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

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Contents

Acronyms

ADS	Alkyl-diol silica
AIF	All ion fragmentation
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure phtoionization
ATCA	2-aminothiazoline-4-carboxylic acid
CBA	Propylcarboxylic acid
ESI	Electrospray
FDA	Food and drug administration
FWHM	Full width at half maximim
HETP	height equivalent to a theoretical plate
HILIC	hydrophilic interaction chromatography
HRMS	High Resolution Mass Spectrometry
HSS	High Strength Silica
ISRP	internal surface reversed-phase

IT-TOF	Ion trap time-of-flight
LLE	Liquid-Liquid Extraction
LOQ	Limit of Quantitation
LSD	Lysergic acid diethylamide
MIP	Molecular Imprinted Polymers
MISBSE	MIPs stir bar sorptive extraction
MS	Mass Spectromtery
MSn	Multiple stage mass spectrometry
NIP	non- imprinted polymer
NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
PE	phosphatidylethanolamines
PPT	Protein precipitation
PRS	Propylsulphonic acid
RAM	Restricted Acces Material
RSD	Relative Standard Deviation
SBSE	Stir Bar Sorptive Extraction
SCID	source collision-induced dissociation
SPME	Solid Phase MicroExtraction
SRM	Selected reaction monitoring
TFC	Turbulent Flow Chromatography
UHPLC	Ultra-High Pressure Liquid Chromatography

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 ultra high pressure liquid chromatography (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. 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. Developments in LC-MS and related techniques has been recently reviewed [1], by giving an overview of recent developments in the last decade about mass analyzers, ionization techniques, fast LC-MS, LC-MALDI-MS, ion mobility spectrometry, as well as emerging mass spectrometric approaches complementary to LC-MS. Additionally, 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, for instance, the bioavailability of many of these drugs, the application of low doses or a fast elimination of the drugs due to excretion or metabolism, between others. 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 [2], but also guaranteeing confirmation of target and the identification of related and new compounds [3].

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 [4-7] or high temperature liquid chromatography [8-10]. 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 [11,12]. Additionally, porous shell columns (packed with sub-3 µm superficially porous particles) are starting to be used for fast chromatographic separations [13-16].

Despite the advances in chromatographic separations techniques, the complexity of biological sample matrices makes direct analysis by HPLC problematic. For instance, irreversible adsorption of proteins in the stationary phase can occur, producing loss of 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 [17]. 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 [18]. 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 [19]. 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 [20].

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 higher chromatographic resolution and separation efficiency can be achieved by UHPLC methods, the likelihood of matrix effects during ionization, 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 [21-23].

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 should be pointed out that "fast analysis" and "fast chromatography" are terms currently used without a formal definition. A certain number of compounds analysed per unit time would be unequivocal analytical parameters. For instance, typical peak widths in routine UHPLC-MS bio-analyses are 3-10 s [24], while peak widths in the fast/ultrafast LC-MS methods are generally in the range 1-3 s [25], but they can be narrower than 1 s under well optimized conditions. However, these parameters are not reported in many cases. In the present review, we have considered analysis times up to 10 min for both fast chromatography and fast analysis. The review 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, sample preparation using 96-, 384-well or even higher density plate formats is a well-established technique that has been successfully adopted for semi-automation of off-line sample procedure in bio-analytical applications [26,27]. 96-well SPE technology and automation workstations significantly improve the assay sample throughput by reducing both time and labor required for generating bioanalytical results compared to the conventional off-line SPE methods [28-31].

Another powerful alternative to the classical off-line SPE is represented by the on-line SPE technology. This technique has revolutionized sample preparation, receiving a lot of interest in recent years because of its ability to dramatically increase assay performance while reducing total analysis time. Furthermore, recent developments and advancements 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 several classes of substances in biological matrices. For instance, it has shown to be very advantageous for the analysis of steroid hormones, insecticides, antibacterial, perfluorinated compounds, therapeutic peptides, immunosuppressant, antidepressant or illicit drugs in a wide range of biological fluids such urine, blood, serum, plasma, saliva, synovial fluid, milk and other tissues (see Table 1, [32-62]).

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.* [63] 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 [43]. 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 [63]. Carryover was not observed even though high levels of the studied compounds were injected [63].

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 [64]. 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 [64], approach carried out in many of the 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 were recently reported for the quantification of free catecholamines in urine [65], 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 [37] 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.* [66] 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 a total run time of 7.5 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 [34]. 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 [55,61,67]. Precisely, system blockage and ion suppression are some of the reasons that keep the injection volumes relatively low, typically < 200 μl [61], which play against achieving higher preconcentration factors and sensitivity [45]. 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 [55]; saliva was diluted and centrifuged [56]; 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 [39,68]. However, most commonly, precipitation of proteins is carried out off-line with organic solvents [36,37,52,59], acid [38,40,59,61]; and/or centrifugation [56] or even SPE [52,69], LLE [39] or purification with an immunoaffinity column [57] 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 [52]. 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 [56]. 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 [38]. 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 [70]. 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 [64,70-74], 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 [56,67,75]; by observing the peak height in absence or presence of matrix [69] or by comparing the slope of the external calibration curve and standard addition curves [59].

