**STATE-OF-THE-ART IN FAST LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY FOR BIO-ANALYTICAL APPLICATIONS.**

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**Abstract**

There is an increasing need of new bio-analytical methodologies with enough sensitivity, robustness and resolution to cope with the analysis of a large number of analytes in complex matrices in short analysis time. For this purpose, all steps included in any bio-analytical method (sampling, extraction, clean-up, chromatographic analysis and detection) must be taken into account to achieve good and reliable results with cost-effective methodologies. The purpose of this review is to describe the state-of-the-art of the most employed technologies in the period 2009-2012 to achieve fast analysis with liquid chromatography coupled to mass spectrometry (LC-MS) methodologies for bio-analytical applications. Current trends in fast liquid chromatography involve the use of several column technologies and this review will focus on the two most frequently applied: sub-2 µm particle size packed columns to achieve UHPLC separations and porous-shell particle packed columns to attain high efficiency separations with reduced column back-pressures.

Additionally, recent automated sample extraction and clean-up methodologies to reduce sample manipulation, variability and total analysis time in bio-analytical applications such as on-line solid phase extraction coupled to HPLC or UHPLC methods, or the use of other approaches such as molecularly imprinted polymers, restricted access materials, and turbulent flow chromatography will also be addressed. The use of mass spectrometry and high or even ultra-high resolution mass spectrometry to reduce sample manipulation and to solve ion suppression or ion enhancement and matrix effects will also be presented. The advantages and drawbacks of all these methodologies for fast and sensitive analysis of biological samples are going to be discussed by means of relevant applications.

**1. Introduction**

The need of high-throughput separations in bio-analytical applications able to cope with the analysis of a large number of analytes in very different and complex matrices has increased considerably in the last years. The main objective of any laboratory, including bio-analytical ones, is to develop reliable and efficient procedures to perform both qualitative and quantitative analysis with cost-effective methodologies with reduced analysis time. High performance liquid chromatography (HPLC) appears as the most common approach to solve multiple analytical problems, as it is able to separate quite complicated mixtures of analytes with different molecular weights as well as different polarities and acid-base properties. However conventional HPLC alone do not solve all the analytical problems related to bio-analytical applications and will not always satisfy the need of reducing the total analysis time in a field with a huge variety of analytes and sample matrices but also with an increased demand on fast analytical results. Challenges in bio-analytical laboratories include development of fast LC-MS methods able to separate closely related compounds (e.g. analytes and metabolites) from endogenous components. For instance, several bio-analytical methods include monitoring of drugs in a variety of biological matrices in order to evaluate their pharmacokinetics, to establish appropriate dosages, or to determine drugs, drugs of abuse and their metabolites in forensic analysis. Many of these methods are required to obtain results very fast in order to take medical, forensic or legal decisions, and at very low concentration levels because of the bioavailability of many of these drugs. The final objective consists of developing bio-analytical methods that meets the rigorous criteria set by validation guidelines in terms of selectivity, accuracy (trueness and precision) and linearity [1], but also guaranteeing confirmation of target and the identification of related and new compounds [2].

Nowadays, there are several approaches in HPLC methods which enable the reduction of the analysis time without compromising resolution and separation efficiency such as the use of monolithic columns [3-6] or high temperature liquid chromatography [7-9]. But among them the main approach, including bio-analytical applications, to achieve high-throughput separations is the use of ultra-high pressure liquid chromatography (UHPLC) using sub-2 µm particle packed columns [10,11]. Additionally, porous shell columns (packed with sub-3 µm superficially porous particles) are starting to be used for fast chromatographic separations [12-15].

Despite the advances in chromatographic separations techniques, the complexity of biological sample matrices makes difficult their direct analysis by HPLC. For instance, irreversible adsorption of proteins in the stationary phase can occur, producing a loss in column efficiency and increase in column backpressure. Therefore, the use of ultra-fast separations is not enough to develop fast analytical methods in bio-analysis, and sample treatment is still one of the most important parts of the analytical process; effective sample preparation is essential for achieving good analytical results. Sample preparation has usually been performed using protein precipitation (PPT), liquid-liquid extraction (LLE) or solid phase extraction (SPE), but these procedures are in general laborious and time-consuming. An ideal sample preparation method would be fast, accurate, precise and keep sample integrity. Over the last years, considerable efforts have been made to develop modern approaches in sample treatment techniques that enable the reduction of analysis time without compromising the integrity of the extraction process [16]. The use of on-line SPE, which minimizes sample manipulation and provides both high pre-concentration factors and recoveries, is an increasingly powerful and rapid technique used to improve the sample throughput and overcome many of the limitations associated with the classical off-line SPE procedure. Higher specificity and selectivity together with satisfactory extraction efficiency can be obtained using sorbents based on molecularly imprinted polymers (MIPs). SPE based on MIPs is a highly attractive and promising approach for matrix clean-up, enrichment and selective extraction of analytes in such kind of complex samples [17]. The use of restricted-access materials (RAM) for direct injection of biological samples appears as a good alternative for selective sample clean-up or fractionation in proteome and peptidome analysis [18]. Another modern trend in sample preparation for bio-analytical applications is the use of turbulent-flow chromatography (TFC) that can be even more efficient at removing proteins based on their size than RAM or SPE [19].

The reduction of the analysis time by combining ultra-fast separations and reduced sample treatments may introduce new analytical challenges during method development. More matrix related compounds may be introduced into the chromatographic system by reducing sample treatment, and although high resolution and separation efficiency can be achieved by UHPLC-MS(/MS) methods, the likelihood of matrix effects, such as ion suppression or ion enhancement, may increase. Additionally, the use of on-line SPE procedures coupled to UHPLC is not a problem-free approach. Conventional on-line SPE systems are not usually compatible with UHPLC and a loss on the chromatographic efficiency may be observed when both methodologies are combined. To solve some of these problems the use of liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) is mandatory and for some applications even high resolution mass spectrometry (HRMS) may be required [20-22].

The aim of this review is to discuss the state-of-the-art in fast liquid chromatography coupled to mass spectrometry and on-line sample preparation techniques for bio-analytical applications. It includes a selection of the most relevant papers recently published (2009-2012) regarding instrumental and column technology in bio-analysis, particularly UHPLC methods with sub-2 µm and novel porous shell particle packed columns. Modern sample treatment procedures such as on-line SPE, the use of MIPs and RAM technology, and turbulent-flow chromatography will also be addressed.

**2. Sample preparation**

**2.1. On-line solid phase extraction**

Laboratory automation and high-throughput analysis have recently become of primary importance to reduce analysis time, costs and variability derived from sample manipulation. With the development of fast chromatographic methods able to separate species in a few minutes with low solvent consumption, it became a priority to shorten conventional sample treatments as well. In this context, recent developments in on-line SPE aspects in combination with the sensitivity and selectivity achieved by MS/MS have made possible the development of faster and precise on-line SPE-LC- and UHPLC-MS/MS methods for both qualitative and quantitative analysis of heterogeneous substances in biological matrices. This technique has shown to be advantageous for the analysis of wide range of analytes, such as steroid hormones, insecticides, antibacterial, perfluorinated compounds, therapeutic peptides, immunosuppressant, antidepressant or illicit drugs in biological fluids as different as in urine, blood, serum, plasma, saliva, synovial fluid, milk and other tissues (see Table 1, [23-65]).

The comparison of diverse purification and determination techniques provides evidences to assess the strengths and limitations of on-line SPE compared to other approaches. For instance, König *et al.* [25] developed an on-line SPE LC-MS/MS method for the determination of the principal psychoactive constituent of cannabis plant and some of its metabolites in human blood for use in forensic toxicology as an alternative to their pre-existing method based on GC-MS. The stationary phase of the trapping and analytical columns were hydrophobic. The on-line method, which was validated, presented limits of detection in the region of 1 µg L-1. Furthermore, the on-line SPE permitted overcoming some downsides of the sample treatment stage previous to the GC-MS analysis such as a laborious sample preparation, long analysis time, and frequent preventive cleaning of the instrumentation, which is particularly critical with GC-MS. This on-line SPE approach was also used for the analysis of one of the metabolites in human urine [37]. In this case, no significant matrix effect was observed, excellent intra- and inter-assay precisions (RSD < 7%) were achieved, with limits of detection in the same range than those observed with the on-line SPE method developed for blood analysis [25]. Carryover was not observed even though high levels of the studied compounds were injected [25].

In a study where LLE, protein precipitation, off-line and on-line SPE were assessed for the analysis of a cephalosporin in plasma, the first two approaches provided low sensitivity and interferences by endogenous compounds [66]. The off-line clean-up provided the best sensitivity and selectivity; however the on-line SPE clean-up offered the shorter analysis time as well as a lower consumption of reagents and still keeping good sensitivity and selectivity. A compromise between the methods tested gave the optimal results: off-line protein precipitation followed by on-line SPE method [66], approach carried out in many of the research works quoted in Table 1. Examples of the advantage of using on-line SPE-LC-MS/MS method in terms of reduction of analysis time was recently reported for the quantification of free catecholamines in urine [64], where it allowed to perform their determination in 3% of the time initially spent with sample preparation and chromatographic separation. Another example of short analysis time is the accurate determination of 3 triazole antifungal drugs in plasma [30] within 3 minutes. To further reduce run time together with an additional increase in the detection sensitivity, on-line SPE systems have also been recently coupled to UHPLC using sub-2 μm particle size columns. For instance, Ismaiel *et al.* [67] developed a selective UHPLC-MS/MS method for the determination of the anti-cancer therapeutic peptide ocreotide in human plasma using on-line ion-exchange SPE with run time below 10 min and LOQ of 25 pg mL-1. Moreover, the on-line removal of phospholipids using column switching and pre-column back-flushing allowed reducing the matrix effect to less than 4%. The direct hyphenation of on-line SPE to UHPLC system has also been reported as a powerful analytical tool for microdosing studies in humans for the clinical development of drug candidates [27]. Furthermore, this study also compared conventional LC-MS/MS method to UHPLC method; the latter approach leads to 5-fold lower injection volume and 1.5-fold higher peaks.

