al 250° C for 15 minutes to remove impurities before sample analysis. The fibers were placed in the headspace above the urine samples for 20 minutes at 50 °C, and then were inserted into the hot GC injector with the adsorbed analytes, which were desorbed for 4 min in splitless modes. After this procedure, the samples were analyzed with GC-MS. This method showed a very high sensitivity, specificity, and accuracy, and might be a good approach in non-invasive prostate cancer screening. [43]

Blood steroid levels are often required for the diagnosis of various endocrinological diseases involving the pituitary-adrenal axis, such as hypercortisolism and Cushing's syndrome. In addition to blood, saliva also represents a biological sample of considerable interest for diagnostic purposes, due to the presence of these hormones in free form with concentration correlated to that present in the blood. Often the saliva concentration of endogenous steroids is very low, and it is necessary to employ a highly sensitive analytical technique.

Bessoneau et al. validated a method for the quantification of endogenous steroids (cortisol, testosterone, and progesterone, E1, E2 and E3) in saliva with in vivo SPME. The extraction method was developed using biocompatible coated SPME blades that were first conditioned for 30 min with 1.5 mL of methanol/water (50:50, *v: v*) in 96-well-plates with agitation; all analytes required a time of 60 min for equilibrium extraction. To guarantee a complete immersion of the coating in the sample and to improve the method sensitivity they have chosen a volume of saliva of 1,2 mL. After extraction, the method involves static washing with deionized water (1.5 mL) to remove matrix components from the surface of the coating and prevent subsequent desorption, no loss of analytes was detected after this step.

The optimal conditions of desorption for all analytes were 90 min in acetonitrile/water 80:20 (*v: v*). The LOQ were 178, 22, 105, 185, 29 and 16 pg mL^{-1} for cortisol, testosterone, progesterone, E3, E2 and E1, respectively. This method has showed advantages in term of high sensitivity, reproducibility, and accuracy for detection of endogenous steroids in saliva. These characteristics makes the method better than the common immunoassays which may lead to erroneous results due to the significant cross-reaction; the validated SPME method had showed better results than liquid-liquid extraction (LLE) thanks to a cleaner extract that prevent matrix effects. Moreover, it was observed that *in vivo* sampling had a better efficiency than *ex vivo* sampling of saliva. [44]

In recent years, a new special application of SPME technique was observed that includes in vivo analysis. This method combines the advantages of SPME technique for sample preparation that requires no or small amount of organic solvent and the advantages of the in vivo sampling that eliminates errors related to degradation or loss of short-life or unstable analytes; moreover, with this sampling method, it was possible to sample over time the same individual (e.g. drug monitoring).

SPME was coupled to GC-MS or LC-MS/MS to separate and detect analytes, or directly to MS. Usually this extraction technique depends on the fiber and matrix characteristics. Different SPME coating fibers has been employed to determine numerous compounds with different volatility and polarity in complex matrices: CAR/PDMS (Carboxen/polydimethylsiloxane) have been used to determine VOCs while PDMS-DVB fibers have been used to determine non-polar or weakly polar VOCs with GC-MS analysis, drugs and metabolites with direct MS and endogenous hydrocarbons in human breath with GC-MS.

Different methods have been developed with this *in vivo* procedure for non-invasive and sensitive determination of different bio analytes in different types of samples in different fields of applications.

Amongst complex matrices used for *in vivo* SPME methods, the easiest to sample is the skin with a direct contact of SPME fiber placed on the skin surface, also breath and saliva can be used to obtain *in vivo* pathophysiological information.

For example, VOCs in human exhaled breath or emanating from human skin are potentially used as biomarkers for different disease diagnosis.