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 (C_4, C_8, C_{18}, 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 [42]. The sample clean-up was performed using a SPE Strata X-CW and the separation was performed by UHPLC-MS/MS. 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 has been made to study and develop new sorbents 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 on-line configurations and higher biocompatibility. Among them, MIPs 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 [76]. 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 [77]. 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 [78]. MIP was used for off-line extraction before LC-MS analysis and its performance compared to that of a conventional C_{18} SPE. Molecularly imprinted SPE showed higher recoveries (~83%) than commercially C_{18} SPE (~65%) with 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 [79], zidovudine and stavudine from human plasma [80], cocaine and its metabolite benzoylecgonine [81], ketamine and norketamine [82] in hair samples, as well as testosterone, epitestosterone [83], and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol from urine samples [84]. 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 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol [85]. The developed method, which has been validated according to Food and Drug Administration (FDA) guideline on bio-analytical method validation [2], 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), were also developed for determination of 2-aminothiazoline-4-carboxylic acid (ATCA) as a marker for cyanide exposure in forensic urine analysis [86]. 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, MS/MS was used to improve the overall selectivity of the method and 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 simulated body fluids [87]. In this study, MIPs were 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 29 ng 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 NIPs. In summary, MIPs 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. RAMs 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 [88-90]. 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 [91], called internal surface reversed-phase (ISRP) materials. Alkyl-diol silica materials (ADS) [92] 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 [93], mercapturic acids in urine [94,95] or multiresidue analysis of xenobiotics in urine [96]. 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 [97]. 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 [98]. The properties of RAM and MIP materials have merged in the RAM-MIP grafted silica synthetized by Wenjuan Xu et al. [99], who by a controlled polymerization technique (see method in [100]) 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 [99].

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 [101]. The optimization of the coupling of a RAM (MSpak) with N-vinylacetamide copolymer as stationary phase with a hydrophilic interaction chromatography (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 [102].

Reusing RAM sorbents after being loaded with biological samples which underwent a minor or no sample treatment [94-96,101,102] proves the efficiency of the technique and shows its superiority to the single use of SPE cartridges. However, despite the advantageous features of RAMs, these materials still have limitations. The elution of the analytes from the RAM to the analytical column is a key step in the purification process; the sorbent could still contain residues of macromolecules at this point if the washing step has not been 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%, but 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 [103]. The assessment of carryover can be carried out directly with the analysis of blanks [96,101,103] or indirectly with the assessment of the recovery through the RAM [102], recovery of the whole analysis including sample treatment, separation and detection [101] or analysis of reference materials [93].

2.3. Turbulent flow chromatography

Turbulent flow chromatography 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 allows improving the radial distribution of the analytes. However, under these conditions, a laminar zone where diffusional forces still dominate the mass transfer process still exists around the stationary phase [104]. Molecules with low molecular weight diffuse faster than molecules with high molecular weight, forcing large molecules to quickly flow to waste while retaining the small ones. The retained compounds are then back-flushed and focused on the analytical column for chromatographic separation, like in an 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.* [105]. Many more studies have been reported in successive years applied to various matrices covering from biological (Table 2, [104,106-118]) 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 [106,119,120]. Besides, this technology has been applied to the analysis of more complex matrices such as hemodialysates [121], edible animal tissues [122] and food samples such as honey [123] and milk [124]. 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 achieved although this can be compensated by the use of

capillary LC leading to the reduction of the amount of sample volume injected [125]. 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 to be similar to SPE followed by liquid chromatography although the extraction in TFC is size-exclusion based, therefore it is mainly applied when there is an interest to separate small analyte molecules from larger matrix molecules [125]; 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 [126]. 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. The analysis time can vary between 2-9 minutes depending on the number of compounds and the number of channels used (multiplexing). Mueller et al. described a reduction of 50% on the analysis of sirolimus and everolimus by using TFC, nevertheless the analysis time reported (5.75 minutes) is longer in comparison to other on-line methods [106]. The authors adopted a slightly longer analysis time in a way to have better robustness, no carryover and extended column lifetime (>600 injections) provided by extending cleaning and equilibration times. On the other hand, real screening methods including a large number of analytes have also been reported where the analysis time can be extended to up 32 minutes [107]. In this case, more than 400 compounds are included in the acquisition list. 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 [109]. The latter helps preventing column clogging.

Mueller *et al.* [107] reported a comprehensive toxicological MS^n 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.* [108] 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.* [109] in the analysis of 25-hydroxyvitamin D2 and D3 in serum.

The sample preparation technique used 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.* [104] 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 [104].

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 [127]. LLE and SPE are effective in terms of producing clean extracts and reducing matrix effect but are alsolaborious and difficult to automate.

TFC can also be used for analytical support of *in vivo* pharmacokinetics and *in vitro* drug metabolism studies [113,128]. Verdirame *et al.* [113] 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 4-fold improvement 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. By using UHPLC, the results of a sample batch can be reported in a few hours rather than a few days as it is needed, for instance, in doping control 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 [129].