On-line SPE methods for bio-analysis provide limited purification in the sense that highly aqueous solvents are used to wash analytes in the trap column. This rinse step is generally not enough when hydrolysis or precipitation of macromolecules are required because the system could get block during the pretreatment [32,57,63]. Precisely, system blockage and ion suppression are some of the reasons that keep the injection volumes relatively low, typically < 200 µl [63], which play against achieving higher preconcentration factors and sensitivity [41]. To isolate the analytes from biological matrices, either straightforward or extensive pretreatment stages have been applied. Urine samples were just filtered and kept in cool conditions [57]; saliva was diluted and centrifuged [58], and serum, plasma and brain microdyalisate samples were injected directly onto the on-line SPE and proteins rinsed with a solution with high water content [33,68]. However, most commonly, precipitation of proteins is carried out off-line with organic solvents [29,30,53,61], acid [31,34,61,63]; and/or centrifugation [58] or even SPE [39,53], LLE [33] or purification with an immunoaffinity column [59] prior to injection of an aliquot of the supernatant into the on-line SPE system. Besides, off-line pretreatment is carried out to increase the lifetime of the costly columns used for SPE [53]. Two consecutive purification steps with on-line SPE cartridges prior dilution and centrifugation of saliva samples provided thorough cleaning and allowed to reuse them 15 times with high precision [58]. The effect of the clean-up on the instrumental sensitivity was assessed by some authors, for instance, 50 injections of 400 µL of deproteinized plasma into a polymeric SPE cartridge resulted in a two-fold reduction of the signal in a MS with off-axis ESI [31]. A novel and promising approach for on-line deproteinization has been carried out with the synthesis of the a polymeric porous monolith poly(N-isopropylacrylamide-co-ethyleneglycol dimethacrylate), which showed LC-UV chromatograms with absence of interferences after the direct injection of spiked urine and plasma [69]. Other approaches to remove macromolecules on-line involving MIPs, RAM and turbulent flow chromatography will be reviewed in the following sections. Chromatograms obtained with LC-UV have given an overview on the on-line purification [66,69-73], technique that unlike MS, also tolerates the presence of phosphate buffers in the mobile phase. When using MS the purification achieved has been assessed by post-column infusion of the study compound in a chromatographic run of blank biological sample and observing the reduction of the signal [32,58,74]; by observing the peak height in absence or presence of matrix [39] or by comparing the slope of the external calibration curve and standard addition curves [61].

Most of the compounds analysed, shown in Table 1, were charged low molecular weight molecules and their determination was carried out with ESI-MS. Ion exchange sorbents could potentially provide higher selectivity for the extraction of these analytes than reversed phase sorbents, which would result in cleaner samples and reduced ion suppression. However, the studies generally opted for sorbents with hydrophobic interaction with the analytes (C4, C8, C18, polydivinyl-benzene (Hysphere GP resin), N-vinylpyrrolidone–divinylbenzene copolymer (Waters HLB)). A small number of works chose ion-exchange or mixed-mode ion-exchange as the purification mechanism. Specifically, functionalized silica with propylcarboxylic acid (CBA) or with propylsulphonic acid (PRS), and polymer based sorbents such as carboxy-divinylbenzene-N-vinylpyrrolidone co-polymer (Oasis WCX), divinylbenzene-based Bond elute Plexa PCX or benzenepropanoic acid (Strata X-CW) are among the sorbents most often used in the works quoted in Table 1 whereas immunosorbents have scarcely been used.

The high versatility and purification potential of on-line methodology has been shown in the fast and quantitative multicomponent analysis in complex biological samples. As an example, a simple on-line laboratory set-up was automated for simultaneous determination of forty-two drugs belonging to different chemical classes in human urine within 11 minutes [36]. The sample clean-up was performed using a SPE Strata X-CW and the separation was performed by UHPLC coupled with tandem mass spectrometry. The validation results on linearity, precision, accuracy, matrix and memory effect were found to be satisfactory, with recovery average greater than 93.8% and LODs/LOQs levels suitable for confirmation tests.

**2.2. Molecularly imprinted polymers (MIPs) and restricted access materials (RAM) technology**

The analysis of compounds in biological fluids is a contest between the analytical demands (best quality parameters and shortest analysis time) and the complexity of the sample.

Due to the drawbacks of the commonly used SPE phases, a great effort in the last years has been made to study and develop new sorbents materials able to increase the overall efficiency of the extraction process from bio-matrices. These new materials try to accomplish the requirements according to present needs, such as selectivity towards target analytes, easy manipulation allowing coupling on-line configurations and higher biocompatibility. Among them, molecularly imprinted polymers (MIPs) and restricted access material (RAM) are currently attracting much interest.

MIPs, also called synthetic antibodies, are polymeric materials possessing an artificially generated three-dimensional network with highly specific and selective recognition sites [75]. These recognition sites are obtained by polymerizing functional and cross-linking monomers around a template molecule, followed by subsequent removal of the template in order to leave a cavity with binding sites complementary to the shape, size and functional groups of the target compound [76]. This technology has grown in popularity over the past few years compared to other techniques such as conventional SPE or immunoaffinity sorbents because of the advantages of being at the same time highly selective, cost-effective, and not suffering from storage limitations and stability problems associated with organic solvents or extreme pH values. An example of the superior features of MIPs when compared to traditional SPE has been recently reported for the extraction of an illicit drug such as lysergic acid diethylamide (LSD) from hair and urine samples [77]. MIP was used for off-line extraction before LC-MS analysis and its performance compared to that of a conventional C18 SPE. Molecularly imprinted SPE showed higher recoveries (~83%) than commercially C18 SPE (~65%) whit a significant improvement in analytical sensitivity. Thus, because of the potential benefits of using this technique, MIP–SPE coupled to LC-MS has been extensively applied for the selective extraction and pre-concentration of a wide range of analytes, such as benzodiazepines [78], zidovudine and stavudine from human plasma [79], cocaine and its metabolite benzoylecgonine [80], ketamine and norketamine [81] in hair samples, as well as testosterone, epitestosterone [82], and 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) from urine samples [83]. However, even if the use of MIP particles as selective sorbents for solid-phase extraction (MIP–SPE) is by far the most common application of MIPs, molecularly imprinted polymers have also been used with satisfactory results as coating agents for stir bar sorptive extraction (SBSE) and solid-phase microextraction (SPME) fibers, or as stationary phase for capillary micro-columns. For instance, a high-throughput on-line microfluidic sample extraction method using capillary micro-columns packed with MIP beads coupled with tandem mass spectrometry was reported for the analysis of urinary NNAL [84]. The developed method, which has been validated according to FDA guideline on bio-analytical method validation [1], has a short run time of 7 min and requires the use of small sample volumes (200 µl), reaching limits of quantitation as low as 20 pg mL-1. MIPs as coating agents for stir bar sorptive extraction (MISBSE) was also developed for determination of 2-aminothiazoline-4-carboxylic acid (ATCA) as a marker for cyanide exposure in forensic urine analysis [85]. The performance of this column-less method, based on MISBSE combined with LC-MS/MS, was demonstrably adequate for the analysis of ATCA at pg µL-1 levels without the use of any derivatization step. Furthermore, tandem mass spectrometry (MS/MS) was used to improve the overall selectivity of the method and to overcome problems associated with matrix interferences due to the possible co-extraction of other urinary acids by the MISBSE procedure. Finally, a somewhat different approach was recently successfully used for the determination of antibiotics drugs in human plasma as well as in synthetic body fluids [86]. In this study, MIPs have been applied as an alternative for selective SPME coating. MIP-coated fibers for SPME were prepared by using electrochemical polymerization of pyrrole and linezolid as template molecules. The developed SPME MIP-coated fibers were then applied to the determination of selected antibiotic drugs such as linezolid, daptomycin and amoxicillin. The method is shown to be rapid, reproducible and with a detection limit for linezolid of 0.029 µg mL-1. Furthermore, the selectivity of the SPME MIP-coated fibers for these antibiotic drugs was assessed by comparing its activity with the non-imprinted polymer (NIP)-coated fibers. As expected, SPME MIP-coated fibers showed higher binding capacity compared with non- imprinted polymer (NIP). In summary, molecularly imprinted polymers appears as a very useful and promising approach for sample extraction and clean-up procedure in bio-analytical applications, where a very high degree of selectivity may be required to reduce analysis time without suffering from problems related to sensitivity of MS detection such as matrix effects.

Restricted access materials in automated sample preparation systems on-line coupled with sensitive techniques such as LC-MS/MS or LC-fluorescence have been an effective strategy to overcome that analytical challenge [87-89]. The use of RAMs simplifies the purification of low molecular weight substances in bio-fluids by physical and chemical diffusion barrier. RAMs have a dual surface configuration; the outer surface employs both size exclusion and hydrophilic shielding to create a non-adsorptive outer surface of the particles and prevent macromolecules accessing the inner surfaces where smaller molecules can be adsorbed by hydrophobic interaction.

The RAMs used today are derived from the first sorbents developed in 1985 [90], called internal surface reversed-phase (ISRP) materials. Alkyl-diol silica materials (ADS) [91] are among the RAM sorbents most frequently used today and are commercially available. ADS have a bimodal function based on diol groups in the outer part and hydrophobic extraction phases (C4, C8 or C18) in the interior. For instance, they have been applied for the analysis of vitamin D and metabolites from serum [92], mercapturic acids in urine [93,94] or multiresidue analysis of xenobiotics in urine [95]. But aside from these well-established sorbents, important steps forward in the development of new RAM sorbents have taken place as it is following described.

The incorporation of restricted access properties to magnetic particles has opened the door to a new modality of sample preparation supports for bio-analysis. Magnetic porous silica microspheres were synthetised through polymerization-induced silica/magnetite colloid aggregation and calcination. The microspheres were subsequently modified with alkyl groups on the internal surface and diol groups on the external surface. These novel materials have been evaluated for the extraction of chemotherapeutic drugs from serum and subsequent determination by LC-UV has shown recoveries generally above 70% and improved cleanliness of the sample compared with conventional SPE or LLE method [96]. Another magnetic RAM, schematized in Figure 1, was prepared by functionalizing magnetite nanoparticles with dodecyltriethoxysilane and non-ionic surfactant (tween). The extraction efficiency was tested for the analysis of estrogens in urine. Salting out effect was found to increase the extraction efficiency, pH was not found to be a critical factor for this application and the addition of an organic modifier reduced their performance [97]. The properties of RAM and MIP materials have merged in the RAM-MIP grafted silica synthetized by Wenjuan Xu et al. [98], who by a controlled polymerization technique (see method in [99]) prepared an advanced material consisting of an internal polymer imprinted with sulfonamides and an hydrophilic external layer of glycerol monomethacrylate that prevented the adsorption of proteins. This advanced RAM has been successfully used in the extraction and clean-up of sulphonamides from milk [98].