The methodology of *in vivo* SPME of VOCs in human skin was used to record the VOC profile in skin cancer (e.g., basal cell carcinoma), fibrotic skin disorders or vascular lesions. In addition, VOCs in human breath are useful in diagnosis of lung cancer, liver disease, myocardial infarction, or diabetes. [45]

3.2 Microextraction by Packed Sorbent (MEPS)

Microextraction by packed sorbent (MEPS) is a miniaturized SPE technique with very interesting potential benefits, such as low solvent volume for analytes elution, small sample volume (10–250 μL), direct injection without further treatments, simple operation, and cost-effectiveness. In this procedure, about 1–2 mg of sorbent (such as C18, C8) is packed in the barrel of a gas–tight syringe positioned between the needle and the barrel as a cartridge. When the sample has passed through the solid bed, the analytes were adsorbed into the sorbent. [38]

Ghimenti et al. validated an innovative method that combined MEPS with ultra-high-performance liquid chromatography coupled to electrospray ionization triple-quadrupole mass spectrometry (UHPLC-ESI-MS/MS) for the simultaneous determination of 8-isoprostaglandin F2α (8-isoPGF2α) and cortisol in saliva samples collected from patients with heart failure. During the optimization of this method, they maximized the extraction efficiency of 8-isoPGF2α and cortisol with dilution of saliva samples with water (ratio 1:5, *v*/*v*) and filtration by syringe filter prior to MEPS extraction to avoid proteins, mucins and other interferences that may cause deterioration of the sorbent and cartridge clogging, without loss of analytes. A LOD of 10 pg/mL was obtained for each target analytes; the coefficient of determination (R^2) was 0.7 and p-value < 0.001.

Compared to other techniques such as enzyme-linked immunosorbent assays (ELISA) which may lead to partially inaccurate results or solid phase extraction (SPE) procedures, which requires a larger volume of samples, a substantial problem for saliva analysis, the obtained results with MEPS showed that this is a good alternative with different advantages. MEPS procedure required low volume of organic solvents (<100 μL/measurement), a reusable sorbent (over 100 extractions with the same cartridge), permits to automate the extraction procedure with reduction of the cost of analysis. In addition, this procedure showed a satisfactory recovery (95–110%) and an adequate limit of detection, which guarantee a reliable determination of low concentration levels of 8-isoPGF2α and cortisol in real saliva samples. The obtained results confirmed that the MEPS method allows a reliable quantification of 8-isoPGF2α and cortisol as new potential non-invasive biomarkers for monitoring patient with heart failure and useful for clinical purposes [46].

Berenguer et al. developed a highly sensitive MEPS/UHPLC method to detect and quantify lipid peroxidation biomarkers such as leukotrienes E4 (LTE4), B4 (LTB4) and 11β-prostaglandin F2α (11⍟PGF2α) related to asthma, in urine of child patients. The targeted analytes were isolated from urine using a semiautomatic miniaturization of solid phase extraction, the MEPS; the LOD for LTB4 and 11⍟PGF2α was respectively 0.04 μg L^{-1} and 1.12 μg L^{-1} while LOQ values ranged between 0.10 μg L^{-1} for the LTB4 and 2.11 μg L^{-1} for 11⍟PGF2α. Compared to other methodologies, this technique had showed more advantages such as lower elution solvent volume consumption, high precision due to the semi-automatic procedure, excellent recoveries and extraction efficiency. Moreover, the extraction procedure is fast with a total analysis time of 31 min. The obtained values for the 11βPGF2α and LTB4 were about 1.8 times higher, and for the LTE4 were about 1.4 times higher in asthmatic patients than in healthy individuals suggesting the potential of these eicosanoids on asthma diagnosis. Consequently, MEPS/UHPLC-PDA represents a promising method for simultaneous detection of these and other eico-

sanoids present in other biological matrices as plasma or saliva of patients with different inflammatory diseases. [47]

3.3 Dispersive Liquid-Liquid Microextraction (DLLME)

Dispersive liquid–liquid microextraction (DLLME) is a type of sample preparation based on liquid phase microextraction (LPME) technique in which the target analytes are extracted from an aqueous solution with small volumes of extraction solvent. In DLLME, the dispersion procedure increases the extraction kinetics by enlarging the exposure surface between the sample and the extractant, then the emulsion was centrifuged and the extractant was isolated. This technique showed several advantages such as easy operation, low sample volume, rapid processing with high recovery without necessity of any appliance. [48]