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 [130], then on Giddings [131], and later on Knox [132] and further interpretations, the efficiency, expressed as the height equivalent to a theoretical plate (HETP, *H*), can be described as:

$$H = A + B/u + Cu = 2\lambda d_p + 2\gamma D_M / u + f(k) d_p^2 u / D_M$$

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, d_p is the particle diameter of the column packing material, D_M 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 [130]. 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 d_p, while *B*- and *C*-terms are both temperature dependent, the *B*-term being directly proportional to D_M while the *C*-term is inversely proportional to D_M but directly proportional to the square of d_p. 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 [24].

Several recent applications of UHPLC-MS methods in bio-analysis using sub-2 µm particle size packed columns are summarized in Table 3 [42,58,59,66,133-157]. 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) [58,59,66,133-144,150,151,153,155-157] and urine [42,138,139,142,146-150] 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 treatment used for palliative therapy. The method allowed the separation of doxorubicin and daunorubicin within 1 min and helped in the better understanding of the factors affecting the delivery and dispersion of doxorubicin within treated tumors during these 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

589 cortisol, cortisone, 11-deoxycortisol, prednisolone and dexamethasone hormones (Figure 2a) as
590 well as the chromatograms obtained after application of the proposed UHPLC-MS/MS method to
591 the analysis of several matrices (Figures 2b-2f). Chromatographic separation of all
592 glucocorticoids in less than 2.5 min was achieved showing limits of quantitation in the range of 1
593 to 5 nmol L^{-1} (depending on the sample matrix) and with intra-assay and inter-assay precisions
594 with RSD values lower than 5 and 10%, respectively, for all compounds in all matrices.
595 Most of the applications are based on reversed-phase separation using the Acquity UPLC
596 BEH C18 column of 1.7 µm particle size with different columns lengths (30, 50 or 100 mm), but
597 other C18 reversed-phase columns such as Zorbax Eclipse XDB-C18 (1.8 µm particle size)
598 [42,59,136,139,150], Shimadzu Shim-pack ODS (1.6 µm particle size) [152] or Hypersil Gold
599 C18 (1.9 µm particle size) [58,144,148,151,157] have also been used. Although not strictly sub-2
600 µm particle size columns, some bio-analytical UHPLC-MS applications can be found using
601 columns with slightly higher totally porous particle sizes. As an example, Tuffal *et al.* [140]
602 reported the use of a Shimadzu Shim-pack XR-ODS II column of 2.2 µm particle size for the
603 UHPLC-MS separation of clopidogrel active metabolite isomers in plasma in less than 7 min.
604 Other stationary phases have also been described for UHPLC-MS bio-analytical applications. For
605 instance, the use of a high strength silica (HSS) column (Acquity UPLC HSS T3, 1.8 µm particle
606 size) was reported by Vanden Bussche *et al.* [149] for the analysis of eight thyreostats in urine,
607 without any derivatisation, in less than 6.5 min. While Jiménez Giron *et al.* [146] used a C8
608 reversed-phase column (Zorbax SB-C8, 1.8 µm particle size) for the UHPLC-High resolution
609 Orbitrap MS screening analysis of diuretic and stimulant compounds in urine for doping control.
610 By screening in full scan MS with scan-to-scan polarity switching more than 120 target analytes
611 could be detected in less than 8 min.
612 Regarding MS detection, triple quadrupole analyzers are instruments of choice for
613 UHPLC-MS bio-analytical applications as can be seen in Table 3. Other MS and HRMS
614 instruments have also been used for some bio-analytical applications, which will be discussed in
615 further detail in section 4.
616
617 **3.2. Fused-core particle packed columns**
618

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 ^{o}C (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 ^{o}C, 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 acceptable 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. 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 the evaluation and the elimination 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.* [106] 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 [107,114], as well as for the analysis of vitamins [109] and tyrosinekinase inhibitors [111]. 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.* [48] 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 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 [3] 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

712 the compound co-elutes with an interfering matrix compound with ions in the MS/MS spectrum
713 matching with those of the target analyte [169-171]. In these cases, false-positive results can be
714 prevented with by further confirmatory analysis, e.g. the use of a third transition or an orthogonal
715 criterion like exact mass measurements. On the other hand, despite its high selectivity and
716 sensitivity the use of SRM acquisition mode in QqQ instruments is limited by the cycle time
717 when dealing with hundreds of compounds, and a significant drawback to this type of analyzers
718 is that only those molecules that have been targeted are detected (missing non-target compounds
719 or even target metabolites). For these reasons, nowadays to solve the problems related to both the
720 cycle time and the target screening method, liquid chromatography coupled to high resolution
721 mass spectrometry (LC-HRMS) is being implemented in bio-analysis. Time-of-flight (TOF) and
722 Orbitrap based technologies are currently the most common analyzers used in LC-HRMS. For
723 instance Fung *et al.* [172] proposed a LC-HRMS method using a quadrupole-time-of-flight (Q-
724 TOF) analyzer for the analysis of prednisone and prednisolone in human plasma operating at a
725 mass resolving power of 10,000 and obtaining mass errors below 6 ppm. On the other hand,
726 Jiménez Girón et al. [146] reported a new screening method based on UHPLC-HRMS using
727 polarity switching for the analysis of 122 targeted analytes in urine by direct analysis. In this
728 work the use of polarity switching acquisition mode in combination with HRMS allowed the
729 possibility to obtain two diagnostic ions. This strategy was used to confirm some diuretics
730 compounds that exhibit high sensitivity in negative mode but were also detectable in positive
731 mode. The use of high resolution mass spectrometry is also especially useful in metabolomics
732 studies where full scan MS spectra and accurate mass measurements are acquired for
733 identification purposes [173]. The use of ultra-high resolution mass spectrometry, operating at
734 mass resolving power higher than 30,000 FWHM is especially important in lipidomics studies
735 due to the complexity of this family of compounds. Taking advantage of the ultra-high resolution
736 provided by an Orbitrap analyser isobaric phosphatidylethanolamines (PE) ether species with 7
737 double bonds (which are common in several model organisms, such as C. elegans) could be
738 differentiated from the PE ester species with two saturated fatty acid moieties having a mass
739 difference of 0.0575 Da (Figure 4, [174]). However, in this case operating at ultra high resolution
740 mass spectrometry has compromised the scan speed and therefore the coupling to fast LC
741 separation.

