High-throughput solid-phase microextraction in multi-well plate format

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Abstract:

Solid-phase microextraction (SPME) was recently successfully automated in multi-well plate format in order to meet the goals of high-throughput clinical, pharmaceutical and environmental analysis. The technique can be used for accurate quantitation of variety of non-volatile analytes such as drugs, metabolites, environmental contaminants and mycotoxins, making it ideally compatible with liquid chromatography-mass spectrometry applications, although recent applications in combination with gas chromatography and ambient mass spectrometry have also been reported. This review will summarize different formats of high-throughput multi-well SPME including fibre, thin-film and in-tip configurations. The advantages and disadvantages of the technique in each format will be critically discussed with the particular focus on its fit within the fields of regulated analysis. The capability of high-throughput SPME will be placed in the context of other micro sample preparation techniques such as micro solid-phase extraction in order to aid the reader in the selection of the most appropriate technique for a given application. New developments of the devices such as monolithic and biocompatible extraction phases will be covered. Finally, selected applications of the technique including the analysis of whole blood samples and automated binding studies will be presented.

Keywords: solid-phase microextraction, thin-film microextraction, in-tip microextraction, high throughput analysis, liquid chromatography-mass spectrometry, 96-well plate format, biocompatible, whole blood analysis, automated binding studies

Highlights:

- Recent strategies for automation of SPME in multi-well plate format are described
- Automated 96-well SPME provides the highest throughput of SPME to date
- Best configuration of automated SPME critically depends on the application objectives
- Whole blood and waste effluents are directly compatible with no sample pre-treatment
- Monolithic and biocompatible coatings address the historical limitations of SPME

Abbreviations:

Desorption electrospray ionization (DESI)

Food and Drug Administration (FDA)

Gas chromatography-mass spectrometry (GC-MS)

Limit of detection (LOD)

Liquid chromatography-mass spectrometry (LC-MS)

Liquid-liquid extraction (LLE)

Lower limit of quantitation (LLOQ)

Microextraction by packed sorbent (MEPS)

Phenylboronic acid (PBA)

Polydimethylsiloxane (PDMS)

Polyacrylonitrile (PAN)

Polystyrene-divinylbenzene (PS-DVB)

Polytetrafluoroethylene (PTFE)

Solid-phase extraction (SPE)

Solid-phase microextraction (SPME)

Thin-film microextraction (TFME)

1.0 Introduction

Sample preparation remains one of the main bottlenecks in modern high-throughput analysis by liquid chromatography-mass spectrometry (LC-MS). With the increased adoption of ultra-high pressure liquid chromatography and other fast chromatographic approaches such as very short

columns, monolithic stationary phases and/or fused-core particle technologies, the chromatographic separation and MS detection steps can be routinely carried out on 1-5 min time scale, with even sub-minute separations reported to date. This speed and increased sample throughput place stricter demands on fast and automated sample preparation methods, in order to meet the desired goals of accurate and efficient analysis of hundreds or even thousands samples per day.

Solid-phase microextraction (SPME) is an equilibrium extraction technique whereby a small amount of extraction phase is immobilized on a solid support and used to extract/preconcentrate analytes of interest [1]. The most popular and widely used configuration of SPME is fibre geometry where extraction phase is immobilized as a thin layer on the outside of fused silica or metal wire support. However, SPME can also easily be performed using other configurations such as coated well, coated stir-bar (known as stir bar sorptive extraction), coated thin film (known as thin-film microextraction (TFME)) and coated capillary column (known as in-tube SPME) as discussed in detail elsewhere [1-3]. The main difference between SPME and more commonly used solid-phase extraction (SPE) methods is the ratio of sorbent versus sample volume [1]. In SPME, an extremely small amount of sorbent is used in comparison to sample volume, so exhaustive extraction analyte does not take place. Typically in SPME, only small portion of the analyte is extracted from the sample, and the amount of the analyte extracted increases with increasing extraction time until equilibrium is established between sample solution and extraction phase when no further increases in the amount extracted will be observed within experimental error. In contrast, the goal of SPE technique is the exhaustive extraction of all the analyte from the sample, so large amount of sorbent is used. Micro-SPE (μSPE) is simply a miniaturized version of SPE, whereby smaller amount of sorbent is used than in conventional SPE in order to be compatible with handling of smaller sample volumes, but the goal still remains exhaustive extraction of all analyte from the samples. Therefore, SPME and μSPE are conceptually distinct and not interchangeable terms. A second important difference between SPME and SPE methods is that most configurations of SPME (except in-tube SPME) rely on an open bed configuration where sorbent is immobilized on the solid support which makes SPME methods more compatible with direct extraction of heterogeneous and particulate-containing samples. However, with the recent developments in various techniques, the lines between exhaustive and microextraction techniques can become increasingly more blurry – for instance,

in simple matrices such as buffer or urine, the extraction efficiency of thin-film configuration of SPME can approach the exhaustive recoveries achieved in μSPE (see section 3.0). The development of new configurations such as disk and pipet tip formats of SPE and in-tip SPME as well as the increased use of SPE-type sorbents to prepare SPME coatings also further blurs the lines between SPME and other solid-phase extraction techniques, making absolute recovery studies mandatory to determine whether exhaustive extraction is achieved in a given application with the selected format of extraction device.

To meet the demands of high-throughput analysis, traditional techniques such as liquid-liquid extraction (LLE) and SPE have been fully automated in multi-well plate format to enable rapid parallel preparation of 96 and/or 384 samples [4]. In contrast, the historical efforts to automate SPME in combination with LC primarily focused on the development of online configurations, mainly in-tube SPME [5-7]. In this configuration, the samples are extracted with a coated capillary column using a series of draw/eject cycles automated via the use of the commercial HPLC autosamplers prior to direct online separation and analysis, and samples are processed in sequential manner [7]. Considering the slow kinetics of extraction and desorption steps in liquid-phase, and the sequential approach to extraction, in-tube SPME cannot provide sample throughput of the magnitude required for demanding high-throughput applications, although excellent performance can be achieved for a wide variety of low- to medium-throughput automated applications [8]. One approach to address the slow liquid-phase kinetics is to perform massively parallel extraction/desorption on a large number of samples, which drastically reduces sample preparation time per sample, permitting easy coupling with even the fastest chromatographic methods. This type of full automation of SPME in multi-well plate format was successfully completed only in 2008 [9,10], and this novel configuration has not yet gained widespread acceptance versus traditional single fibre or in-tube SPME approaches. Thus, SPME as well as other microextraction approaches such as liquid-phase microextraction remain extremely underutilized in the field of high-throughput analysis even though microextraction format can provide significant advantages over classical methods including reduced solvent usage, low cost and reusability, improved selectivity and compatibility with small volume samples.

The objective of this review is to critically discuss the different approaches to automate SPME in multi-well plate format, and present the advantages and limitations of the available configurations, thus extending the discussion of SPME automation beyond brief discussion available in recent general SPME reviews [2,10,11]. The success of the technique relies heavily on the properties of the coatings used to build the SPME device, so critical discussion of progress and challenges in this important research area will be provided. Furthermore, method development strategies when moving from single fiber to multi-fiber configurations will be briefly examined. Finally, recent applications of high-throughput SPME in clinical, pharmaceutical, toxicological and environmental fields will be highlighted including a brief discussion of novel possible future applications such as metabolomics and/or untargeted screening applications.

2.0 96-well plate SPME configurations and devices

To date SPME has been successfully automated in three main configurations (i) fibre, (ii) thin film and (iii) in-tip SPME. Thin-film configuration is commercially available as Concept96 SPME robotic sample preparation station from PAS Technology (Magdala, Germany), while in-tip configuration can be automated using existing laboratory automation systems such as Tomtec Quadra 96 (Hamden, Connecticut, US). Finally, multi-well sample desorption has also been developed for *in vivo* SPME to increase the overall throughput of this type of application.

2.1 Fibre SPME configuration

The earliest proof-of-concept reports of successful manual or semi-automated SPME configurations for use with multi-well plate format relied on a simple array of commercial fibers placed in custom-made devices designed to hold the fibers over the commercial 96-well plate [12,13]. Although this approach performed well, the high cost of single commercial fibers makes the building of 96-fibre device extremely cost-prohibitive. For this reason, the latter study also investigated three alternative low-cost configurations and compared their performance against the array of commercial devices [13]. The authors found that a simple commercial pin-tool replicator provided an excellent metal framework to use as solid support of SPME fibers, while commercially available polydimethylsiloxane (PDMS) medical grade silicone tubing provided an easy-to-prepare and extremely low cost SPME coating. In addition, this study clearly

demonstrated the importance of uniformity of agitation for automated SPME applications, and established the orbital agitation whilst holding SPME device stationary to be the most suitable agitation method (versus sonication and magnetic stirring) for the design of fully automated SPME systems. The main issues with the use of PDMS coatings were extremely long extraction times needed to reach equilibrium due to high thickness of the material, and limited types of analytes that could be extracted using this simple extraction phase.