Novelties in the uses of RAM to solve bio-analytical problems have also taken place. The purification of a protein from the family of cytokines, of about 20 KDa, from plasma with RAM has been carried out despite these sorbents are generally used for retaining low molecular weight compounds. The RAM used in this case was constituted by silica particles with bonded C18 and serum albumin [100]. The optimization of the coupling of a RAM (MSpak) with N-vinylacetamide copolymer as stationary phase with a HILIC column for the analysis of nucleosides in urine, overcoming the compatibility problems between the solvent required for the elution of the RAM the mobile phase used in the separation, represented a step forward in this field of sample preparation [101].

Despite the advantageous features of RAMs, these materials still have limitations. Reusing RAM sorbents after being loaded with biological samples which underwent a minor or no sample treatment [93-95,100,101] prove the efficiency of the technique and shows its superiority to the single use of SPE cartridges. The elution of the analytes from the RAM to the analytical column is a key step in the purification process; on the one hand the RAM could still contain residues of macromolecules at this point if the washing step was not fully effective. To avoid the precipitation of residual macromolecules in the RAM, the amount of organic solvents in the transference is kept low, usually below 15%. A disadvantage of the weak eluotropic strength of the transfer solution is that residual amounts of the analytes can remain in the sorbent and cause false positives. Carryover may be likely to be happen when injecting a hydrophobic analyte in a reversed phase RAM. For instance, carryover of bosentan and metabolites, which are compounds with 4 aromatic cycles, was assessed to happen at about 0.2% of the last injected sample [102]. The assessment of carryover can be carried out directly with the analysis of blanks [95,100,102] or indirectly with the assessment of the recovery through the RAM [101], recovery of the whole analysis including sample treatment, separation and detection [100] or analysis of reference materials [92].

**2.3. Turbulent flow chromatography**

Turbulent flow chromatography (TFC) is widely used in applications were plasma or similar fluids are to be analyzed. This technique allows the direct injection of a liquid sample onto a narrow diameter column (0.5 or 1.0 mm) packed with large particles (30-60 µm) at a high flow rate (higher than 1 mL min-1). Under turbulent flow conditions, there is improved mass transfer across the bulk mobile phase which allow to improve the radial distribution of the analytes. However, under these conditions a laminar zone around the stationary phase particles still exists, where diffusional forces still dominate the mass transfer process [103]. Molecules with low molecular weight diffuse faster than molecules with a high molecular weight, forcing large molecules to quickly flow to waste while retaining the small molecules. The retained compounds are then back-flushed and focused on the analytical column for chromatographic separation, like with the on-line extraction with RAM. The first application of TFC-MS for the direct injection of plasma was described in 1997 by Ayrton *et al.* [104]. Many more studies have been reported in successive years applied to various matrices reaching out from biological (Table 2, [103,105-117]) to environmental and food samples. For example, the successful analysis of immunosuppressants and antibiotics from low volume samples such as ocular fluid (tears) and whole blood has been reported [105,118,119]. Besides, this technology has been applied to the analysis of more complex matrices such as hemodialysates [120], edible animal tissues [121] and food samples such as honey [122] and milk [123]. The major advantage of TFC in comparison to other extraction techniques is the reduction of time consuming preparation steps while similar LOQ, dynamic range, accuracy and precision can be obtained. The main drawback of this technique is probably the low concentration capacity although this can be compensated by the use of capillary LC leading to the reduction of the amount of sample volume injected [124]. In addition, this technique is clearly limited in terms of chromatographic resolution. Therefore, it is common to couple turbulent flow columns to a more conventional analytical column by means of column switching systems. TFC is considered as being similar to solid phase extraction followed by liquid chromatography although the extraction is size-exclusion based, therefore it is mainly applied when there is an interest to separate small analyte molecules from larger matrix molecules [124]; the larger molecules, such as proteins, go directly to waste and the smaller molecules are adsorbed to the retentive stationary phase of the turbulent flow column. Nevertheless, TFC seems to be more efficient at removing proteins than RAM or SPE [125]. In general its simplicity, versatility and automation possibilities are well described in the literature as it dramatically increases the speed of the analysis while maintaining acceptable levels of recovery, efficiency and robustness. Off-line handling of the sample is often limited to centrifugation, for removal of particulates, dilution with internal standard and protein precipitation (PPT) to remove endogenous binding proteins [108]. The latter helps preventing column clogging.

Mueller *et al.* [106] reported a comprehensive toxicological MSn screening method for the analysis of serum and heparinised plasma. This methodology targeted 453 compounds and the results were cross-checked with urine samples to test the performance of the method under realistic clinical conditions as well as to compare the information gathered using different matrices. Pérez *et al.* [107] applied the same principles for bioaccumulation studies of perfluorinated compounds in human hair and urine. Similar results were obtained between radioimmunoassay and TFC by Bunch *et al.* [108] in the analysis of 25-hydroxyvitamin D2 and D3 in serum.

The sample preparation technique has clear effects on the composition of the purified extract. In the case of metabonomic studies, an automated sample preparation methodology in which the sample can be injected directly into the system is appealing. Michopoulos *et al.* [103] reported that even though the use of TFC in metabonomic studies is feasible, different profiles were obtained when comparing TFC and protein precipitation. This was attributed to the greater amount of phospholipids in the protein-precipitated samples, due to fact that TFC would not affect the binding of such compounds to proteins; they would pass through the first column without being retained [103].

The robustness of sample preparation techniques in quantitative assays is crucial. Methods using PPT are simple and cheap but produce relatively dirty extracts which may reduce the lifetime of the chromatographic column, extend the cleaning/maintenance of the mass spectrometer or result in matrix effects [126]. Liquid-liquid extraction and solid phase extraction are efficient producing clean extracts and reducing matrix effects but laborious and difficult to automate.

TFC can also be used for analytical support of *in vivo* pharmacokinetics and *in vitro* drug metabolism studies [112,127]. Verdirame *et al.* [112] reported the advantage of TFC methodologies in terms of sensitivity and throughput in comparison to conventional procedures. In this case, a set up consisting of two parallel turbulent flow and two analytical columns operating independently was used. This configuration allowed a fourfold increase in terms of throughput.

Summarizing, the optimization of the different on-line extraction steps is crucial, as parameters like mobile phase composition, flow rates and extraction time windows will affect recovery and extraction efficiency. In general, TFC provides simplicity, automation, robustness, versatility and high-throughput in bio-analysis.

**3. Trends in chromatography approaches**

**3.1. Ultrahigh pressure liquid chromatography**

Fast chromatography has become a reality in laboratories that require analyzing hundreds of samples per day or those needing short turnaround times. The development of columns packed with sub-2 µm particles and the commercialization of LC systems capable of withstanding pressures as high as 1000 bar lead to a significant increase in the analysis throughput. Using ultrahigh pressure liquid chromatography, the results of a sample batch can be reported in a few hours rather than a few days, for instance in the case of control doping laboratories. Thus, the demand of high sample throughput in short time frames have given rise to high efficiency and fast liquid chromatographic separations in several fields, including bio-analysis, using mainly reversed-phase columns packed with sub-2 µm particles. Moreover, this column technology for UHPLC emerged as a powerful approach particularly because of the ability to transfer existing HPLC conditions directly [128].

In general, fast chromatographic separations can be achieved either by increasing the mobile phase flow-rate, by decreasing the column length or by reducing the column particle diameter. Based on the van Deemter theory [129], then on Giddings [130], and later on Knox [131] and further interpretations, efficiency expressed as the height equivalent to a theoretical plate (HETP, *H*) can be described as:

*H = A + B/u + Cu = 2λdp + 2γDM / u + f(k)dp2u / DM*

where *u* is the linear velocity of the mobile phase, and *A*, *B*, and *C* are constants related to Eddy diffusion, longitudinal diffusion and mass transfer in mobile and stationary phase, respectively, *dp* is the particle diameter of the column packing material, *DM* is the analyte diffusion coefficient, *λ* is the structure factor of the packing material, *γ* is a constant termed tortuosity or obstruction factor and *k* is the retention factor for a given analyte [129]. So basically, HETP depends on three terms, which are the brand broadening due to Eddy diffusion coefficient (*A*-term), longitudinal diffusion coefficient (*B*-term) and the resistance to mass transfer coefficient between the mobile and stationary phases (*C*-term). It is often assumed that *A*-term does not depend on temperature and it is directly proportional to *dp*, while *B*- and *C*-terms are both temperature dependent, the *B*-term being directly proportional to *DM* while the *C*-term is inversely proportional to *DM* but directly proportional to the square of *dp*. So, as lower is the particle diameter of the column packing material higher will be the column efficiency (lower HETP), and high throughput separations will be achieved. The use of small particles will induce a considerable increase in pressure drop, but this inconvenience has been resolved with the availability of new ultra-high pressure resistant LC systems allowing to profit fully from the advantages in using sub-2 µm particle packed columns.

The narrow peaks that will be produced by fast UHPLC separations will require detection systems with a small detection volume and fast acquisition rates in order to keep the high efficiency gained in the separation. Most of the commercial UHPLC instruments available are equipped with modified UV detectors, with flow cell volumes much lower than those for conventional HPLC, in order to ensure the optimal peak capture. However, due the complexity of sample matrices such as biological samples, UHPLC couple to mass spectrometry has become the method of choice in bio-analytical applications in order to guarantee the confirmation of target compounds. Moreover, because UHPLC enhances chromatographic resolution overall, co-elution is reduced, and that, in turn, leads to a decrease of ion suppression, improving MS sensitivity and reliability. However, since UHPLC greatly enhances separation throughput and resolution, base peaks as narrow as 1 s (or even lower) can be obtained creating practical issues for bio-analytical applications. MS instruments are required to work at low dwell times and low inter-channel and inter-scan delays in order to achieve a sufficient amount of data points (e.g., > 15 points per peak) for UHPLC methods to ensure reliable quantitation [132].