However, in some cases the unequivocal identification of target and target-related compounds requires combining the information provided by HRMS and MS/MSexperiments. 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 MS^n 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, 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

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 a high number and variety of samples are being analyzed, and in areas where results must be reported promptly. The state-of-the-art of fast LC-MS for bio-analytical applications have been discussed in this review. 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. 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). The use of HILIC is becoming very popular for bio-analytical applications allowing better separation of highly polar compounds than with reversed-phase chromatography and it will become a complementary tool to explore in the near future for UHPLC bio-analytical applications. Although separation with 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 reducing column backpressure but keeping similar column efficiency than what is achieved in sub-2µm particle size columns. 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. The most recently introduced sample treatment automated methodologies in bio-analytical applications have also been addressed in this review, such as on-line SPE, TFC, MIP and RAM based methods.It should be pointed out that sample preparation techniques must be chosen and optimized on the basis of the method purpose and take into consideration the chromatographic separation that will be used. 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.

TFC 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 a number of applications are expected to be available in the future beyond the bio-analytical field.

MIPs and RAM have attracted much interest in the last years (Table 1). The use of MIP is a very useful approach for some bio-analytical applications because it makes possible to achieve a selective preconcentration ot the 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 among the most environmentally friendly sample treatment procedures. And although some drawbacks exits when using RAM such as the risk of carryover when the sample contains hydrophobic species and the method involves reversed-phase RAM and transfer solutionwith low elutropic strength, advances are taking place in the development of new RAM sorbents. 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 is 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, HRMS, 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 modewill become a powerful tool for bio-analytical applications in the future.

Comprehensive analysis and testing is needed 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 methods but without compromising the reliability and quality of the 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 C_{12}-MNPs. Reproduced from ref. [98], with permission of Elsevier.

Figure 2. Representative UHPLC-MS/MS chromatogram of glucocorticoids. (a) Standard with cortisol, cortisone, 11-deoxycortisol, prednisolone and dexamethasone. (b) Dilute plasma ultrafiltrate with cortisol. (c) Saliva sample with cortisol and cortisone. (d) Plasma ultrafiltrate with cortisol. (e) Plasma sample from a patient collected at 09:00 after administration of 1 mg dexamethasone at 23:00 the previous evening. (f) Urine from a patient on prednisolone treatment. 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}	[32]
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}	[33]
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}	[34]
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}	[35]
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}	[36]
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}	[37]
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}	[38]
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)	[39]
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}	[40]
Tilidine, nortilidine, and bisnortilidine	Serum	OASIS WCX (10mm×1mm)	0.2% formic acid (A) and methanol (B)	LC-ESI-MS/MS	LOQs > 1 ng mL^{-1}	[41]
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 ng mL^{-1} LOQs > 0.66 ng mL^{-1}	[42]
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 ng mL^{-1} LOQs > 5 ng mL^{-1}	[43]
Peptides						
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}	[44]
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)	[45]
Personal care products						
2-ethylhexyl	Human urine	HySphere C18 HD	0.2% formic acid in water (A) and 0.2% formic	LC-ESI-MS/MS	LODs > 0.1 ng	[46]

4-(N,N-dimethylamino)benzoate		(10mm×2mm, 7μm)	acid methanol/ acetonitrile (1/1, v/v) (B)	LC-ESI-TOF/MS	LOQs > 0.3 ng	
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}	[47]
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}	[48]
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	[49]
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}	[50]
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}	[51]
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}	[52]
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	--	[53]
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	--	[54]
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}	[55]
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	[56]
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}	[57]
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	[58]
Perfluorinated compounds	Plasma	Poros HQ (2.1mm×30mm, 10μm)	0.01% NH_4OH solution in 5mM ammonium acetate (A) and 0.01% NH_4OH solution in acetonitrile (B)	UHPLC-ESI-MS/MS	LODs > 3 ng L^{-1} LOQs > 10 ng L^{-1} MDLs > 9 ng L^{-1}	[59]
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}	[60]
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)	[61]
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	[62]