The subsequent study focused on (i) achieving full automation of the entire SPME procedure (ii) increasing the robustness of the system with particular focus on resolving the issues of fibre bending during robotic manipulations and (iii) developing flexible coating procedures that can yield thin coatings and be compatible with a wide variety of sorbents to increase the types of chemistries accessible by automated SPME system [9]. The full automation of SPME procedure was achieved using a newly developed Concept 96 robotic station shown in Figure 1B. The commercial version of this system consists of an XYZ arm capable of manipulating 96-fibre (or thin-film) device, 3 orbital agitators (for pre-conditioning, extraction and desorption), one stationary wash station, XYZ syringe arm for dispensing internal standards or desorption and reconstitution solvents, and 96-well nitrogen blowdown device for evaporation/reconstitution. The use of this robotic station allowed for the automation of all sample preparation steps, including the addition of an internal standard, coating preconditioning, SPME extraction and desorption for a preset amount of time with controlled agitation and an optional evaporation/reconstitution step, all user-programmable with full software control. The typical automated SPME protocol involves the following main steps (i) movement of 96-fibre device to preconditioning position (position 2 in Figure 1B) by changing XYZ coordinates of the main robotic arm (ii) lowering of fibres into wells containing preconditioning solvent by changing Z position of the main arm and computer-controlled agitation for pre-set amount of time at selected agitation speed (iii) dispensing preselected volume of internal standard to sample wells (position 4 in Figure 1B) using syringe arm and agitation for preselected amount of time to mix the contents (iv) positioning of 96-fibre device in extraction position (position 4 in Figure 1B) by changing XYZ coordinates of the main robotic arm (v) lowering of fibres into wells containing samples by changing Z position of the main arm and computer-controlled agitation for pre-set amount of time at selected agitation speed to perform equilibrium or pre-equilibrium extraction

(vi) movement of 96-fibre device to stationary wash station (position 3 in Figure 1B) (vii) lowering and raising 96-fibre device 2-3 times to briefly rinse the fibres by changing Z coordinates of the main arm (10-30s total rinse times) (viii) movement of 96-fibre device to desorption position (position 5 in Figure 1B) by changing XYZ coordinates of the main robotic arm (ix) lowering of fibres into wells containing desorption solvent by changing Z position of the main arm and computer-controlled agitation for pre-set amount of time at selected agitation speed to perform desorption step of SPME (x) (optional) movement of 96-well nitrogen blowdown device to desorption position and activation of nitrogen stream to evaporate the desorption solvent and (xi) reconstitution of the well contents by dispensing selected volume of reconstitution solvent to the wells using syringe arm and computer-controlled agitation for pre-set amount of time at selected agitation speed to redissolve the well contents. Although the original device also contained HPLC injection port for direct online coupling with LC-MS, this feature was subsequently eliminated, as the majority of commercial LC autosamplers can easily handle 96-well plates, thus freeing the unit to prepare new batches of samples while first batch is running.

One critical aspect for achieving the successful full automation of SPME described above was increasing the diameter of the fibre metal support which greatly increased mechanical robustness of the system by eliminating fibre bending, and also improved inter-fibre reproducibility during coating procedure. This optimized multi-fiber device is shown in Figure 1A and relies on thick metal rods (1.55 mm diameter) which were coated by immobilization of a single layer of SPE sorbent using strong UV adhesive (see Section 2.6). Second key aspect of ensuring good performance of automated SPME was good uniformity of agitation throughout the extraction and desorption steps in all wells. Using 850 rpm agitation, it was clearly shown that extraction time required to reach equilibrium was the same for all wells with no well dependence in the amount extracted. This permits the use of this automated system for both equilibrium and pre-equilibrium SPME methods with excellent method precision. Although Concept96 as described above fully automates all steps of SPME, future improvements to the system may include addition of temperature control during extraction and desorption steps, and integration of the system with 96-well plate liquid dispensing robotic equipment to facilitate rapid parallel dispensing of desorption and reconstitution solvents.

2.2 Thin-film microextraction (TFME) configuration

Very soon after the introduction of the multi-fiber device shown in Figure 1A, further modifications into changing the geometry from rod to thin-film were investigated in order to further increase the sensitivity of the system by increasing the surface area of the extraction phase [14,15]. The use of Empore extraction disks (0.5-0.75 mm polytetrafluoroethylene (PTFE) membrane disk saturated with fine bonded silica SPE particles) was found to have good extraction efficiency, but the movement of the extraction phase during the extraction was found problematic and adversely impacted method precision, so this particular configuration was not further pursued [14]. The novel rigid metal-based thin-film design shown in Figure 1C was found to outperform the fiber geometry, and is the preferred design in current commercial automated Concept96 SPME system [15]. This new configuration significantly improved extraction efficiency due to the increased extraction phase volume (~ 2-fold for initial design[15] and ~3.5-fold in finalized design [16]) and increased the initial rate of extraction (~ 2-fold) because of the increased surface area and the blade design of the solid support, both of which contributed to the more effective agitation/mass transfer (Table 1). The time required to reach equilibrium was also slightly shortened with TFME geometry [15]. Thus, although the terminology in literature may cause some confusion TFME or "blade" design of SPME simply implies change in geometry from well-known rod or fibre geometry to thin rectangular flat surface geometry as clearly visible when comparing Figures 1A and 1C, whereas the fundamental principles of the extraction and method optimization remain the same regardless of which configuration is used. All the remaining details of how automation is achieved are exactly the same as described in previous section.

The flat shape and appropriate dimensions (50 mm length, 2.5 mm width, depth 0.7 mm) of TFME configuration have recently enabled a very exciting new application of the technique where offline solvent desorption is replaced with online desorption using desorption electrospray ionization (DESI) for direct MS detection without chromatographic separation [17]. In this approach, individual thin films are taped onto a glass slide and placed on a moving stage platform, and desorption is performed by directing pneumatically assisted spray of solvent (7 µL/min) over approximately 9 mm portion of the coating (Figure 1D). The desorption of one side of the coating is used for the analysis in positive ionization mode, while the second side of

coating is used for negative ionization mode. Although the extraction in this study was performed using manual approach, automated extraction can also easily be employed for future applications. In addition to unprecedented sample throughput, this coupling of TFME with DESI resulted in improved sensitivity when compared to classical SPE-LC-MS using the same sample volume of wastewater effluent. Therefore, coupling of TFME with ambient desorption methods appears to be an extremely promising research direction, with significant expected growth.

2.3 In-tip SPME configuration

Full automation of SPME using an in-tip configuration was also recently successfully developed by Xie *et al.* [18-20]. In this format, SPME fiber is placed inside a disposable pipet tip and held in place with an appropriate polyethylene frit. The first array of devices used commercially available polydimethylsiloxane-divinylbenzene (PDMS-DVB) fibers from Supelco [18] and is also known as fibre-packed in-tip SPME configuration. Subsequent devices utilized monolithic extraction phases prepared *in situ* and which provided enhanced extraction efficiency [19,20]. This configuration is also referred to as sorbent-packed in-tip SPME and is illustrated in Figure 1F. The existing laboratory liquid-handling systems that can handle pipet tips, such as Tomtec Quadra 96, can then easily be user-programmed to automate all steps of SPME extraction in combination with this in-tip format, thus providing easy implementation of SPME without need for dedicated instrumentation (Figure 1F). To implement automated in-tip SPME 96-well sample extraction, desorption, wash and waste plates and reservoirs containing desorption, wash and preconditioning solvents are placed on Tomtec Quadra. The user-written program then directs the automated in-tip SPME sequence including: (i) loading of the in-tip SPME pipet tips, (ii) tip preconditioning (iii) extraction (iv) wash (v) desorption and (vi) nitrogen evaporation and reconstitution. Coating preconditioning, as well as sample extraction and desorption are performed using a series of aspirate/dispense cycles, similar to what is classically used for in-tube SPME configuration. The speed of aspiration was not found to affect the method precision, so the highest speed settings are typically used for fastest throughput [18]. Thus, this SPME configuration successfully eliminates the issues with uniformity of agitation that can be encountered with other multi-well agitation methods. Typically, wash and conditioning steps are performed with 2-3 cycles, while extraction and desorption steps are performed using 10-20 aspirate/dispense cycles.

2.4 96-well plate desorption of *in vivo* devices

Very recently, important developments in the design and applications of *in vivo* SPME devices have been made, with new hypodermic-based devices available commercially from Supelco and useful for a variety of *in vivo* applications including pharmacokinetics and pharmacodynamics, metabolomics and non-lethal environmental monitoring of contaminants in aquatic organisms [10,21,22]. These types of applications also tend to require the analysis of large number of samples, but the desorption step is generally performed manually in small-volume vials using vortex or other suitable agitation. To facilitate processing of large number of *in vivo* probes, several designs have been developed and described with the main idea to facilitate device transport and enable simultaneous desorption of 96 *in vivo* fibers in multi-well plate format [23-25]. The newest prototype device integrates easily with the agitators of Concept 96 robotic station described in section 2.2 or other commercial well-plate agitators [25]. This device consists of (i) base which can house a 96-well plate, (ii) a fiber support and guide system which allows the appropriate placement of the fibers into the centres of the wells of 96-well plate including the exposure of the same length of coating and (iii) a protective cover. Thus, the device can be used for handling, storage, transportation and desorption of *in vivo* probes. Figure 1E shows the device during the desorption step of *in vivo* probes. The comparison of the results for manual extraction versus the use of this device shows excellent repeatability with % RSDs of 6-9% for parallel multi-well desorption versus 11-14% RSD for manual desorption (n=20 fibers) of four drugs tested. This shows reproducible fiber positioning can improve overall method precision in addition to providing ~2-fold savings in time.