Zhou et al. validate an easy method of sample preparation with high-efficiency based on ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction (UA-IL-DLLME), for simultaneous determination of neurotransmitters (NTs) such as γ-aminobutyric acid (GABA), acetylcholine (Ach) and glutamic acid (Glu) in mild cognitive impairment, mild dementia, and moderate dementia patients' urine samples. The analysis of clinical samples showed that some NTs presented significant differences in different dementia stages. This method showed many advantages such as selectivity, stability, sensitivity, and simple procedure of sample preparation, thanks to these characteristics the validated strategy of preparation and analysis might be used also with different type of biological samples. [49]

Table 2. Microextraction techniques used for diagnostic purposes.

Target analytes	Matrix	Extraction technique	Analytical characteristics	Ref.
Dopamine (DA), Serotonin (5-HT), Norepinephrine (NE)	Human urine	SPME-GC-QqQ-MS	DA: LOD 0,59 μg L^{-1}, LOQ 0,81 μg L^{-1} 5-HT: LOD 0,38 μg L^{-1}, LOQ 0,74 μg L^{-1} NE: LOD 13,5 μg L^{-1}, LOQ 21,3 μg L^{-1}	[41]
Homovanillic acid (HVA), vanylmandelic acid (VMA), 5-hydroxyindolacetic acid (5-HIAA)	Human urine	SPME-GC-QqQ-MS	HVA: LOD 1,3 μg L-1, LOQ 2,7 μg L^{-1} VMA: LOD 0,046 μg L-1, LOQ 0,063 μg L^{-1} 5-HIAA: LOD 24,3 μg L-1, LOQ 49,6 μg L^{-1}	[50]
VOCs	Human Breathe	MESI-GC	LOD Acetone 0,4 μg/L LOD Ethanol 0,5 μg/L	[51]
F2-isoprostanes	Human plasma Urine	SPE-GC-MS	LOD (plasma) 0,037 ng/mL LOD (urine) 0,007 ng/mg	[52]
LTB4	Human urine	MEPS-UHPLC-PDA	LOD 0,37 ng/mL LOQ 1,22 ng/mL	[53]

Metanephrine (MN) Normetanephrine (NMN)	Human plasma	MEPS-HILIC-MS/MS	NM LOD 12,4 pg/mL NMN LOD 12,3 pg/mL	[54]
Dopamine (DA), Norepinephrine (NE), Metanephrine (MN), Normetanephrine (NMN), L-3,4-dihydroxyphenylalanine (L-DOPA), Epinephrine (E), Epinephrine-d6 (E-d6), Metanephrine-d3 (MN-d3), Normetanephrine-d3 (NMN-d3), Serotonin (5-HT), Testosterone (T), Epitestosterone (EpiT), Dihydrotestosterone (DHT), 17-hydroxyprogesterone (17-OHP); Androstenedione (A), Progesterone (P4), Epitestosterone-d3 (EpiT-d3)	Human urine	LLE-LC/MS	DA: LOD 1 ng/mL NE: LOD 1 ng/mL MN: LOD 1 ng/mL NMN: LOD 1 ng/mL L-DOPA: LOD 20 ng/mL E: LOD 1 ng/mL E-d6: LOD 1 ng/mL MN-d3: LOD 1 ng/mL NMN-d3: LOD 1 ng/mL 5-HT: LOD 1 ng/mL T: LOD 1 ng/mL EpiT: LOD 1 ng/mL DHT: LOD 1 ng/mL 17-OHP: LOD 1 ng/mL A: LOD 1 ng/mL P4: LOD 1 ng/mL EpiT-d3: LOD 1 ng/mL	[55]
Norepinephrine (NE)	Artificial urine	Rotating-disk sorptive extraction technique (RDSE)	NE: LOD 11,3 μg L-1, LLOQ 34,0 μg L-1	[56]
Epinephrine (EPI), Norepinephrine (NE), Dopamine (DA), Metanephrine (MN), Normetanephrine (NMN), 3-methoxytyramine (3-MT)	Human urine	MEPS-LC-MS/MS	EPI: LOQ 0.167 ng/mL, LOD 0.0800 ng/mL NE: LOQ 0.650 ng/mL, LOD 0.300 ng/mL DA: LOQ 1.53 ng/mL, LOD 0.530 ng/mL MN: LOQ 0.440 ng/mL, LOD 0.176 ng/mL NMN: LOQ 1.10 ng/mL, LOD 0.440 ng/mL 3-MT: LOQ 0.880 ng/mL, LOD 0.176 ng/mL	[57]