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 ng mL^{-1}	[106]
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-MS^n	-	[107]
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}	[108]
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)	[109]
-	Metabonomic studies	Plasma	50 x 0.5 mm, 50 µm Cyclone (Thermo Fisher Scientific)	1.25 mL min^{-1} 10 µL	ESI-MS	-	[104]
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}	[110]
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 ng mL^{-1} [a]	[111]
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}	[112]
-	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	-	[113]
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-MS^n	[b] 10 ng L^{-1} (60%) 100 ng L^{-1} (90%) 1000 ng L^{-1} (100%)	[114]
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}	[115]
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}	[116]
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}	[117]

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}	[118]

[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 °C	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 °C	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 °C	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	[66]
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	Plasma	Acquity UPLC BEH C18	Gradient elution:	ESI(-)	1.3 min	[141]

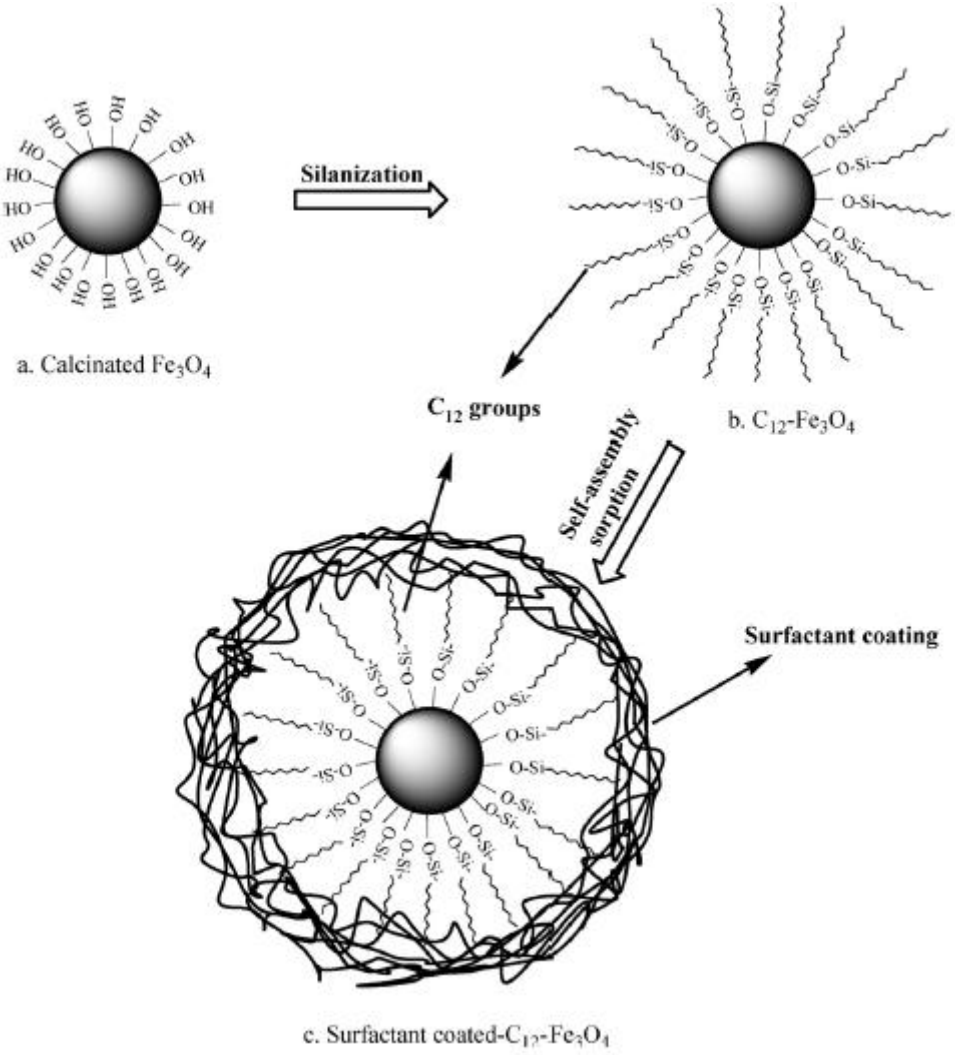
and docosahexenoic acid		(50 mm x 2.1 mm, 1.7 µm) 60 ºC	A) 2 mM ammonium acetate pH 4 B) acetonitrile 1 mL min^{-1}	Triple quadrupole SRM acquisition mode		
Cortisol, cortisone, prednisolone, dexamethasone and 11-deoxycortisol	Plasma, plasma ultrafiltrate, urine and saliva	Acquity UPLC BEH C18 (50 mm 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	[42]
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-	Plasma	Hypersil Gold C18 (50 mm x 2.1 mm, 1.9 µm)	Gradient elution: A) 20 mM ammonium acetate	ESI(+) Linear ion trap MS	4 min	[151]

malarial compound)			B) acetonitrile 0.45 mL min^{-1}	SRM acquisition mode		
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 ^{o}C	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 ^{o}C	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 ^{o}C	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 ^{o}C	Gradient elution: A) 0.01% NH_4OH, 5 mM ammonium acetate in water B) 0.01% NH_4OH in acetonitrile 1 mL min^{-1}	ESI(-) QTrap MS SRM acquisition mode	5 min	[59]
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	[58]
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 MS^2 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% NH_4OH in water (pH 8.5) B) 10 mM ammonium acetate, 0.1% NH_4OH and 0.2% (morpholine or triethylamine) in acetonitrile 0.4 mL min^{-1}	ESI(+) Triple quadrupole SRM acquisition mode	2.5 min	[165]