Several recent applications of UHPLC-MS methods in bio-analysis using sub-2 µm particle size packed columns are summarized in Table 3 [36,60,61,67,133-157]. As can be seen, most of the UHPLC applications using columns packed with sub-2 µm particles are focused in the analysis of mainly plasma (or blood related matrices) [60,61,67,133-144,150,151,153,155-157] and urine [36,138,139,142,146-150] matrices, although applications in other biological samples such as several tissues [153,154], tumor tissues [152,153], faeces [150], human seminal plasma [145], and saliva [142] have also been reported. For instance, Baumgarten *et al.* [152] developed an UHPLC-MS/MS method using a C18 column packed with 1.6 µm particles for the rapid confirmation of doxorubicin drug delivery in liver cancer tissue after a transcatheter arterial chemoembolization (TACE) treatment used for palliative therapy. The method allowed the separation within 1 min of doxorubicin and daunorubicin and helped in the better understanding of the factors affecting the delivery and dispersion of doxorubicin within treated tumors during TACE treatments. McWhinney *et al.* [142] developed an UHPLC-MS/MS method for the laboratory routine analysis of glucocorticoid hormones in several matrices such as plasma, plasma ultrafiltrate, urine and saliva. As an example, Figure 2 shows the chromatographic separation of cortisol, cortisone, 11-deoxycortisol, prednisolone and dexamethasone hormones (Figure 2a) as well as the chromatograms obtained after application of the proposed UHPLC-MS/MS method to the analysis of several matrices (Figures 2b-2f). Chromatographic separation of all glucocorticoids in less than 2.5 min was achieved showing limits of quantitation in the range of 1 to 5 nmol L-1 (depending on the sample matrix) and with intra-assay and inter-assay precisions with RSD values lower than 5 and 10%, respectively, for all compounds in all matrices.

Most of the applications are based on reversed-phase separation using the Acquity UPLC BEH C18 column of 1.7 µm particle size with different columns lengths (30, 50 or 100 mm), but other C18 reversed-phase columns such as Zorbax Eclipse XDB-C18 (1.8 µm particle size) [36,61,136,139,150], Shimadzu Shim-pack ODS (1.6 µm particle size) [152] or Hypersil Gold C18 (1.9 µm particle size) [60,144,148,151,157] have also been used. Although not strictly sub-2 µm particle size columns, some bio-analytical UHPLC-MS applications can be found using columns with slightly higher totally porous particle sizes. As an example, Tuffal *et al.* [140] reported the use of a Shimadzu Shim-pack XR-ODS II column of 2.2 µm particle size for the UHPLC-MS separation of clopidogrel active metabolite isomers in plasma in less than 7 min. Other stationary phases have also been described for UHPLC-MS bio-analytical applications. For instance, the use of a high strength silica (HSS) column (Acquity UPLC HSS T3, 1.8 µm particle size) was reported by Vanden Bussche *et al.* [149] for the analysis of eight thyreostats in urine, without any derivatisation, in less than 6.5 min. While Jiménez Giron *et al.* [146] used a C8 reversed-phase column (Zorbax SB-C8, 1.8 µm particle size) for the UHPLC-High resolution Orbitrap MS screening analysis of diuretic and stimulant compounds in urine for doping control. By screening in full scan MS with scan-to-scan polarity switching more than 120 target analytes could be detected in less than 8 min.

Regarding MS detection, triple quadrupole analyzers are instruments of choice for UHPLC-MS bio-analytical applications as can be seen in Table 3. Other MS and HRMS instruments have also been used for some bio-analytical applications, which will be discussed in further detail in section 4.

**3.2. Fused-core particle packed columns**

The recent commercialization of fused-core (also known as porous shell) particle technology presents a new option for HPLC bio-analytical applications in order to achieve fast chromatographic and high efficiency separations. Today, columns packed with porous shell particles consisting of silica particles of a 1.7 µm fused core and 0.5 µm layer of porous silica coating, creating a total particle diameter of 2.7 µm, are available under the brand name HALO (Advance Materials Technology) or Ascentis (Sigma-Aldrich). Other particle diameter sizes are also available such as in the case of Kinetex (Phenomenex) columns with a 1.9 µm fused core and 0.35 µm layer of porous silica coating, obtaining 2.6 µm particles and Accucore (Thermo Fisher Scientific) columns with also a total particle diameter of 2.6 µm. This fused-core column technology, with a solid silica inner core surrounded by a porous silica shell has a shortened diffusion path which allows rapid mass transfer and thus reduced axial dispersion and peak broadening [158]. The reduction in axial diffusion makes possible working at higher flow- without losing chromatographic performance [159]. So, fused-core silica particles offer the possibility to improve chromatographic column efficiency over fully porous particles, and exhibit efficiencies that are comparable to sub-2 µm porous particles, but with lower backpressures. For instance, Figure 3 shows the chromatographic separation of bromo-guanosine, labetalol, reserpine and a selected drug compound obtained with two conventional particle size columns (Luna C18(2) HST 2.5 µm and Luna PFP 3 µm), a sub-2 µm particle size column (Acquity BEH C18 1.7 µm) and a fused-core column (Ascentis Espress C18 2.7 µm) [160]. The fused-core column showed similar peak widths than the other columns (similar column efficiency) at approximately 75% of the maximum specified backpressure for this column, even after more than 1500 injections of protein precipitated plasma extracts. It should be noted that the most popular UHPLC stationary phase material, Acquity BEH C18 1.7 µm column, operates at high backpressure (>700 bars) even with the column oven set at 65 oC (combined with an efficient mobile phase pre-heating device in-line prior to the UHPLC column). Higher pressures were obtained when the column oven temperature was set at 40 oC, leading to concern regarding the robustness of the system for application in the successful conduct of thousands of analyses of extracted plasma samples.

The use of porous shell column technology is a relatively recent trend in chromatographic separations and only a few papers about bio-analytical applications are described in the literature, and some of the most recent ones have been included in Table 4 [160-165]. As can be seen, all the applications are dealing with C18 reversed-phase separations. As in the case of columns packed with sub-2 µm particles, triple quadrupole instruments are usually selected for UHPLC-MS applications. For instance, Song *et al*. [165] proposed an UHPLC-MS/MS method using a 2.7 µm fused-core column and a triple quadrupole instrument for the analysis of imipramine and desipramine antidepressants in protein precipitated rat plasma samples, and a separation within 2.5 min was achieved at a flow rate of 0.4 mL min-1, with good intra-run precisions and accuracies (within 14.4 and 14.7% at the LOQ level for both analytes). However, other MS instruments such as quadrupole linear ion traps for the analysis of oseltamivir and oseltamivircarboxylate in dried blood spots [163], or even high resolution mass spectrometry using a TOF MS instrument for the UHPLC-MS analysis of isoliquiritigenin metabolites in urine [162] or a linear ion trap-Orbitrap HRMS instrument for the analysis of glutathione-trapped reactive metabolites in plasma [161] have also been reported.

**4. Mass spectrometry in bio-analysis**

LC-MS has proven to be a powerful technique in bio-analysis. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most common ionization sources used in LC-MS. Nevertheless, ESI operating in both negative and positive modes is in general the most selected ionization source in bio-analytical science (Table 3 and 4). However, many studies have reported difficulties with reproducibility and accuracy when analyzing small quantities of analytes in complex samples such as biological fluids. Because of the specificity of the MS methods, analysis times in LC–MS assays are often reduced significantly by researchers, due to the misconception that chromatographic separation and sample preparation can be minimized or even eliminated. However, LC–MS by itself does not guarantee selectivity. Disregarding sample clean-up, especially when complex matrices are involved, will lead to poor performance. Thus, careful consideration must be given to evaluating and eliminating matrix effects when developing any assay. Ion suppression is one the major problems in LC-MS with atmospheric pressure ionization (API) sources, especially with ESI. Ion suppression occurs due to the competition among several ions during ion evaporation [166]. Nowadays, as it is reported in this review, fast liquid chromatography and fast sample analysis are commonly used to reduce the analysis time and determine the maximum number of compounds in the same run. However, important matrix effects can be present and its evaluation is necessary to obtain accurate quantitation results. Generally, in order to reduce matrix effects, one strategy can be to improve the sample preparation procedure which is in conflict with the fast and non-selective sample treatment procedures that are demanded. Recent breakthroughs in sample clean-up have been achieved to exploit on-line SPE and TFC with column switching systems, where matrix components are diverted to waste before the elution step, hence the amount of undesirable compounds reaching the LC-MS system is reduced. Another way to reduce the matrix effect is to optimize the chromatographic separation and increase the chromatographic resolution. In this way, and as it was commented in the section 3.1 and 3.2 the use of sub-2 µm particle size columns and porous shell columns is increasing. Nevertheless, important matrix effects can still be observed and the use of alternative ionization sources less sensitive to matrix effects such as APCI and atmospheric pressure photoionization (APPI) is proposed. For instance, Mueller *et al.* [105] developed a TFC-LC-MS/MS method using APCI as ionization source for the analysis of sirolimus and its derivative everolimus, two immunosuppressive agents, in whole blood in order to reduce the matrix effects observed when ESI was used. APCI ionization source has been also used for the multi-screening of drugs and metabolites in serum and plasma [106,113], as well as for the analysis of vitamins [108] and tyrosinekinase inhibitors [110]. On the other hand, some results indicate that APPI is less susceptible to ion suppression and salt-buffer effects than ESI and APCI [167]. For instance, Borges *et al.* [45] developed a LC-APPI-MS/MS method for the analysis of ethinylestradiol in human plasma. In this study the use of APPI provided better sensitivity than ESI and in addition no significant matrix effects were observed making possible the analysis of ethinylestradiol at low concentration levels. On the other hand, this ionization technique has been successfully used for the analysis of a broad spectrum of non-polar lipids such as steroids, (glycol-)sphingolipids, and phytosterols [168]. However, until now there are only few publications regarding the use of APPI in bio-analysis and much work needs to be done to evaluate its potential in this area.

Triple quadrupole (QqQ) instruments operating in selected reaction monitoring (SRM) mode are the most common analyzers used in bio-analysis (Table 3 and 4). As a compromise between sensitivity, acceptable chromatographic peak shape, and the confirmation purposes established by 2002/657/EC directive [2] two SRM transitions are currently monitored. However, in some cases the use of only two transitions could result in false-positive or false-negative confirmations when the compound co-elutes with an interfering matrix compound with ions in the MS/MS spectrum matching with those of the target analyte [169-171]. In these cases, false-positive results can be prevented with by further confirmatory analysis, e.g. the use of a third transition or an orthogonal criterion like exact mass measurements. On the other hand, despite its high selectivity and sensitivity the use of SRM acquisition mode in QqQ instruments is limited by the cycle time when dealing with hundreds of compounds, and a significant drawback to this type of analyzers is that only those molecules that have been targeted are detected (missing non-target compounds or even target metabolites). For these reasons, nowadays to solve the problems related to both the cycle time and the target screening method, liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) is being implemented in bio-analysis. Time-of-flight (TOF) and Orbitrap based technologies are currently the most common analyzers used in LC-HRMS. For instance Fung *et al.* [172] proposed a LC-HRMS method using a quadrupole-time-of-flight (Q-TOF) analyzer for the analysis of prednisone and prednisolone in human plasma operating at a mass resolving power of 10,000 and obtaining mass errors below 6 ppm. On the other hand, Jiménez Girón et al. [146] reported a new screening method based on UHPLC-HRMS using polarity switching for the analysis of 122 targeted analytes in urine by direct analysis. In this work the use of polarity switching acquisition mode in combination with HRMS allowed the possibility to obtain two diagnostic ions. This strategy was used to confirm some diuretics compounds that exhibit high sensitivity in negative mode but were also detectable in positive mode. The use of high resolution mass spectrometry is also especially useful in metabolomics studies where full scan MS spectra and accurate mass measurements are acquired for identification purposes [173]. The use of ultra-high resolution mass spectrometry, operating at mass resolving power higher than 30,000 FWHM is especially important in lipidomics studies due to the complexity of this family of compounds. Taking advantage of the ultra-high resolution provided by an Orbitrap analyser isobaric phosphatidylethanolamines (PE) ether species with 7 double bonds (which are common in several model organisms, such as C. elegans) could be differentiated from the PE ester species with two saturated fatty acid moieties having a mass difference of 0.0575 Da (Figure 4, [174]).