2.5 Critical comparison of fibre SPME, TFME and in-tip automated SPME configurations

The differences in the types of configurations recently developed for multi-well SPME also require a critical discussion of the advantages and disadvantages of each format and the types of applications most suitable for a given format. When comparing in-tip versus fibre SPME or TFME, both techniques have very similar sample throughput with ~100-250 min required for all steps of sample preparation, if equilibrium extraction is used. TFME is currently the only format that is fully commercially available with both fully automated robotic station with full computer-

software control and commercial 96-TFME devices. On the other hand, in-tip SPME uses existing laboratory automation already available in numerous laboratories for liquid handling, which permits easier adoption as no dedicated SPME instrumentation is required. However, 96-well in-tip SPME devices are not currently available commercially, thus requiring custom in-house preparation. Secondly, in-tip configuration is not suitable for complex samples containing cells and particulates and/or very viscous samples: sample filtration and/or dilution is recommended for such samples. TFME and fibre configurations, on the other hand, are fully compatible with viscous and complex samples; even whole blood [9] or waste water samples [17] can be analyzed without any sample pre-treatment. In-tip fibers are typically single use, while open-bed configuration fibers or thin films are reusable. Reusability can be a significant benefit from the point of view of keeping the cost of analysis per sample down. However, reusability can also decrease the overall sample throughput, as portion of the sample preparation time must be spent on adequately cleaning the extraction phase to eliminate carryover. Typical cleaning procedures include 30-min cleaning step in methanol/water which also serves as sample preconditioning step for the next extraction [9], or a separate second desorption step (40-min) aimed to remove any remaining analytes with stronger solvents than typically used for preconditioning [16,26]. When coupling SPME with DESI, carryover from the thin films was removed using five consecutive 15 min sonication steps in alternating methanol and acetonitrile solvents [17]. The carryover issue is also particularly problematic for untargeted applications such as metabolomics, where it is impossible to validate that any washing step employed is sufficient to remove traces of every single possible metabolite regardless of its chemical properties and/or polarity. For such demanding applications, single-use fibers are clearly preferred so in-tip SPME configuration is more promising for this type of analysis. Another important microextraction advantage that current automated Concept 96 configuration of TFME has lost is the compatibility with small sample volumes. Typically 0.8-1.8 mL sample volumes are used to meet the requirement that the entire length of the coating must be fully immersed in sample throughout the extraction procedure. This means that TFME is no longer ideally suited to sample-limited situations, and in-tip SPME is much more suited for such applications with sample volumes as low as 0.1 mL used successfully. Similarly, large volumes are also required for desorption in TFME, so analyte pre-concentration is not really achieved, unless a time-consuming evaporation/reconstitution step is added which drastically reduces sample throughput.

One exception to this is the direct desorption of TFME using DESI-MS [17], where the sensitivity of TFME exceeded that of SPE for the preparation of same sample volumes due to pre-concentration factors achieved with the use of direct desorption directly in MS source. Taking into consideration that the reduction in solvent use and compatibility with small volume extraction are the two of main driving forces for adoption of SPME in high-throughput laboratories versus classical techniques such as SPE and LLE, in-tip SPME configuration appears better suitable to meet these demands, while TFME offers improved analytical sensitivity and better compatibility with complex heterogeneous samples. The requirements of low cost and improved selectivity are easily met by both types of configurations. Figure 2 summarizes the main advantages of each approach.

2.6 Development of coatings for automated SPME: requirements and current status

The chemical and physical properties of the coatings employed in the device design largely dictate sensitivity and selectivity ultimately achievable by SPME. The main requirements for the development of coatings for the automated multi-well systems are good mechanical robustness, good solvent compatibility with commonly used desorption solvents, low thickness to enable fast mass transfer and excellent inter-fiber reproducibility to permit accurate and precise quantitative analysis. To keep the low cost of analysis it is also preferable for the coatings to be reusable, if possible, especially for fibre and thin-film configurations where metal solid-support framework may be relatively costly to manufacture. In addition, for dealing with complex matrices such as biological fluids, food samples or wastewater and effluents, the compatibility of coating with the presence of biological material is crucial with the primary focus to minimize the adsorption of biomolecules which can impact the analyte mass transfer into the coating. Finally, the availability of numerous coating chemistries would facilitate easy adoption of the technique for various applications.

However, despite the extensive research efforts in the optimization and development of new coatings with huge improvements achieved within the past decade as summarized in recent reviews [10,27,28], the commercial availability of SPME coatings both in single-fibre and high-throughput formats lags significantly behind other solid-phase techniques such as SPE. For example, for high-throughput TFME, only the biocompatible octadecyl silica (C_{18}) and mixed-

mode octadecyl silica (C_{18}) with strong cation exchange group coatings are currently available commercially [17], while single manual fibre coatings for LC applications include about five chemistries, some of which are not fully compatible with solvent desorption due to polymer swelling [29]. Even the more recently introduced microextraction by packed sorbent (MEPS) allows the user access to wider selection of sorbent chemistries including reversed-phase, ion-exchange, mixed-mode and normal-phase materials than is available for SPME technique. This issue presents an extremely serious shortcoming in the adoption of SPME in high-throughput laboratories as many users are simply not interested in producing their own in-house coatings and presents a major obstacle for current SPME technology. Table 2 summarizes all the coatings employed to date in automated SPME applications, and briefly describes their main properties and preparation procedures. Among all of the coatings developed to date, three main trends predominate: (i) design of biocompatible coatings for use in complex biological samples (ii) design of thin coatings to improve mass transfer kinetics for increased sample-throughput and (iii) design of monolithic coatings for enhanced extraction efficiencies. Notably, none of the coatings reported to date successfully incorporates all three characteristics, opening up additional avenues of research for future coating improvements.

2.6.1. Design of biocompatible coatings for repeated use in complex biological samples

Polyacrylonitrile (PAN) is a known biocompatible polymer with high chemical and mechanical stability, making it an excellent choice for effective sorbent immobilization in automated SPME. The use of this polymer as adhesive and as top layer covering sorbent particles, provides a surface that minimizes protein adsorption, thus effectively eliminating the issue of fibre "fouling" often encountered with the use of non-biocompatible coatings in complex matrices [30]. Although first developed for *in vivo* applications, PAN-based coatings represent promising coatings for high-throughput applications as they provide very high fibre reusability as shown in Table 2.

Mirnaghi *et al.* recently compared three different methods for preparation of thin-film coatings for Concept 96 systems: dipping, spraying and brush-painting using octadecyl (C_{18}) PAN coating as the model system for the evaluation [31]. The spraying of the particle-polymer slurry using flask type sprayer with nitrogen gas resulted in the most robust reusable coatings with the highest

physical stability. The mean extraction efficiency of diazepam over 70 uses was 94% (4 % RSD) for spraying method, versus 32% (147% RSD) for brush painting and 16% (230% RSD) for dipping methods, indicating no extraction phase loss over repeated uses. In fact, the sprayed coating could also be used for 70 extractions in human plasma with no detectable loss of extraction efficiency, and even up to 140 uses could be achieved with small decreases in extraction efficiency and method sensitivity while still maintaining adequate method precision. The enhanced stability of sprayed coating over other preparation methods was attributed to improved attachment of multiple thin layers of the coating, accomplished by coating 10 successive thin layers followed by instant thermal curing at each step. The resulting optimum coatings were 60 μm thick. Thinner coatings resulted in significantly decreased physical stability even when optimized spraying method was used. The coatings had good chemical stability, and performed well in pH range 2-10, while pH values outside of this range resulted in significant loss of coating.