Figure 1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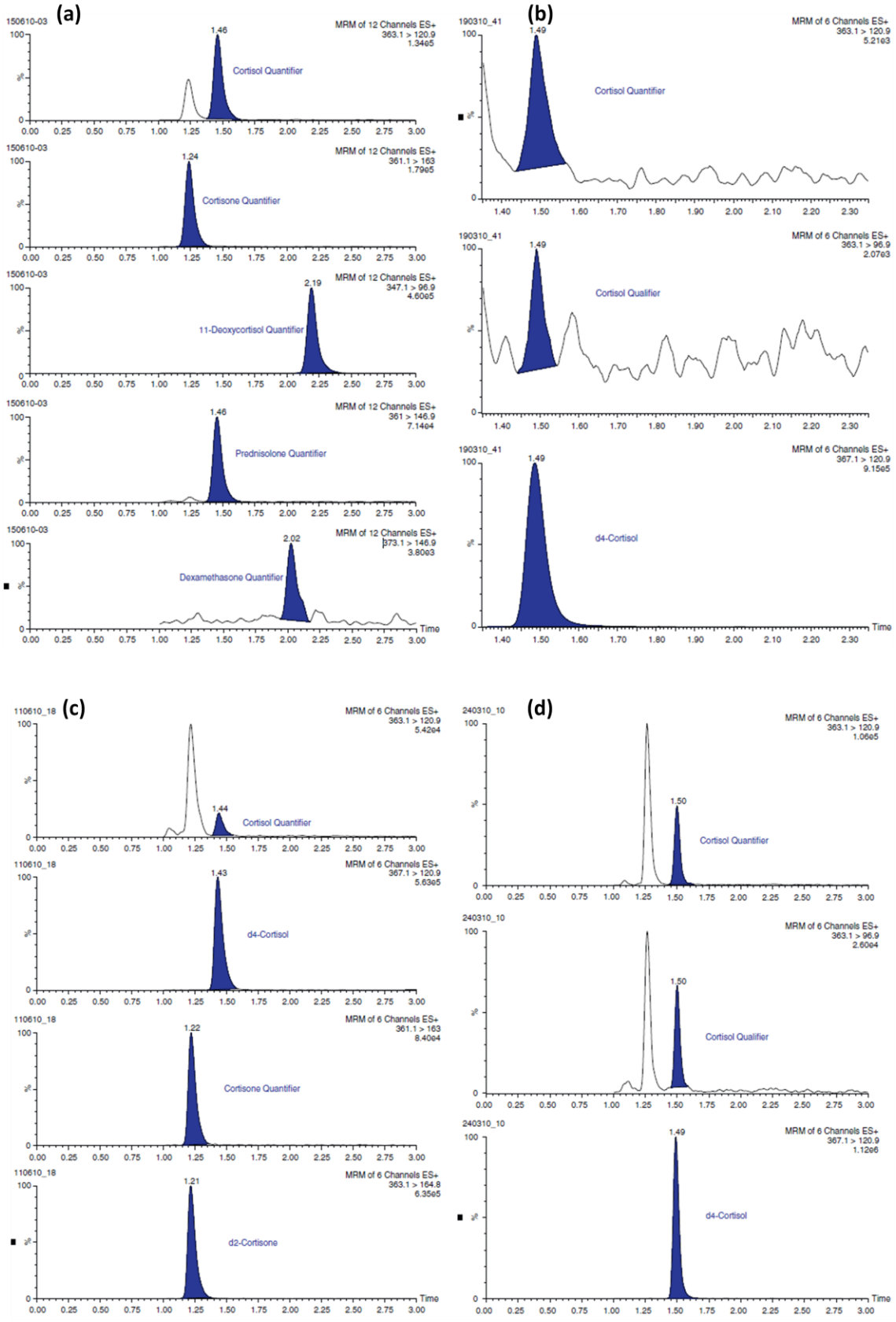
Self-assembly sorption
Surfactant coating
c. Surfactant coated-C_{12}-Fe_3O_4