3.4 Fabric Phase Sorptive Extraction (FPSE)

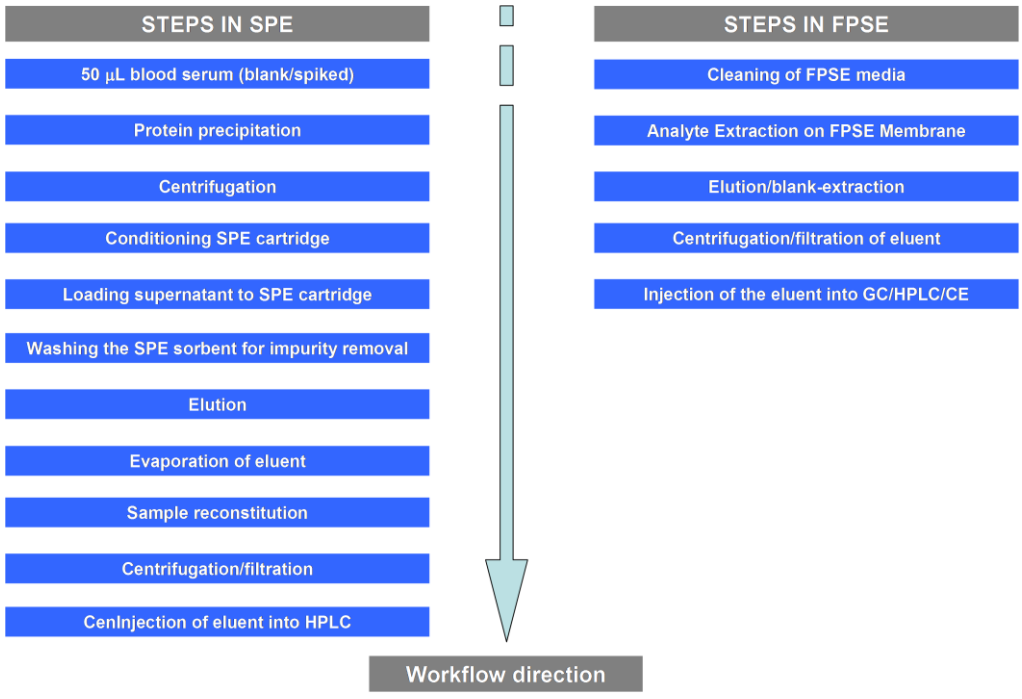
Fabric phase sorptive extraction (FPSE) is an innovative technique developed by Kabir and Furton [58] that offers substantial simplification of the selective extraction of the target analytes from biological samples. Now-a-days, thanks to the new analytical methods based on FPSE, it is possible to determine low concentrations of analytes with no/minimal sample pretreatment. In addition, the several steps in the treatment of complex biological matrices (for example whole blood, urine, saliva etc.) such as protein pre-

cipitation, sonication, elution of extract in an organic solvent, solvent evaporation and sample reconstitution often the cause of most of the errors during the analytical process can be eliminated from the sample preparation workflow when FPSE is used. [59]