However, in some cases the unequivocal identification of target and target-related compounds requires combining the information provided by HRMS and tandem mass spectrometry (MS/MS) experiments. Moreover, accurate mass measurements and elemental composition assignment are essential for the characterization of small molecules. For instance, the accurate mass measurements of the product ions generated in MSn experiments facilitate the elucidation of unknown compounds structures, making attractive the use of hybrid mass spectrometers such as Q-TOF, ion-trap - time-of-flight (IT-TOF), linear ion-trap quadrupole – Orbitrap (LTQ-Orbitrap) and quadrupole-Orbitrap (Q-Exactive). In this way, a new concept in tandem mass spectrometry, “all ion fragmentation” (AIF) experiments, have been recently introduced. This acquisition mode enables the combination of the fragmentation of all generated ions entering into the collision cell with the full scan MS data, allowing retrospective data evaluation for unknown substances in any untargeted approach, and consequently providing an extra confirmation strategy. AIF acquisition mode has become highly important in some bio-analytical applications such as in doping control. For instance, Thomas *et al.* [157] developed a LC-HRMS(/MS) method for the analysis of some prohibited drugs in dried blood spots for doping control with AIF acquisition mode. This strategy was also followed by Zhu *et al.* [161] in the screening of glutathione-trapped metabolites in human plasma where AIF, non-selective in-source collision-induced dissociation (SCID) fragmentation and HRMS were used. Study in which the putative metabolites could be confirmed and their structures elucidated with the corresponding high resolution full scan and high resolution MS/MS data acquired using a LTQ-Orbitrap velos instrument.

**Conclusions and future perspectives**

There is a growing demand for reliable, fast and efficient chromatographic procedures to perform both qualitative and quantitative analysis in bio-analytical field but achieving cost-effective methodologies with reduced analysis time. Fast or ultra-fast separation methods appear as a good tool to satisfy the necessity of reducing the total analysis time in bio-analysis where an increasing number and variety of samples is expected, and in areas where results must be obtained fast. Additionally, the number of target and non-target compounds is also increasing in some of these areas, especially when addressing drug development and doping control issues. The state-of-the-art of fast liquid chromatography coupled to mass spectrometry for bio-analytical applications have been discussed in this review. The advantages and drawbacks of two of the most employed column technologies in bio-analysis, i.e. sub-2µm particle size and porous-shell particle packed columns, have been discussed. Nowadays, UHPLC technology is the most convenient approach to achieve modern, high throughput, efficient, economic and fast LC separations for bio-analytical applications using sub-2µm particle size packed columns. This column technology provides the highest reduction in analysis time maintaining very high column efficiency. However, although different stationary phases i.e. reversed phase, hydrophilic interaction liquid chromatography (HILIC), fluorinated columns, etc. are available in sub-2µm particle size columns, most of the bio-analytical applications are still focused mainly in C18 or C8 reversed-phase columns (Table 3). HILIC is becoming very popular for bio-analytical applications allowing better separation of highly polar compounds than reversed-phase chromatography and it will become a complementary tool to explore in the near future for UHPLC bio-analytical applications. Although the use of columns packed with sub-2µm particles requires special instrumentation because of the high pressures achieved, instruments adapted to operate at these pressures are commercially available. However, this drawback can also be compensated with the use of porous shell columns, which can be employed in any HPLC or UHPLC instrument because the fused-core particle design allows to considerably reduce column backpressure but keeping similar column efficiency than what is achieved in sub-2µm particle size columns. Moreover, as in the case of sub-2µm particle size columns, several stationary phases are also available with porous shell column technology, although only few applications using C18 reversed-phase columns have been recently reported in bio-analysis (Table 4). From this point of view, columns packed with porous shell particles seems to be a more advantageous approach to easily achieve fast LC separations even when using conventional LC instrumentation, and it will also become a field to explore in the next years for bio-analytical applications as an alternative to sub-2µm particle size columns.

Despite the important advances in fast liquid chromatography able to separate species in a few minutes with low solvent consumption, sample extraction and clean-up treatments must be carefully developed to reduce total analysis time. Laboratory automation and high-throughput analysis have become of primary importance to reduce analysis time, costs and variability derived from sample manipulation. The most recently introduced sample treatment automated methodologies in bio-analytical applications have also been addressed in this review, such as on-line SPE methods, turbulent-flow chromatography and the use of MIP and RAM materials. Many current sample preparation techniques are focusing on the reduction of sample manipulation and the number of treatment steps prior to analysis. However, it should be pointed out that sample preparation techniques must be chosen and optimized regarding the method purpose and in consideration of the chromatographic separation. In some cases, a simple and fast sample treatment procedure will not be compatible with a fast liquid chromatographic separation as problems concerning matrix related interferences or matrix effect may arise. In this context, recent developments in on-line SPE aspects in combination with the sensitivity and selectivity achieved by MS/MS have made possible the development of faster and precise on-line SPE-LC- and UHPLC-MS/MS methods for both qualitative and quantitative analysis of heterogeneous substances in biological matrices.

Tubulent flow chromatography appears as a very useful approach for sample treatment in bio-analytical applications where plasma or similar fluids need to be analyzed by removing proteins based on their size better than restricted access materials or SPE procedures. For these reasons, TFC is one of the modern approaches in sample treatment procedures that is becoming more popular and several applications will be available in the future and not only in the bio-analytical area.

New materials are being developed in order to increase the overall efficiency of the extraction process from bio-matrices, the selectivity towards target analytes and allowing easy manipulation and the on-line coupling with higher biocompatibility. Among these materials, MIPs and RAM are attracting much interest in the last years (Table 1). MIP materials are a very useful approach for some bio-analytical applications because it allow preconcentration while providing the highest selectivity for target analytes. One of the main advantages of MIPs is the possibility to prepare selective sorbents pre-determined for a particular substance or a group of structural analogs, which will become very useful for some specific applications. On-line RAM (together with TFC) are solvent-less techniques and although using mobile phase for sample elution, are some of the most environmentally friendly sample treatment procedures. And although some drawbacks are present when using RAM materials such as limitations in sample treatment after being reused several times with biological samples or the presence of carryover for some hydrophobic analytes in reversed-phase RAM due to the weak eluotropic strength of typically used transfer solutions, several advances are taking place with RAM technology. For instance, the incorporation of restricted access properties to magnetic particles are providing supports for new and relevant bio-analytical applications.

Regarding mass spectrometry, the use of triple quadrupole instruments monitoring two SRM transitions continues to be the most common approach used for bio-analytical applications. Nevertheless, in several cases the use of only two transitions resulted in false-positive or false-negative confirmations. Moreover, one of the major drawbacks in bio-analysis when using QqQ analyzers is that only targeted molecules are being detected, missing important information for some bio-analytical applications. Nowadays, high resolution mass spectrometry, either using TOF or Orbitrap analyzers, is being implemented in bio-analytical analysis to solve these problems. The possibility of working at high resolving power together with accurate mass measurements makes these instruments ideal to facilitate identification of unknown compounds which is essential for some bio-analytical applications. In this way, Orbitrap instruments working in AIF mode, which enables the combination of the fragmentation of all generated ions entering into the collision cell with full scan MS data and making possible retrospective data evaluation for unknown substances, will become a powerful tool for bio-analytical applications in the future.

As reviewed, there are many methodologies to choose from in the literature. Comprehensive analysis and testing is needed in order to evaluate these methodologies applied into bio-analytical applications. It is necessary that all steps in analytical method development, sample treatment (extraction and clean-up), chromatographic separation and detection, are developed and optimized in alignment, focusing in the reduction of the total analysis time in order to achieve fast but reliable analytical methods that guarantees quality analytical results, especially in a field with direct implications for human health such as bio-analysis.

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**Figure Captions**

Figure 1. Schematic presentation of a synthetic route of surfactant-coated C12-MNPs. Reproduced from ref. [97], with permission of Elsevier.

Figure 2. Representative UHPLC-MS/MS chromatogram of glucocorticoids. The mass charge ratio of the precursor and product ion, as well as the ion count for each acquisition is shown. (a) Standard with 25 nmol L-1 cortisol, cortisone, 11-deoxycortisol, prednisolone and 5 nmol L-1 dexamethasone. (b) Dilute plasma ultrafiltrate with 0.6 nmol L-1 cortisol. The signal to noise ratio was approximately 4:1. (c) Saliva sample with cortisol and cortisone levels of 2.1 nmol L-1 and 14.7 nmol L-1, respectively. (d) Plasma ultrafiltrate with 6.3 nmol L-1 of cortisol. (e) Plasma sample from a patient collected at 09:00 after administration of 1 mg dexamethasone at 23:00 the previous evening. Plasma cortisol was 133 nmol L-1, cortisone 28 nmol L-1 and dexamethasone 9.0 nmol L-1. (f) Urine from a patient on prednisolone treatment. Urine cortisol was 17 nmol L-1, cortisone 111 nmol L-1 and prednisolone 221 nmol L-1. Reproduced from Ref. [142], with permission of Elsevier.

Figure 3. Gradient UHPLC-MS/MS chromatograms of the (i) bromo-guanosine, (ii) labetalol, (iii) reserpine and (iv) drug compound SB243213A test mix on several possible columns for generic gradient UHPLC-MS bio-analysis. All columns were of 50 mm x 2.1 mm I.D.: (a) Luna HST C18(2) 2.5 µm, (b) Luna PFP 3 µm, (c) Acquity BEH C18 1.7 µm (n=4 chromatograms overlaid) and (d) Ascentis Express C18 2.7 µm (n=4 chromatograms overlaid). Reproduced from Ref. [160], with permission of Elsevier.