One historical limitation of SPME in direct extraction mode is poor extraction efficiency of polar compounds, so many current coating development efforts focus on addressing this shortcoming. As shown in Table 2, an interesting option to improve extraction of polar compounds and other species of interest such as mycotoxins, is carbon tape, which is commonly used to immobilize samples in microscopy [32]. The double-sided nature of this tape provides an easy and low-cost way to prepare in-house an array of 96- fibers or thin films, and excellent extraction efficiencies were observed for ochratoxin A in urine with almost exhaustive recoveries (66%) even when using pre-equilibrium extraction times. The main disadvantages of this coating are long extraction times to reach equilibrium necessitating use of short pre-equilibrium extractions and the observed fouling of extraction phase in biological matrices, so single-use of the coating is recommended for complex matrices. A recent comprehensive study of 42 different types of SPME coatings for the extraction of 36 metabolites of varying polarity, identified several new coating types particularly useful to further improve the extraction of polar compounds including mixed-mode silica-based or polymeric sorbents, polar-modified polystyrene-divinylbenzene (PS-DVB) polymeric sorbents and phenylboronic acid (PBA) coatings [33]. These coatings were prepared using strong solvent-compatible Loctite adhesive, however the coating robustness and reusability when using this type of procedure decreases as the particle size of the sorbent

increases. This procedure performs the best for ~ 5 µm size particle immobilization, while larger particles (40-80 µm PS-DVB and PBA particles) may detach upon repeated use. Thus, a subsequent study improved immobilization procedure and robustness of PS-DVB and PBA coatings using previously described PAN spray coating procedure[26]. In addition, this procedure also improved the biocompatibility of the coatings by using PAN biocompatible polymer as adhesive for immobilization, and spraying an additional thin layer of PAN on top of the coating to ensure complete coverage of sorbent. This approach resulted in very strong and reusable coatings, capable of >100 extractions in plasma matrix without any loss in efficiency. Although, PAN-PS-DVB performed equivalently or better than PAN-PBA coating for 5 compounds tested in this study as shown in Table 3 (no coating volume correction) and required 2-fold shorter extraction times to reach equilibrium (60 min versus 120 min), the compounds selected for the comparison did not include sufficiently wide range of chemistries. With the broader range of analytes included in the initial study, significantly higher recovery of some compounds was achievable with PBA, showing it can have complementary selectivity to existing common SPME coatings [33]. Therefore, additional research is needed to better understand the extraction capability of this new PBA coating and whether or not the top layer of PAN affects the extraction of polar compounds. It is also important to note that PBA coating can extract not only cis-diol containing analytes, but may have more broader applicability as general SPME sorbent via hydrogen bonding, ionic, charge transfer, Van der Waals and pi-pi interactions [26,33]. Also, contrary to expectations for this affinity-type coating, the influence of extraction and desorption pH seems to have limited influence on the performance of this coating in SPME format, although this may be compound-dependent and deserves further investigation. PBA coating showed about 2-fold lower carryover than PS-DVB, and interestingly for both coatings there was no evidence that polar compounds could reach equilibrium faster than non-polar compounds, in contrast to *in vivo* hypodermic devices where 5 min was found sufficient to reach equilibrium for a high number of polar metabolites using vortex agitation [33]. The results also clearly demonstrate that the use of polymeric sorbents such as PS-DVB can eliminate the need for preconditioning thus further increasing sample throughput in SPME methods [26]. In summary, the availability of both PAN-PS-DVB and PAN-PBA coatings in format compatible with high-throughput automated multi-well SPME presents an important advance and extends the capability of the technique towards much more polar classes of compounds for the first time.

2.6.2 Design of thin coatings to improve mass transfer kinetics for increased sample-throughput

One of the major problems that remains in the development of SPME coatings is the reproducible production of very thin coatings, which are preferable from the point of view of short extraction and desorption times. As can be seen in Table 2, only three types of coating employed for automated SPME have thickness below 10 μm, opening up new research opportunities in this area. For example, a recent study reporting 8 μm thick silicate-entrapped porous coatings that can be custom-modified using on-fibre derivatization and that enable equilibrium extraction times as short as 2 min appears to be a particularly promising direction in the development of thin coatings [34]. The fibers had carryover of <2% for a range of drugs tested. Thin porous films are also preferred for coupling with DESI for improved extraction efficiency and better resulting method sensitivity with 25 μm commercial thin films used for this application [17].

Although to date very few successful sol-gel coatings for coupling to LC were reported due to problems with solvent stability, the usefulness of sol-gel procedures to prepare robust reusable thin coatings was investigated by Mirnaghi *et al.* [31]. The speed of coating (1 mm/sec) and very specific drying procedure with gradual increase in temperature were found to produce the most robust high-quality coatings without shrinkage or cracking of the coating phase, while achieving good solvent compatibility. The viscosity of sol preparation (controlled through sol composition and aging time) and the coating speed during the dipping method, accurately controled the thickness of this type of coating, thus permitting reproducible preparation of very thin coatings ($\leq$ 10 μm). The surface morphology of these coatings is rougher than corresponding PAN-based coatings which may be problematic for some types of analyses. However, three extractions in whole blood showed no evidence of red blood cell attachment to the surface. The reusability of coating over 20 uses in plasma was successfully established, but protein adsorption on the surface was noted, making this type of coating less than ideal for the analysis of biological samples unless further refinement to the coating procedure is introduced.

2.6.3 Design of monolithic coatings for enhanced extraction efficiencies

Monolithic SPME coatings are another very attractive type of coating for automated SPME systems due to their enhanced mass transfer kinetics and easy preparation with a wide variety of chemistries [19,20]. Monolith preparation is usually carried out via bulk free-radical copolymerization of a monovinyl monomer and a cross-link monomer in the presence of porogenic solvent and an appropriate initiator with the aid of thermal or photo irradiation. The porosity and surface area of the resulting polymer are critically controlled by the type and amount of porogenic solvent and the crosslinker percentage presenting opportunities to prepare highly porous coatings of prime interest in SPME. Recently, photopolymerization technique using ethylene glycol dimethacrylate, dimethoxy-α-phenylacetophenone and 1-decanol was fully optimized for the full automation of the coating procedure in 96-well plate format using a Tomtec Quadra 96 robotic station and compact UV lamps [19]. To prepare the tips, an array of 96 in-tip SPME fiber modules was prepared by placing a piece of GC capillary tubing (0.02" outer diameter) inside commercial polypropylene pipet tips with a 2-3 mm of tubing protruding from the narrow opening of the tip while the top portion of the tubing is held in place using a polyethylene frit. This array of tips is then loaded onto Tomtec Quadra station, and the rest of the coating procedure is performed simultaneously for the entire 96-array of devices by aspirating 10 µL of polymerization solution mixture followed by fast 10-min UV curing at 365 nm using UV lamps placed on Tomtec Quadra in a second position. The design of the devices simplifies the coating procedure as the placement of capillary introduces a flow channel through the polymer, and this capillary GC tubing is removed after coating preparation. Another nice feature of the proposed coating procedure is the ability to embed SPE type sorbents during polymerization procedure, for example authors successfully embedded Oasis HLB 60 µm SPE particles to further enhance extraction efficiency of the coating. The resulting coatings provided excellent extraction efficiencies of 20.2% for drug candidate tested in plasma [19] and 24-29% for vitamin D derivatives [20]. The direct comparison of monolithic in-tip SPME versus commercial PDMS-DVB SPME and Varian C18 µSPE showed monolithic phase recoveries can approach those of µSPE methods. For example, the absolute recovery using PDMS-DVB fibre was 3.3 and 12.3% for oxazepam and diazepam respectively, while for monolithic phase the recoveries were 13.7 and 46.5% versus 26.4% and 73.9% recoveries obtained for non-optimized µSPE method. These high recoveries were attributed to the extremely high surface area available for extraction in the monolithic coating, and represent some of the highest extraction efficiencies obtained by SPME

in blood-derived fluids. In addition, this study provides the first automated coating procedure for SPME, addressing an important need for simple and rapid preparation of large numbers of SPME devices. Subsequent studies also demonstrated that high recoveries of polar zwitterionic drugs such as impinem, cilastatin and MK-4698 could be obtained with proposed monolithic coatings with absolute recoveries ranging from 9.3-16% for the three compounds studied while the recoveries obtained with commercial PDMS-DVB or lab-made silicate-entrapped porous C_{18} coatings were below 2% for all three compounds [35].

3.0 Method development considerations for high-throughput 96-well plate SPME

Currently, there is an increased interest in developing general purpose SPME protocols, and the use of multi-well or in-tip geometries imposes practical limits in terms of sample volumes and desorption solvent volumes needed to develop an SPME application thus permitting easier standardization. The focus in this type of applications is expected to slowly shift towards more rational method design with special focus placed on decreasing the time required for all steps in order to achieve the best possible sample throughput. A detailed protocol summarizing the best practices and considerations during method development of automated SPME/TFME methods including automated binding studies has been published elsewhere [36].

In contrast to traditional single-fibre method development, high-throughput automated multi-fibre SPME development also requires evaluation of two additional parameters: inter-fibre reproducibility and uniformity of agitation. Depending on the type of coating procedure used, some coatings may have acceptable inter-fibre variability in the amount of extraction phase immobilized, so further corrections may not be mandatory. Table 2 summarizes typical inter-fibre reproducibility achievable using different coating procedures. For the coatings with poorer (>5% RSD) inter-fibre reproducibility, the use of an appropriate calibration method is recommended to ensure best accuracy and precision. The recommended calibration methods for this purpose are the traditional calibration approaches of internal standard calibration or the use of constant fibre correction factor if dealing with highly reusable coatings. The newer calibration method of in-fibre standardization was not found to perform well with either in-fibre or in-tip SPME configurations [9,35]. Secondly, the evaluation of agitation uniformity is absolutely critical for fibre SPME and TFME applications. Generally, the highest speed not causing spilling

is preferred for all geometries including TFME and *in vivo* devices. For TFME, the agitation speed of 1000 rpm (2.5 mm amplitude) to 1200 rpm (1 mm amplitude) represents the best compromise between extraction kinetics and prevention of spilling [26]. Method precision improves when using higher agitation speeds and longer extraction times, thus indicating that uniformity of agitation at very short extraction times may not be suitable even when using highly reproducible orbital agitation [15]. For instance, using agitation speed of 850 rpm, extraction times ≥ 10 min resulted in good method precision while at 500 rpm speed acceptable precision could only be obtained when using ≥ 20 min extraction times [15]. This indicates that although automated SPME is very suitable for use in pre-equilibrium conditions, the extraction times selected must be sufficiently long to establish uniform agitation in all wells, so this parameter must be carefully considered during the method development, especially for pre-equilibrium methods. The effect of agitation speed on method precision was also noted for *in vivo* probe desorption, where the use of 500 rpm agitation resulted in much poorer method precision with RSD ≥ 15%, while the use of 1200 rpm resulted in RSDs ≤ 7.0% [25]. In contrast, in-tip configuration relies on highly reproducible agitation using aspirate/dispense cycles so this parameter does not require further optimization during in-tip SPME method development.