Briefly, when the FPSE membrane is immersed into the aqueous sample, the analyte(s) permeates through the sol-gel sorbent like an SPE disk and establishes intermolecular interaction. However, at the same time, the analyte(s) continue to accumulate on the FPSE membrane based on the partition coefficient between the sol-gel sorbent and the sample matrix. Therefore, this novel technique combines both the exhaustive extraction principle and the equilibrium driven-extraction principle by design. [60]

Samanidou et al. used three FPSE membranes in particular, sol-gel poly(dimethyldiphenylsiloxane) (sol-gel PDMDPS), sol-gel poly (tetrahydrofuran) (sol-gel PTHF) and sol-gel poly (ethylene glycol) (sol-gel PEG) to determinate benzodiazepines from human blood serum. The authors demonstrated the simplicity of this extraction, in terms of the number of operations in comparison with other innovative techniques, like SPE. [59]

Fig. 2 Comparison between solid phase extraction (SPE) and fabric phase sorptive extraction (FPSE)



As shown above, Locatelli M. et al. integrate the FPSE with HPLC-DAD for separation and quantification of anastrozole, letrozole and exemestane from whole blood, plasma, and urine. The first step was the creation of sol-gel sorbent coated membrane: selection of appropriate substrate and its pre-treatment, design of the most appropriate sol solution to obtain maximum selectivity, coating the pre-treated fabric substrate with sol-gel sorbent and conditioning and cleaning the FPSE membrane. Membranes were cut to have an identical surface area with an internal diameter of 1 cm, they were washed first with acetonitrile: methanol (50:50, *v*/*v*) and then with milliQ water. Subsequently, the conditioned membranes were inserted into the sample (diluted whole blood, plasma, and urine) and were left for 30 minutes on the rotator. After this time, the analytes extracted were eluted from FSPE membranes using methanol, and the eluant was centrif-

ugated. An aliquot from obtained supernatant was directly injected into the HPLC-DAD for the analysis. [22]

Tartaglia et al. used eight different FPSE membranes for preliminary experiments to extract antidepressant drugs from conventional biological matrices (plasma and urine) and unconventional biological matrices (human saliva). For the optimization of the extraction process, all the parameters were gradually optimized to obtain the maximum extraction efficiency of the compounds. After the activation of the membranes, the initial general conditions have been tested: each support was immersed in 500 µL of standard solution for 30 minutes under stirring and then each membrane was immersed in 150 µL of MeOH for 30 min for analyte back-extraction. Subsequently, other parameters were optimized: extraction time, elution solvents and mixtures, back extraction time and volume. After selection of optimal condition using standard solutions, the optimization was performed on the biological complex matrices mentioned previously. [61]

In the same way, Locatelli et al. have optimized all parameters to obtain the maximum extraction of twelve azole antimicrobial drugs residues from human plasma and urine. They have investigated three different FPSE membranes, and different dimensions of these, to study the optimum sample volume and the optimum extraction media dimension. [36]

Kabir A. et al. have used this simple and powerful technique combined with HPLC-DAD for simultaneous monitoring of three anti-inflammatory drugs (ciprofloxacin, sulfasalazine, and cortisone) using in treatment of inflammatory bowel disease. They have tested five different FPSE membranes coated with different sorbent and different extraction parameters to develop an extraction procedure for molecules that are characterized by a wide range of LogP. [31]
Gazioglu I. et al. have combined FPSE-extraction with HPLC-fluorimetric detention to quantify simultaneously Febuxostat and Montelukast in human blood. [62]
Recently FPSE extraction was also used by Tiris G. et al. to extract an anti-viral drug from breast milk [63] and was also applied for *in vivo* non-invasive exhaled breath aerosol sampling to monitoring the exposure to xenobiotics by Locatelli M. et al. [64-66]