Figure 4. LTQ Orbitrap analysis of isobaric species PE 18:0/18:0 and PE O-16:1p/22:6. The time needed for performing one scan at given resolution settings is highlighted in boxes. Reproduced from Ref. [174], with permission of Thermo Scientific.

**Table 1. Use of on-line SPE procedures in bio-analytical applications.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Target compounds** | **Sample** | **SPE Stationary phase** | **Elution Solvents** | **Analytical technique** | **Analytical features** | **Reference** |
| *Pharmaceuticals and drugs* | | | | | | |
| FTY720, FTY720-P | Blood | HySphere C18 HD 7mm | Dimethylhexylamine solution (A) and acetonitrile/isopropanol  (80:20, v/v) (B) | LC-ESI-MS/MS | LOQs > 0.08 ng mL-1 | [23] |
| Sirolimus  and everolimus | Blood | Zorbax Extend-C18  (2.1 mm×12.5 mm, 5 µm) | Methanol (A) and water (B) both containing 0.1% formic acid  in 2 mmol L−1 ammonium formate | LC-ESI-MS/MS | LODs > 0.1 ng mL-1  LOQs > 0.2 ng mL-1 | [24] |
| Δ9-tetrahydrocannabinol and major metabolites | Blood | Polar RP  (20mm×2.0 mm) | 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) | LC-ESI-MS/MS | LODs > 0.18 ng mL-1  LOQs > 0.44 ng mL-1 | [25] |
| Ac‐SDKP peptide | Plasma | TSK BSA‐ODS  (4.6mm×35 mm) | 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) | LC-ESI-MS/MS | LOQs > 0.1 ng mL-1  LODs > 0.05 ng mL-1 | [26] |
| Pharmaceutical compound and its metabolites | Plasma | Gemini C18  (2mm×10mm, 5 µm) | 5mM ammonium formate and 0.2% formic acid in water/acetonitrile (80:20, v/v) | UHPLC-ESI-MS/MS | LOQs > 5 ng mL-1 | [27] |
| Fluoxetine | Plasma | Kromasil C18  ( 1mm×5mm, 5 µm) | Acetonitrile and 0.05 mol L−1 ammonium formate buffer (25:75, v/v) | Capillary LC-ESI-TOF-MS | LODs > 3 ng mL-1  LOQs > 5 ng mL-1 | [28] |
| Amlodipine | Plasma | HySphere C8 EC-SE (2 mm× 10) | After treatment with acetonitrile , 0.1% formic acid in water (A) and methanol (B). | LC-ESI-MS/MS | LOQ 0.10 ng mL-1 | [29] |
| Fluconazole, itraconazole, posaconazole and voriconazole (antifungal drugs) | Plasma | Waters Oasis HLB column  (2.1×20 mm, 25 μm) | Acetonitrile/water 80/20 (A), 2 mM ammonium formiate (B) | LC-MS/MS | LOD 2–10 ng mL-1 | [30] |
| Pharmaceuticals | Plasma | Oasis® HLB cartridge column (2cmL×2.1mmID, 25µm) | 0.1% ammonium acetate (A) and 0.1% methylamine in  methanol (B) | LC-MS/MS | LOD 19-100 pg ml-1 | [31] |
| General unknown screening (drugs) | Plasma and serum | HySphere Resin GP cartridges (2.0mm×10mm, 7µm) | 10 mM ammonium  formate at pH 3.0 (A), 90% acetonitrile, 10% 10 mM  ammonium formate at pH 3.0 (B) | LC-MS/MS | LOD ≤100µg L-1 | [32] |
| Pharmaceutical Remoxipride (dopamine D2 receptor antagonist) | Plasma and Brain homogenate and dialysate | Oasis®weak cationic  exchange (WCX) cartridges | Acetonitrile with 16mM acetic acid (7:3, v/v,  0.1% TFA, pH 2). | LC-MS/MS | LOQ 0.5 ng mL-1 (plasma)  LOQ 0.25-1.8 ng mL-1  ( Brain homogenate and dialysate) | [33] |
| Indacaterol | Serum | Oasis MCX,  (1mm×  10 mm | After acidification, centrifugation, 0.03% ammonia (A) and methanol (B). Vinj 150 µl. | LC-ESI-MS/MS | LOQ 10 ng L-1 | [34] |
| Tilidine, nortilidine,  and bisnortilidine | Serum | OASIS WCX  (10mm×1mm) | 0.2% formic acid (A) and methanol (B) | LC-ESI-MS/MS | LOQs > 1 µg L-1 | [35] |
| Forty-two therapeutic drugs and drugs of abuse | Serum | SPE Strata X-CW  (2mm×20mm, 25 μm) | 0.5% HCOOH in water (A) and 0.5% HCOOH  in acetonitrile (B) (90/10, v/v) | UHPLC-ESI-MS/MS | LODs > 0.20 µg L-1  LOQs > 0.66 µg L-1 | [36] |
| 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid | Serum | HySphere C8 | 0.1% formic acid (A) and acetonitrile (B) | LC-ESI-MS/MS | LODs > 0.25 µg L-1  LOQs > 5 µg L-1 | [37] |
| Six corticosteroids | Liver and fat | Alltima C18  (2.1mm×7.5mm, 5µm) | 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) | LC-ESI-MS/MS | LOQs > 0.4 fmol | [38] |
| *Peptides* | | | | | | |
| Peptide drug FE 202158 | Plasma | C4(1.0mm×50mm) packed+  a cyano column  (2.1mm×50mm) | Formic acid (A), acetonitrile (B) (50:50) | LC-MS/MS | LOQ 5 pg mL-1 | [39] |
| Sifuvirtide (anti-HIV peptide) | Plasma | C18 SPE column (50mm×2.1mmi.d., 2µmfrits | 0.2% formic acid in water (A), acetonitril/methanol (1:1) (B) (70:30) | LC-MS/MS | 6.1 ng mL-1 | [40] |
| Aggrecan fragments (peptide) | Urine and Human sinovial fluid | home-prepared immunoaffinity columns and macro peptide-trapping column  (Michrom Bioresources, Auburn, CA, USA) | 0.1% formic acid in water (A) and 5% acetonitrile in water (B) | LC-MS/MS | LOD 2.5 pg mL-1  (Urine)  LOD 10 pg mL-1 (human synovial fluid) | [41] |
| *Personal care products* | | | | | | |
| Triclocarban and its oxidative metabolites | Human urine and serum | LiChrospherTM RP-18 ADS (25 mm × 4 mm, 25 µm particle size, 60 ˚A pore size) | Acetonitrile/water  (50/50, v/v) | LC-ESI-MS/MS | LODs > 0.01 ng mL-1  LOQs > 0.03 ng mL-1 | [42] |
| 2-ethylhexyl  4-(N,N-dimethylamino)benzoate | Human urine | HySphere C18 HD  (10mm×2mm, 7µm) | 0.2% formic acid in water (A) and 0.2% formic acid methanol/ acetonitrile (1/1, v/v) (B) | LC-ESI-MS/MS  LC-ESI-TOF/MS | LODs > 0.1 ng  LOQs > 0.3 ng | [43] |
| *Endogenous compounds* | | | | | | |
| Tryptophan, kynurenine and 3-hydroxykynurenine | Plasma | Isolute PRS (propylsulphonic acid based strong cation  exchange) (10mm×1mm) | 50 mM ammonium formate  (pH 3) | LC-ESI-MS/MS | LODs > 1 nmol L-1  LOQs > 23 nmol L-1 | [44] |
| Ethinylestradiol | Plasma | Hysphere C18 HD  7µm | Methanol/water (75/25, v/v) | LC-APPI-MS/MS | LODs > 0.08 pg mL-1  LOQs > 5 pg mL-1 | [45] |
| 8-oxo-7,8-dihydro-2′-deoxyguanosine | Plasma, urine and saliva | Inertsil ODS-3 column  (50 mm×4.6 mm, 5 µm) | 50 mL L-1 methanol containing 1 mL L-1 formic acid (A) and 500 mL L-1 methanol containing 1 mL L-1 formic acid (B) | LC-ESI-MS/MS | LODs > 2 fmol | [46] |
| Aldehyde products | Plasma and urine | ISOLUTE CBA  (10 mm ×2.1 mm, 40 µm) | 99% water + 1% acetonitrile + 0.2% formic acid (A) and 5% water + 95% acetonitrile + 0.2% formic acid (B) | LC-ESI-MS/MS | LODs > 0.5 nM | [47] |
| Eicosanoid inflammation  biomarkers | Serum | HySphere C18 (EC)  (10mmx2.0mm, 8 μm) | methanol/water/  acetonitrile/acetic acid (76/22/2/0.02, v/v) | LC-ESI-MS/MS | LODs > 0.09 pg mL-1;  LOQs > 3 pg mL-1 | [48] |
| Prostanoids | Serum | Hysphere C8 (EC)  (10mm×2.0mm, 8µm) | Water/acetonitrile containing  0.02% formic acid (66:34, v/v) | LC-ESI-MS/MS | LODs > 2.3 pg  LOQs > 7.5 pg | [49] |
| Testosterone | Serum | Poros R1/20 (C4)  (30 mm×2.1 mm, 20 µm) | 60% methanol in water (pH 2.8) (A) and 100% methanol (pH 4.6) (B) both containing  2 mmol L−1 ammonium acetate and 0.1% formic acid (70:30, v/v) | LC-ESI-MS/MS | LODs > 0.15 nmol L-1  LOQs > 0.25 nmol L-1 | [50] |
| Folate catabolites | Serum, urine and breast milk | Hysphere MM anion exchange  (10mm×2mm, 25–35 µm) | 20mM ammonium formate in 80:20  (v/v) acetonitrile–water (pH 7.3) | LC–ESI-MS/MS | LODs > 0.3 pmol mL-1  LOQs > 1.1 pmol mL-1 | [51] |
| Serotonin | Serum | Oasis WCX  (10 mm×1 mm) | 100 mM ammonium formate (pH 3) (A) and acetonitrile (B) | LC-ESI-MS/MS | LOQs > 0.9 nmol mL-1 | [52] |
| 25-Hydroxyvitamin D | Serum | Waters 5 \_m X-Terra  (2.1 mm×20 mm C18) | (98:2) methanol:water  0.1% formic acid and 2 mM ammonium acetate(A),  water with 0.1% formic acid and 2 mM ammonium acetate (B) | LC-MS/MS | LOQ 3 nmol L-1 | [53] |
| N3-methyladenine | Urine | Inertsil ODS-3  (33mm×2.1mm i.d., 5 µm) | 3% methanol containing 0.1% trifluoroacetic acid (v/v) (A) and 75% methanol containing 0.1% trifluoroacetic acid (v/v) (B) | LC-ESI-MS/MS | LODs > 0.035 ng mL-1  LOQs > 0.1 ng mL-1 | [54] |
| Cortisol, cortisone and metabolites | Urine | POROS R1/20  (2.1mm×30 mm, 20 μm particle size) | methanol/acetonitrile (50:50, v/v) (A), and  water and methanol (50:50, v/v) (B), both  containing 0.1% formic acid | LC-ESI-MS/MS | -- | [55] |
| Methylmalonic acid | Urine | Waters Oasis HLB  (2.1×20 mm,25 µm) | 1 g L−1 formic acid-acetonitrile/10 mM ammonium formiate 60:40 (v/v) | LC-ESI-MS/MS | -- | [56] |
| Pthalate metabolites | Urine | Merck C18 trap cartridge  (2.0 x 55-mm, 3µm, , | 0.001% formic acid in H2O (A), metanol (B) | LC-MS/MS | LOD 0.2-2 ng mL-1 | [57] |
| Cortisol and cortisone | Human saliva | HySphereTM C18 HD  (2 mm 10mm, 7 μm SPE cartridge | 0.1% (v/v) formic acid in water (A),0.1% (v/v) formic acid in methanol (B) (50:50) | LC-MS/MS | LOQ Cotisol 0.75 nM  Cortisone 0.50 nM | [58] |
| 8-iso-PGF2α (indicator of lipid peroxidation) | Exhaled breath condensate | C18 Inertsil ODS  (33.3mm×4.6mm, 5µm) column | 1% acetonitrile, v/v with 0.1% formic acid)(A), 90% acetonitrile, v/v with 0.1% formic acid (B) (85:15) | LC-MS/MS | LOD 1 pg mL-1 | [59] |
| *Pollutants* | | | | | | |
| Chlorpyrifos and cypermethrin | Plasma | Hypersil GOLD C8  (20mm×2.1 mm, 1.9µm) | 20 mM ammonium acetate aqueous solution and  methanol (10:90, v/v) | LC-ESI-MS/MS | LODs > 0.01 ppb | [60] |
| Perfluorinated compounds | Plasma | Poros HQ  (2.1mm×30mm, 10µm) | 0.01% NH4OH solution in 5mM ammonium acetate (A) and 0.01% NH4OH solution in acetonitrile (B) | UHPLC-ESI-MS/MS | LODs > 3 ng L-1  LOQs > 10 ng L-1  MDLs > 9 ng L-1 | [61] |
| Polyfluorinated  compounds | Serum | Polaris C18 HD  (2mm 10mm) | 20mM ammonium  acetate (pH 4) in water (A), acetonitrile (B) | LC-MS/MS | LOD 0.1- 0.2 ng mL-1 | [62] |
| Polyfluorinated  compounds | Serum and human breast milk | Oasis® HLB,  (2.1mm×20mm 25µm) | 38% 2mMammonium acetate buffer adjusted  to pH 5 with acetic acid (A), 62% methanol (B) | LC-MS/MS | 0.1 to 0.4µg L-1 (serum)  0.02 to 0.15µg L-1  (human breast milk) | [63] |
| Free catecholamines | Serum | Bond Elut PBA  (2mm×10mm) | 100mM ammonium formate (pH 3) | LC-ESI-MS/MS | LOQs > 1.47 nmol L-1 | [64] |
| Intermediates of the tricarboxylic acid cycle | Pig and mouse heart tissue | Phenomenex C8 SPE cartridge  (4 mm x 2 mm, 5 µm) | 98% water, 2% acetonitrile and 0.1%  formic acid (A), (98% acetonitrile, 2% water and 0.1% formic acid (B) | LC-ESI-MS | LOD 12-1000 nM | [65] |