Figure 2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(d)
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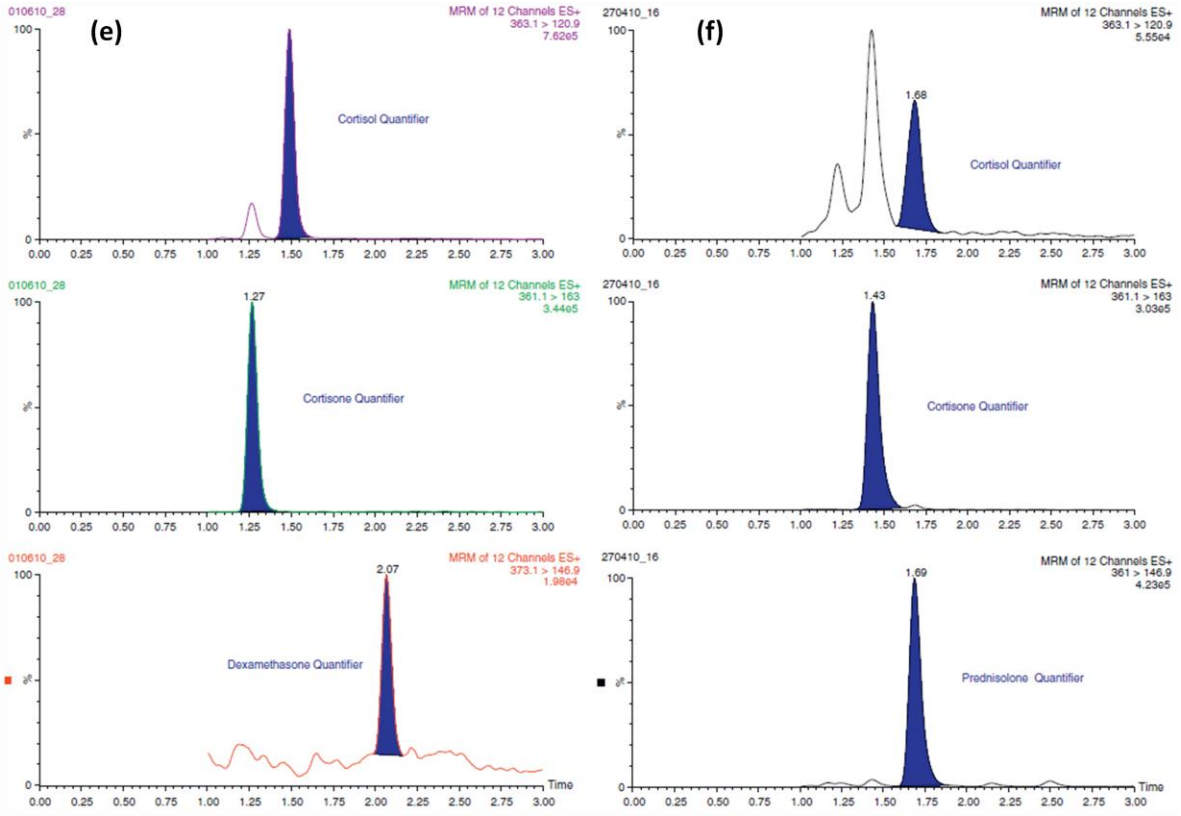
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Cortisone Quantifier
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Prednisolone Quantifier
Time