The evaluation of absolute recovery should be investigated during the development of any SPME application, but is often not reported in literature. For high-throughput applications, especially TFME, the increase in the extraction phase volume often in fact permits exhaustive extraction of moderately to highly non-polar compounds in simple matrices such as buffer or water, as illustrated in Table 3 for the extraction of diazepam, oxazepam and caffeine using PAN-PS-DVB coating. In complex matrices, the absolute recovery of SPME decreases if the analyte of interest is bound to biomolecules present in the complex matrix, because the amount of analyte extracted by SPME is proportional to free concentration. For example, in Table 3 absolute recovery for diazepam drops to 5.3% due to high degree of binding to plasma proteins, while for sucrose the absolute recovery remains the same in both matrices, since no binding of sucrose takes place. The knowledge of absolute recovery then helps dictate whether the technique can be used for the determination of free concentrations and/or binding studies. For studies where such parameters are not of interest, absolute recovery by SPME in biological fluids can be enhanced by disrupting

drug binding via addition of salt, solvent or pH change to improve limits of detection achievable by SPME.

4.0 Current applications of high-throughput SPME

To date high-throughput SPME/TFME has been successfully applied in a variety of applications including clinical, pharmaceutical, toxicological, food and environmental analysis. Other applications, not exploited to date, could include tissue analysis after homogenization or analysis of non-volatile components or contaminants in food commodities, leaving many opportunities for increased development of automated SPME applications. In future, multi-well SPME configurations can also be used beyond targeted analyses applications such as described in this review to encompass the newly emerging field of metabolomics. For instance, metabolite profiling of plasma or blood can easily be migrated to the high-throughput platform in future for fast preparation of large number of samples [33].

4.1 Clinical and bioanalytical applications

In an early proof-of concept investigation of the feasibility of SPME in pharmaceutical drug discovery, the performance of SPME was compared with LLE using the same drug candidate and 0.25 mL plasma samples [12]. Intra-day precision of LLE ranged from 0.8-3.3% RSD with accuracy at all levels within ± 2%, while for SPME precision ranged from 0.5-6.9% RSD with accuracy at all levels ±5%. Both methods were successfully validated in the range of 1-500 ng/ml according to Food and Drug Administration (FDA) guidelines [37]. The comparison of concentration-time profiles obtained using SPME and LLE after single-dose administration of 25 mg of drug candidate showed excellent agreement between the two techniques. Overall, LLE and SPME were both suitable for the analysis of the proposed drug candidate in clinical samples, but SPME reduced solvent usage, eliminated the need for evaporation/reconstitution step needed for LLE and had overall higher sample throughput if using a full 96-fibre device. The main disadvantages found in this study were high carryover (>10%) requiring extensive coating clean-up prior to reuse, limited number of coating chemistries available and high cost of building 96-fibe device using commercial fibers.

Subsequent studies of the performance of high-throughput SPME in pharmaceutical discovery focused on in-tip SPME configurations and illustrated the promise of the technique in a variety of applications. For example, in-tip SPME was applied for peroxisome proliferator activated receptor modulated drug candidate (MK-0533) undergoing clinical investigation [18]. The intra-day precision was 13.7% RSD at lower limit of quantitation (LLOQ) and ranged between 1.0 to 9.1% RSD at all other concentration levels, while intra-day accuracy was within ±8% across the entire linear dynamic range of the method. The proposed method showed excellent agreement with validated LLE method routinely used for this analysis as shown in Figure 3. In-tip SPME was also successfully validated for the determination of drug candidate MK-0974 in human plasma with intra-day accuracy and precision ranging from 97.5 to 104.3%, and 3.0 to 13.0% RSD, respectively [19]. The method performance was compared to µSPE using the same HLB sorbent material, and excellent agreement was obtained between the two methods for the analysis of clinical samples and calculation of individual and mean pharmacokinetic parameters after oral administration [35]. These types of head-to-head comparisons clearly reiterate that SPME is a feasible alternative approach for routine drug analysis in pharmaceutical industry. In-tip SPME was also successfully used to develop a method for the determination of vitamin D3 in human serum with derivatization [20]. Intra-day accuracy ranged from 92.8 to 104.8% while precision (including derivatization) ranged from 2.2 to 10.9 % RSD. Unfortunately, the results of this study illustrate one of the recurrent limitations of SPME and other microextraction techniques when the analysis of ultratrace analyte concentrations is required, as SPME was not able to match the sensitivity achievable by LLE (0.5 ng/mL) with LOQ of 5 ng/ml for SPME. However, SPME method used 4-fold lower volume of human serum, had lower absolute matrix effects versus automated LLE method, considerably higher sample throughput (2 hr per 96 samples) versus both manual (9 hr per 96 samples) and automated LLE methods (3 hr per 96 samples). SPME also reduced solvent use by about 5-fold versus automated LLE method, and remarkable 34-fold versus manual LLE method. Thus, although SPME method was not found suitable for the determination of vitamin D3 time concentration profiles after administration of low doses, it provides a simple and rapid alternative for pharmacokinetic studies at higher doses.

Automated TFME and Concept96 were also recently used in clinical practice for the high-throughput determination of tranexamic acid concentrations in patients undergoing

cardiopulmonary bypass surgery [38,39,39]. LLOQ of the proposed method was 1 µg/mL and was suitable for therapeutic drug monitoring of this common antifibrinolytic agent for high-risk surgeries. This LLOQ could be achieved despite the 4-fold dilution to reduce the consumption of clinical samples and the use of C_{18} coating not ideally suited for the extraction of polar compounds such as tranexamic acid. More surprisingly, the automated TFME (270 min for 96 samples) provided better LLOQ than protein precipitation (10 µg/mL), and ultrafiltration (2.5 µg/mL) methods, and the gains in sensitivity were even higher considering the 4-fold matrix dilution used for TFME. Intra- and inter-day accuracy was ±10% and precision was ≤ 12% at all concentration levels tested. The mean recovery of the method was 2.38 ± 0.18%. The correlation coefficient between TFME and protein precipitation method was 0.82, with no statistically significant differences found between the two methods using t-test ($p > 0.45$). The Bland-Altman analysis of solvent precipitation versus TFME also demonstrated good agreement of the two techniques across clinically-relevant concentration range, showing that the two methods can be used interchangeably [40]. The method was subsequently used for evaluating inter-patient variability in drug concentrations and the suitability of the proposed dosage model [41]. This clinical application also represents the first successful reported high-throughput application of automated SPME to highly polar molecule, showing that the range of compounds accessible with this technique has been successfully expanded in recent years. Also, the results of this study establish for the very first time the suitability of automated SPME for high-throughput clinical analysis representing an important first step to wider adoption of this technique in clinical laboratories. Although not important for this particular application, since tranexamic acid has negligible plasma-protein binding, automated SPME/TFME can also be used to determine both free and total concentrations of analytes in a single sample and using a single analysis, thus providing significant time savings over traditional approaches where second method, such as ultrafiltration is necessary if free drug concentration is of interest [42]. Such determinations of free drug concentration are particularly important in therapeutic drug monitoring applications of the drugs with narrow therapeutic index, a possible new area of application for automated SPME methods.

Furthermore, automated SPME in fiber or thin film formats permits the analysis of whole blood or other complex samples without any need for sample pre-treatment. For instance, the

automated multi-fibre SPME of four benzodiazepines from whole blood with EDTA anticoagulant was fully validated, with intra-day accuracy 87-109% for all compounds and method precision ranging from 2-14% RSD and inter-day accuracy and method precision ranging from 87-115% and 1-12% RSD respectively [9]. LLOQs obtained in the study were 4 ng/mL, suitable for therapeutic drug monitoring of benzodiazepines, and sub-ng/mL LLOQs are possible if using TFME configuration [15,16] and/or newer generation of triple quadrupoles for MS analysis for applications where increased sensitivity is required. The entire automated SPME procedure required 100 min for the preparation of 96 samples, matching well the capabilities of multi-well SPE and LLE methods.