**Table 2. Use of turbulent flow chromatography in bioanalysis. Selected references from 2009-up-to-date.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Target compounds** | **Class** | **Matrix** | **TFC Column** | **Flow-rate**  **Injection Volume** | **Detection** | **LOQ** | **Reference** |
| Sirolimus and everolimus | Immunosuppressant | Whole blood | 50 x 0.5 mm, 50 µm  Cyclone  (Thermo Fisher Scientific) | 2.0 mL min-1  50 µL | APCI-MS/MS | 0.5 µg L-1 | [105] |
| 453 Drugs | General - toxicological screening | Urine and serum | 50 x 0.5 mm, 60 µm  Cyclone and C18 XL  (Thermo Fisher Scientific) | 2.0 mL min-1  100 µL | APCI-MSn | - | [106] |
| Perfluorinated compounds | Perfluorinated compounds | Human hair and urine | 50 x 0.5 mm, 60 µm  Cyclone and C18 XL  (Thermo Fisher Scientific) | 1.5 mL min-1  20 µL | ESI-MS/MS | 0.06-13.34 ng g-1 | [107] |
| 25-OH D2/D3 | Vitamins | Serum | Cyclone P  50 x 1.0 mm  (Thermo Fisher Scientific) | 4.0 mL min-1  100 µL | APCI-MS/MS | 4.6 nmol L-1 (25-OHD3) 3.0 nmol L-1 (25-OHD2) | [108] |
| - | Metabonomic studies | Plasma | 50 x 0.5 mm, 50 µm  Cyclone  (Thermo Fisher Scientific) | 1.25 mL min-1  10 µL | ESI-MS | - | [103] |
| Busulfan | Alkylating antineoplastic agent | Plasma and serum | Cyclone P  50 x 0.5 mm  (Thermo Fisher Scientific) | 1.5 mL min-1  50 µL | ESI-MS/MS | 0.15µmol L-1 | [109] |
| 9 TKIs and metabolites | Tyrosine kinase inhibitors | Plasma and serum | 50 x 0.5 mm, 50 µm  Cyclone  (Thermo Fisher Scientific) | 2.0 mL min-1  100 µL | APCI-MS/MS | 1-10 µg L-1 a | [110] |
| Creatinine | Creatinine | Serum | 50 x 0.5 mm  Cyclone MCX  (Thermo Fisher Scientific) | 1.5 mL min-1  20 µL | ESI-MS/MS | 0.20 mg dL-1 | [111] |
| - | Pharmacokinetics | Plasma and hepatocytes | 50 x 0.5 mm, 60 µm  Cyclone  (Thermo Fisher Scientific) | 1.25 mL.min-1  20 µL | ESI-MS/MS | - | [112] |
| 356 Drugs and metabolites | General - toxicological screening | Urine | 50 x 0.5 mm, 60 µm  Cyclone and C18 XL  (Thermo Fisher Scientific) | 2.0 mL min-1  100 µL | APCI-MSn | b 10 ng L-1 (60%)  100 ng L-1 (90%)  1000 ng L-1 (100%) | [113] |
| Amitriptyline Desipramine  Imipramine  Nortriptyline | Tricyclic Antidepressants | Serum | Cyclone P  50 x 0.5 mm  (Thermo Fisher Scientific) | 2.0 mL min-1  10 µL | ESI-MS/MS | 6-18 ng mL-1 | [114] |
| Metanephrine and Nometanephrine | Metanephrines | Plasma | 50 x 0.5 mm  MCX-2  (Thermo Fisher Scientific) | 2.0 mL min-1  100 µL | ESI-MS/MS | 6.3-12.6 pg mL-1 | [115] |
| Verticine  Verticinone  Isoverticine | Drugs | Plasma | 20 x 2.1 mm, 25 µm  Oasis HLB  (Waters) | 4.0 mL min-1  50 µL | ESI-MS | 0.12-0.595 ng mL-1 | [116] |
| 19 drugs and metabolites | Drugs | Urine | 50 x 0.5 mm  Cyclone P and Cyclone P  (Thermo Fisher Scientific) | 2.0 mL.min-1  100 µL | ESI-MS/MS | 5-25 ng mL-1 | [117] |

a Reported as limit of accurate measurement (signal at least five times the SD of the background noise)