Figure 3

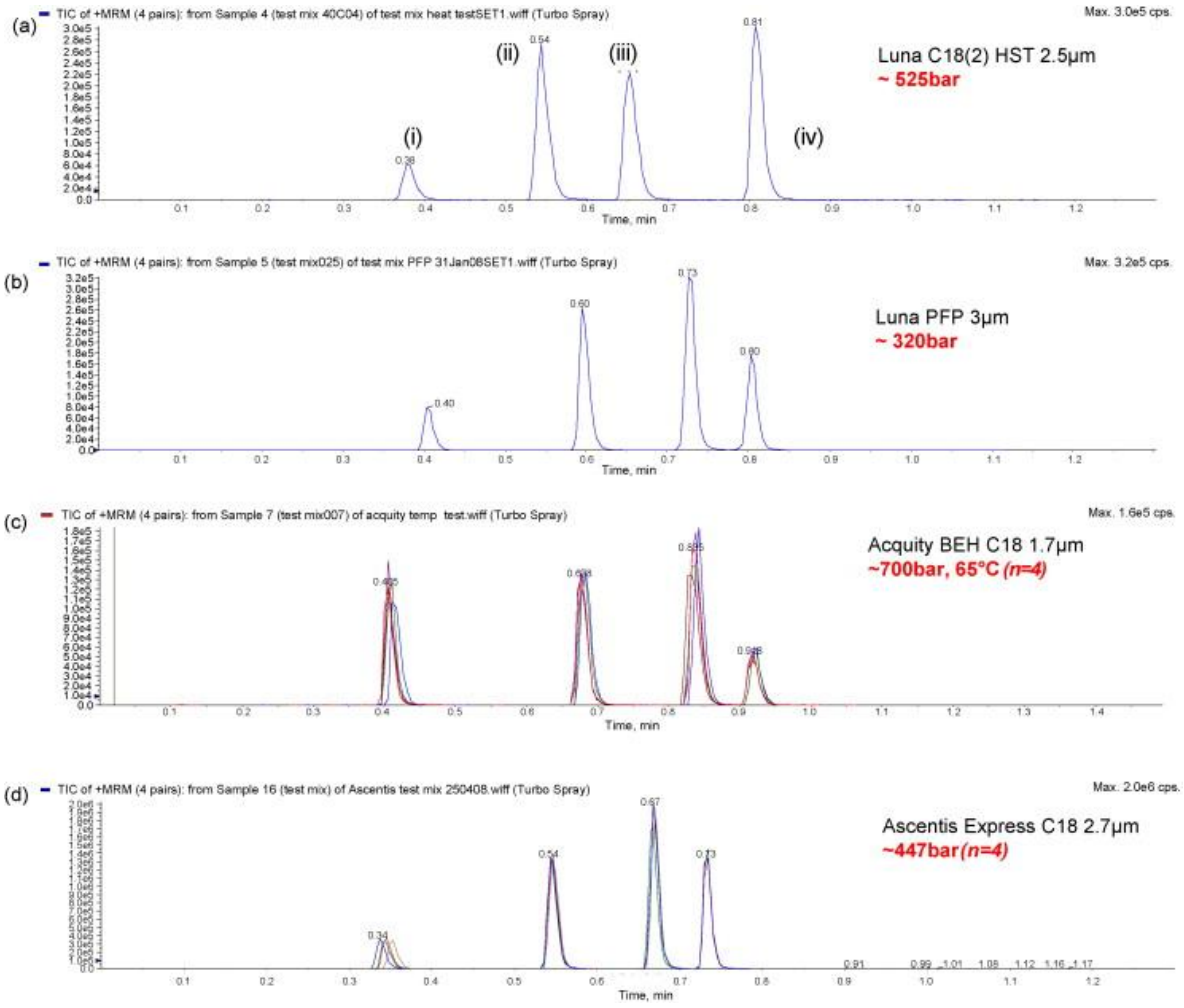
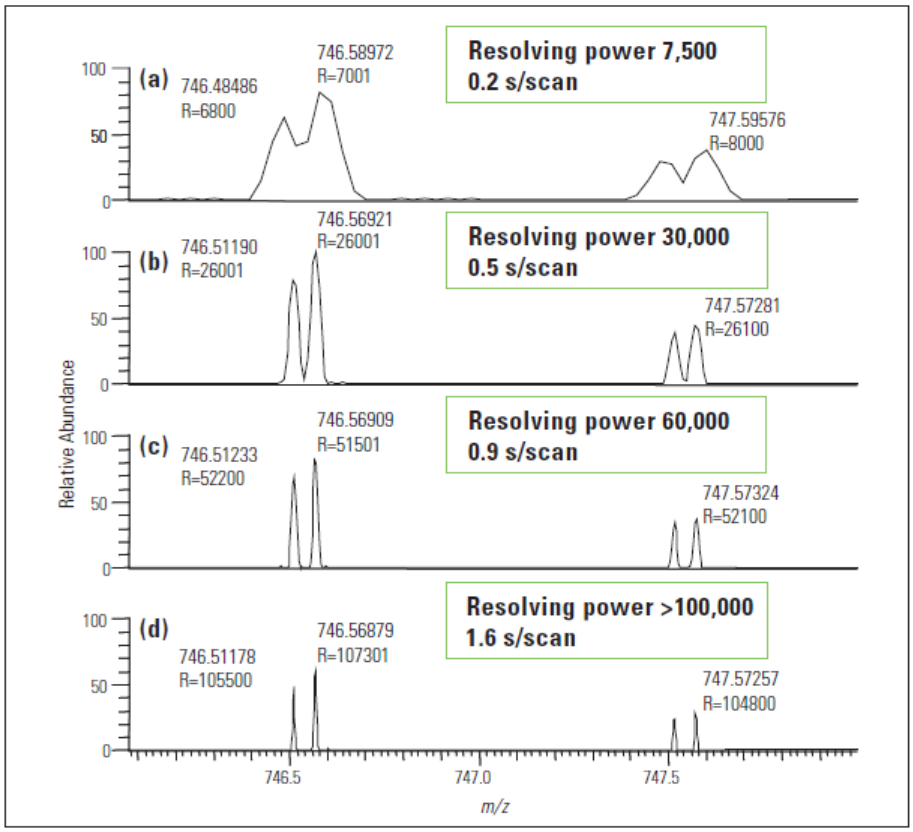

(a)
TIC of +MRM (4 pairs): from Sample 4 (test mix 40C04) of test mix heat testSET1.wiff (Turbo Spray)
Max. 3.0e5 cps.
(i)
(ii)
(iii)
(iv)
Luna C18(2) HST 2.5µm
~ 525bar
Time, min
(b)
TIC of +MRM (4 pairs): from Sample 5 (test mix025) of test mix PFP 31Jan08SET1.wiff (Turbo Spray)
Max. 3.2e5 cps.
Luna PFP 3µm
~ 320bar
Time, min
(c)
TIC of +MRM (4 pairs): from Sample 7 (test mix007) of acquity temp test.wiff (Turbo Spray)
Max. 1.6e5 cps.
Acquity BEH C18 1.7µm
~700bar, 65°C (n=4)
Time, min
(d)
TIC of +MRM (4 pairs): from Sample 16 (test mix) of Ascentis test mix 250408.wiff (Turbo Spray)
Max. 2.0e6 cps.
Ascentis Express C18 2.7µm
~447bar(n=4)
Time, min

Figure 4


(a)
746.48486
R=6800
746.58972
R=7001
747.59576
R=8000
Resolving power 7,500
0.2 s/scan
(b)
746.51190
R=26001
746.56921
R=26001
747.57281
R=26100
Resolving power 30,000
0.5 s/scan
(c)
746.51233
R=52200
746.56909
R=51501
747.57324
R=52100
Resolving power 60,000
0.9 s/scan
(d)
746.51178
R=105500
746.56879
R=107301
747.57257
R=104800
Resolving power >100,000
1.6 s/scan
Relative Abundance
100
50
0
746.5
747.0
747.5
m/z