The automated SPME can also be useful for large-scale toxicological and epidemiological studies of human exposure to various contaminants. For example, automated SPME with carbon tape coating was successfully validated to determine the concentrations of an ubiquitous mycotoxin, ochratoxin A, in human urine [32]. Excellent sensitivity (0.7 ng/mL LLOQ) was achieved while the method was extremely simple and required only pH adjustment of urine prior to extraction. In contrast, most other analytical methods to date for this mycotoxin required much more costly and time-consuming multiple extraction and/or immunoextraction procedures, so automated SPME presents a much faster lower-cost alternative to classical approaches. It is also perfectly suited for high-throughput screening with capability to prepare >1500 samples per day (75 min per 96-samples). Mean intra-day accuracy and precision ranged from 93.5 to 113.7% and 2.1-11.5% RSD, while inter-day figures of merit ranged from 91.0-109.1% and 4.4-14.3% RSD respectively, thus easily meeting the requirements for regulated analysis recommended by FDA [37].

4.1.1 Ionization suppression and matrix effects

One of the major challenges in the development of bioanalytical methods by LC-MS is to reduce or eliminate matrix effects which can cause ion suppression/enhancement of the analyte signal of interest [43,44]. SPME methods can help to address this challenge due to non-exhaustive nature of the extraction, which reduces the amounts of co-eluting interferences such as phospholipids, thus reducing the extent of ionization suppression in complex samples. Thus, SPME represents a useful alternative when simple low-cost methods such as protein precipitation with solvent do not provide sufficiently clean sample extracts and sufficient selectivity for the needs of a given

application. For example, in-tip SPME showed moderate sample clean-up with ~2-fold decrease in the amount of phospholipids co-extracted from plasma versus solvent precipitation, while LLE provided the cleanest extract in a recent comparison [35]. The direct comparison of SPME and SPE also showed differences in the composition of co-extracted lipids, with SPE performing better for the removal of more hydrophobic phosphocholine lipids rather than lysophosphatidylcholine lipids, showing that the preferred method may strongly depend on the nature of analyte and co-eluting interferences. Phospholipid presence in SPME can be further reduced by using acetonitrile versus methanol as SPME desorption solvent. Furthermore, absolute matrix effects were evaluated for both in-tip and fibre/TFME configurations for a wide variety of analytes ranging from highly polar to highly non-polar, and no absolute matrix effects were observed with the values falling between 80-120% form nominal values when comparing the signal of standard spiked into matrix post-extraction versus neat standard signal intensity [19,26,36,38]. The comparison of monolithic in-tip SPME versus C18 µSPE showed presence of absolute matrix effects for the latter method for the determination of benzodiazepines in human plasma, while the two SPME methods showed no absolute matrix effects for all analytes except for oxazepam-monolithic coating combination [35]. In general, significant absolute matrix effects are not often observed for SPME methods in combination with LC-MS unless the analyte elutes in the region of anticoagulant or stabilizer [33,35] or co-elutes with an interference present in solvents used for desorption [29]. The use of HILIC rather than reversed-phase methods also increases the likelihood of encountering noticeable ionization suppression due to increased chromatographic co-elution in HILIC conditions [33,35]. For example, Xie reported significant matrix effects for cilastatin with >25% deviation from nominal concentration using both in-tip SPME and solvent precipitation methods of sample preparation [35]. Omitting the chromatographic separation step is also likely to result in noticeable absolute matrix effects for SPME, but even in this case SPME (50% reduction in signal) was found to outperform SPE (90% reduction in signal) in a recent DESI-MS study [17]. The presence of relative rather than absolute matrix effects is more likely to be problematic for SPME methods as small variations in sample composition, pH and ionic strength could slightly affect the amounts of analyte extracted and co-extracted interferences. However, extensive evaluations of relative matrix effects by comparing slopes of the calibration lines constructed in different lots of biological matrix indicate excellent performance of SPME with respect to this parameter, with slope RSDs of 1.4%

[12], 2.1% [9], 3% [19] 4.2% [20] and 4.6% RSD [32] depending on the analyte and matrix under study, all of which indicate methods free of relative matrix effects [43,44]. One exception to this general trend is HILIC LC-MS method for the determination of impinem, cilastatin and MK-4698 drugs in rat plasma where relative matrix effects of 10.1%, 8.8% and 10.4% were reported, and SPME performed more poorly than solvent precipitation (slope RSDs of 3.1-6.0%) [35]. Using post-column infusion experiments, the observed ion suppression and relative matrix effects were attributed to drug co-elution with endogenous phospholipids and/or stabilizers added to the samples due to the unstable nature of the drugs studied, depending on the retention time of the drug. In such cases, the use of deuterated internal standards is mandatory to achieve good method performance, and therefore, detailed evaluation of matrix effects remains an important parameter for investigation during development of any bioanalytical method including SPME-based methods.

4.2 Automated binding studies

SPME can also be used to perform automated binding studies both (i) to determine binding affinity between a particular receptor and ligand and/or (ii) the extent of overall binding in a complex matrix such as % plasma-protein binding, extent of sorption of pollutants to humic substances or bioaccumulation factors [45,46]. The main principle for this type of application is that the amount of analyte extracted by SPME/TFME is proportional to free concentration, and thus SPME avoids the need to physically separate bound from unbound analyte as is done in classical methods of equilibrium dialysis and ultrafiltration. Among the very few automated methods reported for drug binding studies, 96-well equilibrium dialysis method requires 8-hr to reach equilibrium, severely limiting sample throughput [47] while high-throughput ultrafiltration assay lacks accuracy and precision for highly bund drugs [48]. Properly designed SPME methods can successfully address both of these limitations [36] and can be performed both under negligible or significant depletion conditions as discussed in detail elsewhere [45]. The automation of binding studies by SPME permits all points of binding curve to be prepared simultaneously, and 8-12 ligands can be studied simultaneously within 96-well format, providing drastic increase in sample throughput versus manual binding studies relying on a single fibre. An automated study of diazepam binding to human serum albumin accurately determined binding constant (K=9.1×10^5 ± 3×10^5 l/mol) for high affinity binding site for diazepam in excellent

agreement with gold standard method of equilibrium dialysis (K =17.49×10^5 ±6.26×10^5 l/mol and K = 11.59×10^5 in two independent studies) [49]. In addition, numerous reports of manual binding studies by SPME exist for a diverse range of drugs and other compounds exhibiting both low and high binding, including ibuprofen, warfarin, verapamil, propranolol, caffeine, estradiol, isosorbide dinitrite and chlorhexidine just to name a few [45,46,50-52]. Binding constants for multiple binding sites can also be acquired using SPME provided that a sufficient number of data points over a wide concentration range is acquired for the ligand [45], and the data obtained by SPME can be highly complementary to other methods such as spectroscopic techniques [53]. Considering the importance of ligand-receptor binding studies in various fields including drug discovery, bioaccumulation, toxicology and ecology, it can be expected that the use of automated SPME methods for this type of applications may increase in future years.

4.3 Environmental applications of multi-well SPME devices: coupling to GC-MS and DESI-MS

Considering its inherent compatibility with the analysis of complex heterogeneous samples, automated TFME was recently employed to monitor selected pharmaceuticals in wastewater effluents from pilot-scale municipal treatment plants and wastewater-influenced river samples in combination with LC-MS [2,54]. The proposed method achieved limits of detection (LOD) of 2-13 ng/L for carbamazepine, fluoxetine, sertraline, and paroxetine with good precision (<16% RSD), showed good agreement with accepted SPE method and was found suitable for monitoring of wastewater effluents. The absolute recoveries ranged from 71 to 88% depending on the analyte, demonstrating nearly exhaustive recoveries achievable by TFME in some applications. Overall sample preparation time included 30 min preconditioning, 70 min equilibrium extraction and 60 min desorption.

Although multi-well SPME configurations have been primarily developed to increase the throughput in combination with LC-MS studies, GC applications can also be envisaged for this type of technology if developing very high-throughput applications for the analysis of species of sufficiently low volatility. For example, the study by Hutchinson *et al.* showed that multi-well SPME using PDMS silicone tubing immobilized on rods is suitable for the analysis of less volatile PAHs from water such as anthracene and fluoranthene with remarkable method precision of 1.8% for n=96 extractions with the use of deuterated standard in 60-min total sample

preparation time [13]. However, significantly poorer figures of merit were obtained for more volatile species such as napthalene and fluorene with 37.9% RSD for naphthalene. In another study, Bagheri *et al.* used 96-well plate set-up for the extraction of selected organophosphorus and triazole pesticides (diazinon, penconazol, tebuconazol, bitertanol, malathion, phosalone and chlorpyrifos-methyl) from cucumber [55]. The equilibrium extraction (40-min) was performed using manual 96-fibre set-up with PDMS silicone tubing (1.0 cm) placed on stainless steel tubing to serve as the SPME extraction phase, while custom-made PTFE 96-well plate block was used in lieu of commercial multi-well plates for extraction and desorption. Desorption (5-min) was performed using 600 µL of acetonitrile, followed by evaporation to dryness using stream of nitrogen and reconstitution in 20 µL of n-octane. The carryover was less than 0.6% when using 5-min desorption times. The method precision was found satisfactory, with the inter- and intra-day RSDs less than 15.4%. LODs ranged from 8-60 µg/kg, and LLOQs were sufficient to meet regulations of European Commission.