b Reported as limited of identification

**Table 3. UHPLC bio-analytical applications using sub-2 µm particle size packed columns.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Target compounds** | **Sample matrix** | **Column / Stationary phase/Temperature** | **Mobile phase / Flow-rate** | **Mass spectrometry** | **Analysis time** | **Reference** |
| JAK2 inhibitor CYT387 | Plasma | Acquity UPLC BEH C18  (30 mm x 2.1 mm, 1.7 µm)  40 oC | Gradient elution:  A) 0.005% formic acid solution in water  B) 0.05 % formic acid solution in methanol  0.6 mL min-1 | HESI(+)  Triple quadrupole  SRM acquisition mode | 1.3 min | [133] |
| Ketoconazole | Plasma | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm) | Isocratic elution:  Water:acetonitrile 44:56 (*v/v*) with 0.1% ammonium hydroxide and 10 mM ammonium bicarbonate  1.0 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 20 s | [134] |
| Vitamin D metabolites | Plasma | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm | Gradient elution:  A) 0.1% formic acid in water (5 mM methylamine)  B) 0.1% formic acid in methanol  0.3 mL min-1 | ESI(+)  Triple quadrupole-linear ion trap  SRM acquisition mode | 2.2 min | [135] |
| Anticancer PR-104 and metabolites | Plasma | Zorbax Eclipse XDB-C18  (50 mm x 2.1 mm, 1.8 µm) | Gradient elution:  A) 0.01% formic acid in water  B) acetonitrile  0.5 mL min-1 | Positive ESI-APCI combined mode  Triple quadrupole  SRM acquisition mode | 3.1 min | [136] |
| Small-molecule complements in Biological system | Plasma | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm)  40 oC | Gradient elution:  *Extracts reconstituted in formic acid*  A) 0.1% formic acid in water  B) 0.1% formic acid in methanol  *Extracts reconstituted in ammonium bicarbonate*  A) 6.5 mM ammonium bicarbonate in water, pH 8  B) 6.5 mM ammonium bicarbonate in methanol:water 95:5 (*v/v*)  0.35 mL min-1 | ESI(+) and ESI(-)  Linear ion trap MS  SRM acquisition mode  *Accurate mass measurements*  Hybrid LTQ-FTICR M  (Resolving power 50,000 FWHM) | 8 min | [137] |
| Pravastatin  Pravastatin lactone | Plasma  Urine | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm)  35 oC | Gradient elution:  A) 1 mM ammonium acetate pH 4.0  B) acetonitrile  0.2 mL min-1 | ESI polarity switching mode  Triple quadrupole  SRM acquisition mode | 2 min | [138] |
| Tectorigenin, irigenin and irisfloretin | Plasma  Urine | Zorbax SB-C18  (50 mm x 2.1 mm, 1.8 µm) | Gradient elution:  A) 0.1% formic acid in water  B) acetonitrile  0.4 mL min-1 | ESI(-) and ESI(+)  Triple quadrupole  SRM acquisition mode | 6 min | [139] |
| Octreotide | Plasma | Acquity UPLC BEH C18  (100 mm x 2.1 mm, 1.7 µm) | Gradient elution:  A) 0.1% formic acid in water  B) 0.1% formic acid in acetonitrile  0.25-0.3 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 5 min | [67] |
| Clopidogrel active metabolite isomers | Plasma | Shim-pack XR-ODS II  (75 mm x 2.0 mm, 2.2 µm) | Gradient elution:  A) 2 mM ammonium acetate-0.2% formic acid in water  B) 2 mM ammonium acetate-0.2% formic acid in acetonitrile  0.5 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 7 min | [140] |
| Eicosapentaeonic acid and docosahexenoic acid | Plasma | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm)  60 oC | Gradient elution:  A) 2 mM ammonium acetate pH 4  B) acetonitrile  1 mL min-1 | ESI(-)  Triple quadrupole  SRM acquisition mode | 1.3 min | [141] |
| Cortisol, cortisone, prednisolone, dexamethasone and 11-deoxycortisol | Plasma, plasma ultrafiltrate, urine and saliva | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm) | Gradient elution:  A) 2 mM ammonium acetate – 0.1 % formic acid in water  B) 2 mM ammonium acetate – 0.1% formic acid in methanol  0.4 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 2.5 min | [142] |
| Nitrofuran metabolites | Plasma | Acquity UPLC BEH C18  (100 mm x 2.1 mm, 1.7 µm)  65 oC | Gradient elution:  A) 0.5 mM ammonium acetate in water  B) methanol  0.5 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 3 min | [143] |
| Testosterone and testosterone enanthate | Plasma | Hypersil Gold C18  (50 mm x 2.1 mm, 1.9 µm)  45 oC | Gradient elution:  A) 2 mM ammonium formate in water  B) methanol  0.5 mL min-1 | HESI(+)  Triple quadrupole  SRM acquisition mode | 4.5 min | [144] |
| Anandamide and related acylethanolamides | Human seminal plasma | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm) | Gradient elution:  A) 2 mM ammonium formate containing 0.1% formic acid and 5% acetonitrile  B) 0.1% formic acid in acetonitrile  0.7 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 3 min | [145] |
| Diuretic and stimulant compounds | Urine | Zorbax SB-C8  (50 mm x 2.1 mm, 1.8 µm) | Gradient elution:  A) 1 mM ammonium acetate – 0.001% acetic acid in water  B) 1 mM ammonium acetate - 0.001% acetic acid in methanol  0.3 mL min-1 | ESI polarity switching mode  HRMS Orbitrap | 8 min | [146] |
| Anabolic steroids | Urine | Acquity UPLC BEH C18  (100 mm x 2.1 mm, 1.7 µm) | Gradient elution:  A) 5 mM ammonium formatted pH 3 in water  B) 0.1% formic acid in acetonitrile  0.4 mL min-1 | HESI(+)  Triple quadrupole  SRM acquisition mode | 8 min | [147] |
| Cardiovascular drugs, polyphenols and metabolites | Urine | Hypersil Gold C18  (100 mm x 2.1 mm, 1.9 µm)  25 oC | Gradient elution:  A) 0.1% formic acid in water  B) acetonitrile  0.65-0.7 mL min-1 | ESI(+) and ESI(-)  Triple quadrupole-linear ion trap  SRM acquisition mode | 8 min | [148] |
| Therapeutic drugs and drugs of abuse | Urine | Zorbax Eclipse XDB-C18  (50 mm x 4.6 mm, 1.8 µm)  55 oC | Gradient elution:  A) 0.5% formic acid in water  B) 0.5% formic acid in acetonitrile  0.9 mL min-1 | ESI(+)  QTrap MS  SRM acquisition mode | 6 min | [36] |
| Thireostats | Urine | Acquity UPLC HSS T3  (high strength silica particles)  (100 mm x 2.1 mm, 1.8 µm)  25 oC | Gradient elution:  A) 0.1% formic acid in water  B) 0.1% formic acid in methanol  0.3 mL min-1 | HESI(+)  Triple quadrupole  SRM acquisition mode | 6.5 min | [149] |
| Rifamicyn isonicotinyl hydrazone (HYD) | Plasma, urine and faeces | Zorbax Eclipse C18  (50 mm x 4.6 mm, 1.8 µm) | Gradient elution:  A) 10 mM ammonium acetate in water  B) acetonitrile  0.5 mL min-1 | ESI(+)  Linear ion trap MS  SRM acquisition mode | 5 min | [150] |
| 2-*tert*-butylprimaquine (anti-malarial compound) | Plasma | Hypersil Gold C18  (50 mm x 2.1 mm, 1.9 µm) | Gradient elution:  A) 20 mM ammonium acetate  B) acetonitrile  0.45 mL min-1 | ESI(+)  Linear ion trap MS  SRM acquisition mode | 4 min | [151] |
| Doxorubicin and daunorubicin | Liver tumors | Shimadzu Shim-Pack ODS  (50 mm x 2.0 mm, 1.6 µm) | Gradient elution:  A) 5 mM ammonium acetate (pH 3.5) in water  B) acetonitrile  0.5 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 1.1 min | [152] |
| Anti-tumoral alkyl lysophospholipid edelfosine | Plasma, tissue, tumor and lipid nanoparticulate systems | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm)  50 oC | Isocratic elution:  1% formic acid aqueous solution:methanol 5:95 (*v/v*)  0.5 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 4 min | [153] |
| Neurotransmitters | Brain tissue | Acquity UPLC BEH C18  (50 mm x 2.1 mm, 1.7 µm) | Gradient elution:  A) 0.05% formic acid and 1 mM heptabluorobutyric (HFBA) acid in water  B) methanol  0.2 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 4 min | [154] |
| Atorvastatin and its metabolites | Serum | Acquity UPLC BEH C18  (100 mm x 2.1 mm, 1.7 µm)  35 oC | Gradient elution:  A) 0.5 mM ammonium acetate (pH 4.0) in water  B) acetonitrile  0.25 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 3.5 min | [155] |
| Emodin | Plasma from digestive segments | Acquity UPLC BEH C18  (100 mm x 2.1 mm, 1.7 µm)  35 oC | Gradient elution:  A) 0.1% formic acid in water  B) methanol  0.3 mL min-1 | ESI(-)  Triple quadrupole  SRM acquisition mode | 4 min | [156] |
| Perfluorochemicals | Plasma and serum | Zorbax Eclipse XDB-C18  (50 mm x 4.6 mm, 1.8 µm)  55 oC | Gradient elution:  A) 0.01% NH4OH, 5 mM ammonium acetate in water  B) 0.01% NH4OH in acetonitrile  1 mL min-1 | ESI(-)  QTrap MS  SRM acquisition mode | 5 min | [61] |
| Chlorpyrifos and cypermethrin | Cord blood plasma | Hypersil Gold C18  (50 mm x 2.1 mm, 1.9 µm) | Gradient elution:  A) 20 mM ammonium acetate in water  B) methanol  0.3 mL min-1 | HESI(+)  Triple quadrupole  SRM acquisition mode | 5 min | [60] |
| Prohibited drugs in doping control | Dried blood spots | Hypersil Gold C18  (50 mm x 2.1 mm, 1.9 µm) | Gradient elution:  A) 0.2% formic acid in water  B) acetonitrile  0.2 mL min-1 | HESI(+) and HESI(-)  Quadrupole-Orbitrap  Full scan and all-ion fragmentation full scan acquisition modes | 11 min | [157] |

**Table 4. Bio-analytical applications using porous shell column technology.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Target compounds** | **Sample matrix** | **Column / Stationary phase/Temperature** | **Mobile phase / Flow-rate** | **Mass spectrometry** | **Analysis time** | **Reference** |
| Glutathione-trapped reactive metabolites | Plasma | Kinetex C18  (100 mm x 2.1 mm, 2.6 µm) | Gradient elution:  A) 0.1 % formic acid in water  B) 0.1% formic acid in acetonitrile  0.4-0.6 mL min-1 | HESI(+) and HESI(-)  Linear ion trap-Orbitrap HRMS  SIM acquisition mode and  FTMS or ITMS data dependent scan of MS2 spectra | 10 min | [161] |
| Isoliquiritigenin metabolites | Urine | Agilent Poroshell EC-C18  (100 mm x 2.1 mm, 2.7 µm)  40 oC | Gradient elution:  A) 0.1% formic acid in water  B) 0.1% formic acid in acetonitrile  0.4 mL min-1 | ESI(+) and ESI(-)  TOF MS | 12 min | [162] |
| Oseltamivir and oseltamivircarboxylate | Dried blood spots | Ascentix Express C18  (100 mm x 2.1 mm, 2.7 µm)  30 oC | Gradient elution:  A) 0.1% formic acid in water  B) 0.1% formic acid in methanol  0.35 mL min-1 | ESI(+)  QTrap MS  SRM acquisition mode | 3.5 min | [163] |
| Bromo-guanosine, labetalol, reserpine and SB243213A (drug compound) | Plasma | Ascentix Express C18  (50 mm x 2.1 mm, 2.7 µm)  40 oC | Gradient elution:  A) 0.1% formic acid in water  B) 0.1% formic acid in acetonitrile  1.1 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 0.8 min | [160] |
| Drugs of abuse | Oral fluid | Kinetex C18  (100 mm x 2.1 mm, 2.6 µm)  40 oC | Gradient elution:  A) 0.05% formic acid and 5 mM ammonium formiate in water  B) 0.1% formic acid in methanol:acetonitrile 1:1 (*v/v*)  0.5 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 8 min | [164] |
| Imipramine and desipramine antidepressants | Plasma | Halo fused-core C18  (30 mm x 2.1 mm, 2.7 µm) | Gradient elution:  A) 10 mM ammonium acetate and 0.1% NH4OH in water (pH 8.5)  B) 10 mM ammonium acetate, 0.1% NH4OH and 0.2% (morpholine or triethylamine) in acetonitrile  0.4 mL min-1 | ESI(+)  Triple quadrupole  SRM acquisition mode | 2.5 min | [165] |