The amazing benefits of FPSE as a new generation sample preparation technique were further manifested in a study presented by G. Sidiropoulou et al. [67] where FPSE was combined with UPLC-ESI-MS/MS to monitor the presence of residual antiviral drugs including amantadine, memantine, and rimantadine in human urine. Sol-gel poly(propylene glycol)-poly(ethylene glycol)-poly(propylene glycol) coated FPSE membrane reached extraction equilibrium in just 20 min, thanks to the rapid mass transfer of the target analytes from the bulk of the sample to the FPSE membrane due to the engineered affinity of the sol-gel sorbent towards the target analytes via different intermolecular interactions and the sponge-like porous structure of the sol-gel sorbent. A matrix matched calibration curve was developed in the range of 5-100 ng ml^{-1} to aid the quantitative analysis of the target analytes in unknown clinical samples. The FPSE combined with UPLC-ESI-MS/MS method provides LOD values in the range of 0.2-0.8 ng/mL and LOQ value of 5 ng/mL for all target analytes. It is worth mentioning that, unlike other sample preparation techniques, FPSE didn't require any kind of pretreatment of urine sample prior to the extraction of analytes on FPSE membrane.

Another interesting study presented by K. Mazaraki et al. [68] where the researchers investigated the presence of traces of six beta-blockers possesing widely varied logP values (from 0.1 to 3.1) in human serum and urine using FPSE as the sample preparation technique followed by UHPLC-ESI-MS/MS as the chromatographic technique. Sol-gel CW 20M sorbent coated on 100% cotton cellulose was identified as the most efficient

FPSE membrane for the target analytes. After optimizing all the FPSE parameters, the FPSE-UHPLC-ESI-MS/MS composite method provided LOD values for the selected beta-blockers from 0.3-2 ng/mL and the LLOQ values were calculated as 50 ng/mL in both serum and urine samples. The method was linear in the broad range of 50-5000 ng/mL.

G. Tris et al. [63] described an FPSE method followed by HPLC-UV analysis designed to monitor Favipiravir, a promising antiviral agent for the treatment of corona virus, in human plasma and breast milk samples. Overall chromatographic run was only 5 min. The LOQ values of the validated method were 0.2 μg/mL in plasma and 0.5 μg/mL.

By eliminating the protein precipitation and other sample pretreatment steps from the sample preparation workflow, FPSE has established itself as a rapid, simple, and green sample preparation technique for forensic toxicology. Recently, M. Locatelli et al. [69] have proved the operational simplicity and performance superiority of FPSE in processing post-mortem samples including whole blood and cerebrospinal liquor collected during autopsy to monitor seven common antidepressant drugs. Sol-gel CW20M coated FPSE membrane was identified as the best FPSE sorbent for the selected drugs with an optimum extraction time of 20 min, back-extraction time of 5 min and 150 μL of methanol as the eluent. The overall chromatographic run time was 20 min. The LOQ values were 0.1 μL/mL for all drug compounds except Venlafaxine, which was 0.2 μL/mL.

The performance superiority, simplicity, and easiness in application of this novel technique have been demonstrated in many articles and reviews, in extractions from complex sample matrices.

4. Conclusions

As can be seen from the manuscript, last several years were important to develop new strategies and techniques to apply in bioanalytical studies. The principal aim was to maximize the performance with these samples in term of efficiency and operational steps and speed, complying with green chemistry principles and the possibilities to use them for different applications. It is important to emphasize the diversification of the samples, from conventional samples as whole blood or urine to unconventional like saliva or breast milk, but at the same time duration of the sample preparation workflow, efficiency, reproducibility, and the potential reuse of the sample preparation device to reduce the overall analytical cost. Moreover, the wide range of samples can be applied in different clinical analysis, useful for research's progress. Clinical analysis includes different studies like pharmacokinetics studies or studies that may help in a specific treatment regimen or, also, studied that diagnose some pathologies. Therefore, the employment of different matrices means different approaches in terms of preparation and pre-treatment, and it follows the possibilities to differ specific studies. For complex sample matrices, the principal issue can be the pre-treatment and extraction of the sample, in fact often the pre-treatment is the principal cause of error. This is the primary rationale as to why we discussed about new techniques, easy for operators, simple to execute and capable of reducing overall sample preparation time and number of steps involved.

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