The coupling of TFME with direct online desorption by DESI-MS for environmental monitoring of carbamazepine and triclosan in wastewater effluents was also reported [17]. The proposed method showed good agreement with traditional SPE- LC-MS analysis, but SPME offered the advantages of short analysis times, low-cost, higher sample throughput and possibility of miniaturization. The method had excellent accuracy and precision, and method sensitivity of TFME exceeded that of SPE-LC-MS method when using the same sample volumes, thus showing excellent promise of the technique in environmental monitoring applications. Even more importantly, untargeted pharmaceutical screening could also be carried out with successful detection of beta blockers, UV filters, insect repellent, non-steroidal anti-inflammatory drugs and various surfactants. Finally, the potential of TFME to monitor temporarily high concentrations of contaminants in effluent streams was also evaluated, showing that TFME could be potentially used for process monitoring provided hourly analysis is performed. However, temporarily high concentrations could not be successfully detected over longer time periods as TFME would respond to new analyte concentrations fairly rapidly, an important advantage of SPME often exploited in other types of applications such as pharmacokinetic studies [56], but a disadvantage in this type of process monitoring application.

5.0 Future perspective and new opportunities

In summary, automated multi-well SPME/TFME provides the highest throughput of SPME to date. It is suitable for a wide variety of applications to non-volatile analytes including drugs, metabolites, vitamins, environmental contaminants, food and plant components and even untargeted screening and metabolomics applications. The main advantages for implementation of SPME in high-throughput analysis are (i) reduced use of solvents (ii) clean sample extracts minimizing the potential for ionization suppression (iii) low-cost (iv) similar throughput to what is achievable by SPE and LLE and better throughput than for online SPE methods (v) the ability to handle complex heterogeneous samples such as whole blood or waste effluents without sample pre-treatment (vi) the ability to obtain both free and total concentrations from a single analysis and/or perform binding studies and (vii) excellent compatibility with new ambient mass spectrometry methods such as DESI. The main disadvantages of the automated SPME are similar to those of all microextraction methods: (i) strict control of extraction conditions is required including control of pH, ionic strength and temperature to achieve best method precision, (ii) highly sensitive analytical instrumentation is needed for detection to compensate for non-exhaustive analyte recovery and (iii) analytical sensitivity of SPME in direct extraction mode is generally lower than for classical methods thus making it an unsuitable choice for the development of methods requiring extremely high sensitivity. Furthermore, due to open-bed configuration, this technique is not suitable for volatile analytes due to evaporative losses. Finally, there is an inherent lack of data demonstrating reproducible and robust implementation of automated multi-well SPME for routine analysis due to very recent development of this particular configuration and extremely limited commercial availability of suitable coating chemistries for all SPME configurations. Until this latter issue is addressed, widespread adoption of SPME in direct extraction mode for high-throughput analysis cannot be expected.

Current multi-well SPME configurations described in this review are capable of providing sample throughput equivalent to about 0.7-2 min per sample, and although this represents the highest throughput of SPME achievable to date, it still may not be enough to compete with the rapid progress in other techniques. For example, monolithic pipet tip SPE was recently reported with cycle times of 2-6 min for 96 samples [57,58]. This places increasing demands on finding innovative solutions to further speed up automated SPME. Such drastic improvements in speed of automated SPME can be accomplished through a number of ways such as the use of pre-

equilibrium extraction times, polymeric coatings which do not require preconditioning step, very thin coatings to reduce extraction and desorption times, use of disposable coatings to eliminate the need for extraction phase cleaning and perhaps more revolutionarily moving the technique back to micro-scale or nano-scale dimensions to speed up extraction kinetics and permit fast analysis of minute sample volumes. Other opportunities in adoption of SPME in high-throughput analysis include automated binding studies, whole blood analysis, applications requiring improved sample clean-up and improved therapeutic drug monitoring assays by simultaneous determination of both free and total drug concentrations, the types of applications that require excessive sample preparation times by classical approaches or the use of multiple analytical runs. Finally, the coupling of automated TFME with ambient desorption techniques such as DESI opens up new opportunities for the design of extremely high-throughput and highly sensitive applications of enormous interest in environmental, food and clinical analysis.

Acknowledgements

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List of Figures

Figure 1 Different multi-well automated formats of SPME (A) multi-fiber SPME [9] (B) Concept96 robotic station equipped with (1) 96-TFME device whereby (2, 4, 5) are orbital agitators used for preconditioning, extraction and desorption respectively, (3) is stationary wash station, (6) is 96-well nitrogen blowdown device, (7) is syringe arm and (8) is XYZ arm used to position TFME or nitrogen blowdown devices over the correct multiwell plates placed in positions 2-5 [36] (C) detailed picture of TFME device shown in (B) [16] (D) TFME coupling with DESI where (1) is an electronic sprayer, (2) is inlet capillary (3) is TFME device secured to the stage (4) is rotating stage (5) is sample table moveable in XYZ directions (6) is gas supply and (7) is solvent supply. DESI is directed over two 9-mm lengths of TFME coating and the resulting ions are analyzed in MS after collection into inlet capillary [17] (E) *in vivo* SPME 96-well desorption device shown positioned on an orbital agitator[25] and (F) detailed picture of an in-tip SPME device and (G) in-tip SPME device shown attached on commercial Tomtec Quadra station to carry our extraction and desorption steps of SPME[19]. Figures were reprinted with permission of the appropriate publisher from the references specified in brackets.

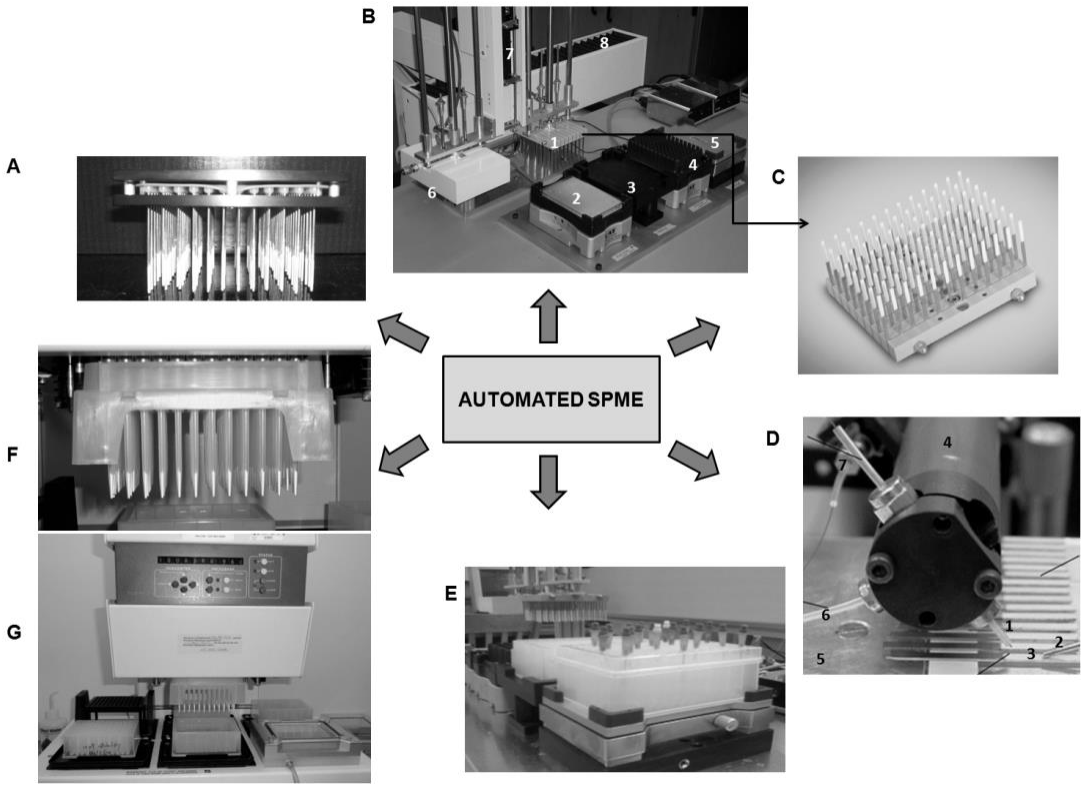

Figure 2 Comparison of the main advantages and disadvantages of in-tip versus TFME configurations of high-throughput SPME

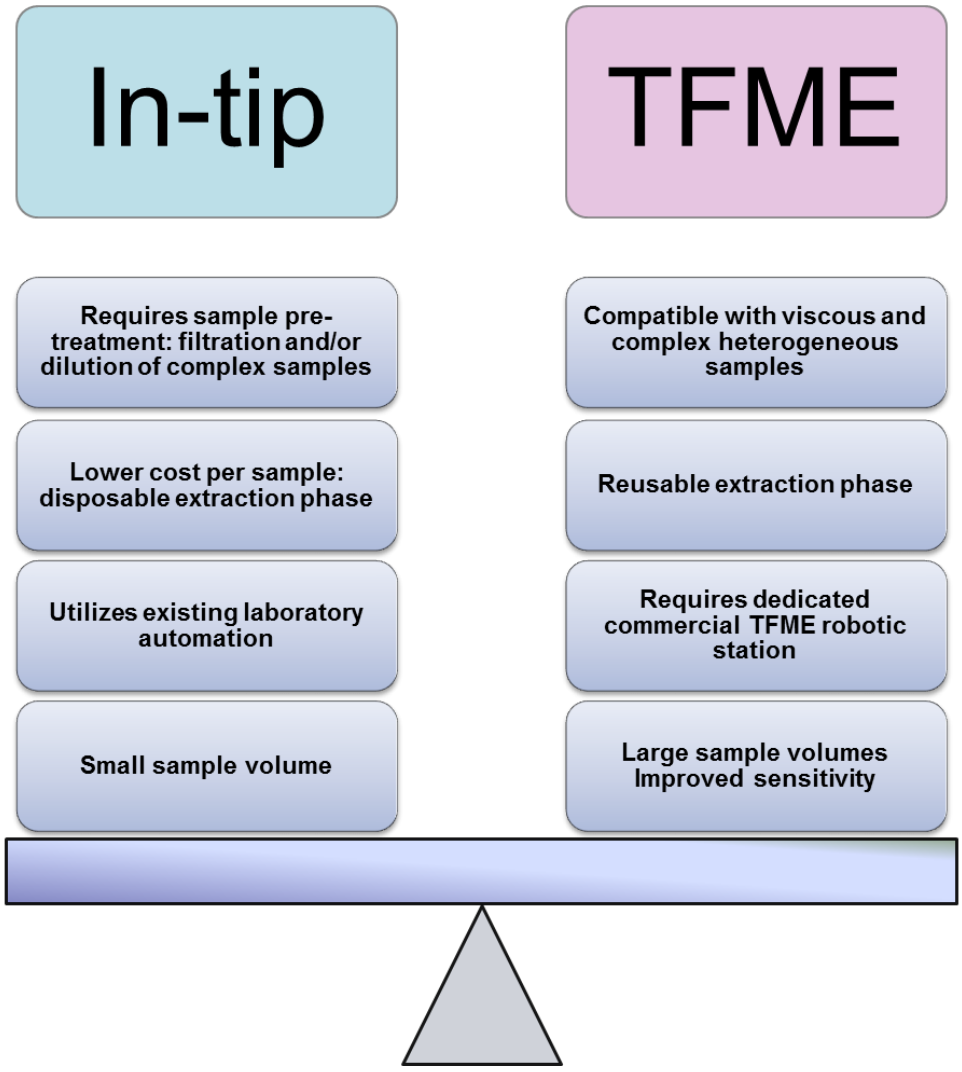

Figure 3 Concentration-time profile of MK-0533 drug candidate in plasma of pooled healthy subjects after single-dose administration of 75 mg of drug using LLE and in-tip SPME techniques. Figure reprinted from ref. [18] with permission of Elsevier.

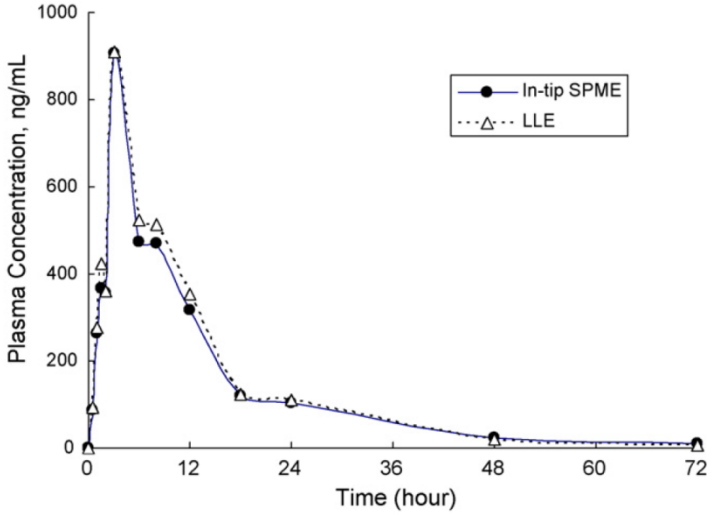

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Table 1 Comparison of the extraction rates (from linear portion of extraction time profile) and absolute recoveries using thin-film (TFME) and fiber SPME methods on Concept 96 for the extraction of benzodiazepines in PBS buffer (n=96) Table reprinted from ref. [15] with permission of American Chemical Society. Copyright 2009.

Parameter	**Diazepam**	**Nordiazepam**	**Lorazepam**	**Oxazepam**
Recovery (%) TFME	51	45	17	20
Recovery % SPME	30	26	12	10
Extraction rate TFME (ng/min)	6.2	6.6	2.2	1.6
Extraction rate SPME (ng/min)	3.4	3.5	1.3	0.94

Table 2 Summary of coatings and coating procedures employed to date in automated multi-well SPME systems

Coating type	Coating chemistry	Coating thickness (µm)	Automated coating procedure	Inter-fibre reproducibility (% RSD of n=96 fibers)	Coating reusability	Extraction of polar compounds	Extraction of non-polar compounds	Refs
Fiber SPME and TFME coatings								
Polydimethyl siloxane (PDMS)	Biocompatible Silicone tubing placed over metal rods	165	no	10% (LC-MS) 6.8-10.6% (GC-MS)	yes (> 50 uses)	no	yes	[9,13,28,55]
Octadecyl or C16 with an embedded amide group	Silica particles immobilized using Loctite 349 strong UV adhesive	5-6	no	7-12% (SPME) 7-11% (TFME)	yes (>15 uses)	no	yes	[9,15,36,49]
Carbon tape	Double-sided tape	N/A	no	13-15%	yes/no (depends on carryover and type of matrix)	yes	Yes but problematic desorption for very high log P compounds	[32]
Empore C18 disks (commercial sorbent disks C18 silica particles embedded in PTFE)	Cut and immobilize with pins	0.5 mm	no	4-6%	yes	no	yes	[14]
Silicate-entrapped porous C4, C8, C18 and C30	On-fiber derivatization of entrapped 5 µm silica particles using organosilane chemistry	8	no	18.6% (n=6)	yes (>100 uses)	no	yes	[34]
Octadecyl-PAN	Biocompatible 5 µm silica particles immobilized using PAN	60	no	5-7 %	yes (~ 70 uses in plasma and >140 uses in PBS)	no	yes	[16,38,54]

Octadecyl-silica glass	Sol-gel	10	no	3.8-8.5% (n=12)	yes (>100 uses in PBS and >20 in plasma)	no	yes	[31]
Polar-modified polystyrene-divinylbenzene (PS-DVB) - PAN	Biocompatible PS-DVB with weak anion exchange group 80 µm silica particles immobilized using PAN	230		4.4-8.3% (n=6)	Yes (>100 uses)	yes	yes	[26]
Phenylboronic acid (PBA) -PAN	Biocompatible PBA 40 µm irregular acid-washed silica particles immobilized using PAN	185	no	5.6-9.8% (n=6)	Yes (>100 uses)	yes	yes	[26]
PDMS-DVB	Commercial (Supelco Inc.)	60	Commercial procedure	Not evaluated	yes	no	yes	[12]
In-tip SPME coatings								
PDMS-DVB	Commercial (Supelco Inc.) fibers secured inside disposable pipet tips using custom procedure	60	Commercial procedure	Not evaluated	yes	no	yes	[18]
Methacrylate monolithic polymer with embedded oasis HLB sorbent particles	UV photopolymerization SPE sorbents may be embedded such as Oasis HLB 60 µm particles	n/a	yes	15.4	no (disposable)	yes	yes	[19,20]

Table 3 Percent absolute recovery for PAN–PS–DVB and PAN–PBA 96-blade SPME coatings for equilibrium extraction (100 ng/mL diazepam, oxazepam, caffeine and riboflavin, and 300 ng/mL sucrose spiked in PBS (pH = 7.4), and 300 ng/mL of all five compounds spiked in human plasma, n = 6 and four experiments). Abbreviated table reprinted from ref. [26] with permission of publisher.

Analyte	**log *P***	**pK_a**	**PAN–PS–DVB %PBS recovery**	**PAN–PS–DVB %Plasma recovery**	**PAN–PBA %PBS recovery**	**PAN–PBA %Plasma recovery**
Diazepam	2.82	3.4	98.1 ± 4.2	5.3 ± 0.6	74.1 ± 5.5	1.5 ± 0.2
Oxazepam	2.24	12.4	97.4 ± 3.3	6.7 ± ± 0.5	50.1 ± 2.3	1.8 ± 0.2
Caffeine	−0.07	10.4	98.9 ± 5.4	30.2 ± ± 2.1	33.7± 2.7	15.1 ± 1.5
Riboflavin	−1.46	10.2	71.4 ± 2.9	42.6 ± 3.3	44.6 ± 3.9	19.5 ± 1.8
Sucrose	−3.70	12.6	3.5 ± 0.3	3.6 ± 0.4	4.0 ± 0.4	3.8 ± 0.5