

Review

In-Tube Solid-Phase Microextraction Directly Coupled to Mass Spectrometric Systems: A Review

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Abstract: Since it was introduced in 1997, in-tube solid-phase microextraction (in-tube SPME), which uses a capillary column as extraction device, has been continuously developed as online microextraction coupled to LC systems (in-tube SPME-LC). In the last decade, new couplings have been evaluated on the basis of state-of-the-art LC instruments, including direct coupling of in-tube SPME to MS/MS systems, without chromatographic separation, for high-throughput analysis. In-tube SPME coupling to MS/MS has been possible thanks to the selectivity of capillary column coatings and MS/MS systems (SRM mode). Different types of capillary columns (wall-coated open-tubular, porous-layer open-tubular, sorbent-packed, porous monolithic rods, or fiber-packed) with selective stationary phases have been developed to increase the sorption capacity and selectivity of in-tube SPME. This review focuses on the in-tube SPME principle, extraction configurations, current advances in direct coupling to MS/MS systems, experimental parameters, coatings, and applications in different areas (food, biological, clinical, and environmental areas) over the last years.

Keywords: in-tube SPME; state-of-the-art coupling of instruments; selective capillary coating; recent applications in different areas



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1. Introduction

Sample preparation is an important step when analyzing complex samples (e.g., food, biological, and environmental samples) by chromatography—in these samples, the target analytes coexist with higher concentrations of interferents (exogenous and endogenous compounds) [1,2]. The main purposes of the sample preparation step include removing the interfering species and isolating and pre-concentrating the target analytes (trace levels). Thus, this step increases the selectivity and sensitivity of chromatographic methods [3,4].

Unfortunately, interferents can coelute with the target analytes, or they can irreversibly adsorb onto the vanguard pre-column. Consequently, the backpressure of the liquid chromatography (LC) system increases and/or ion formation is suppressed during electrospray ionization in mass spectrometry analysis (ESI-MS), decreasing the analytical sensitivity of the method [5,6].

Conventional offline sample preparation techniques generally require laborious procedures, which make them a time-consuming step of the analytical process (corresponding to 80% of the total analysis time). Additionally, the target analytes can be lost during multistep sample preparation [7]. Modern sample preparation techniques have focused on selective new sorbent materials, miniaturization, and automation of the analytical systems. These innovations reduce organic solvent consumption and sample volume and favor high-throughput performance of analytical systems [8]. In this context, in-tube solid-phase microextraction (in-tube SPME) is worthy of mention.

In-tube SPME was initially proposed by Eisert and Pawliszy in 1997 [9] to overcome difficulties inherent to SPME fibers (first generation), including low sorption capacity, limited effectiveness for weakly volatile or thermally labile compounds, and stability

against solvents used for high-performance liquid chromatography (HPLC). In-tube SPME was developed to allow SPME to be directly coupled to HPLC systems online. In these coupled systems, the target analytes can be directly transferred to the chromatographic system, so that the extraction, pre-concentration, injection, separation, and detection steps can be performed in a single and automated step [6].

The classic in-tube SPME system uses a segment of an open-tubular fused-silica capillary column with stationary phase coating as extraction device. The target analytes are extracted from the diluted sample and pre-concentrated (adsorption or absorption) into the stationary phase on the basis of the distribution coefficient (equilibrium process) between the sample solution phase and the SPME stationary phase. Then, the extracted analytes can be directly transferred to the analytical system. However, the analytes are not exhaustively extracted. Indeed, in many cases only a small fraction of the analytes is extracted for analysis [3,7].

In-tube SPME can be easily coupled to HPLC by using an online valve-switching technology, which automates the extraction and analysis of the target analytes; alternatively, in-tube SPME can also be coupled to a gas chromatography (GC) analytical system. Direct coupling of in-tube SPME to mass spectrometry systems (in-tube SPME-MS), without the chromatographic separation step, has also been possible thanks to the selectivity of capillary column coatings and MS/MS systems in the Selected Reaction Monitoring mode (SRM mode) [8,10–13].

Several excellent review articles have discussed different aspects of the in-tube SPME technique, its variables, and applications. For example, the review articles of Kataoka et al. about the in-tube SPME technique discussed the developments and applications of in-tube SPME [8,14,15]. Queiroz et al. [3] described selective capillary column coatings for in-tube SPME coupled to HPLC or LC/MS for bioanalysis. Later, Queiroz et al. [7] described the fundamental theory and current advances in in-tube SPME, as well as its use in environmental, clinical, and food analyses. Moliner-Martinez et al. [16] reviewed the advances in in-tube SPME coupled to different chromatographic systems, the preparation of new in-tube SPME extractive phases, and their main applications. Fernandez-Amado et al. [17] discussed the strengths and weaknesses of in-tube SPME and critically described the parameters used during in-tube SPME. Yamini et al. [18] reviewed the modifications and applications of magnetic nanomaterials for in-tube SPME. Xu et al. [19] focused on the development and application of in-tube SPME for analyzing proteins from biological samples and highlighted the use of selective, tailored sorbents in tube, including antibodies, aptamers, molecularly imprinted polymers (MIPs), and boron affinity materials (BAMs).

This review focuses on the in-tube SPME principle, extraction configurations, direct coupling to MS/MS systems, experimental parameters, coatings, applications, and current and future challenges.

2. Instrumental Configuration of the In-Tube SPME System

Among the instrumental configurations developed for in-tube SPME systems, the most used are based on draw/eject cycles and the flow-through extraction mode (Figure 1).

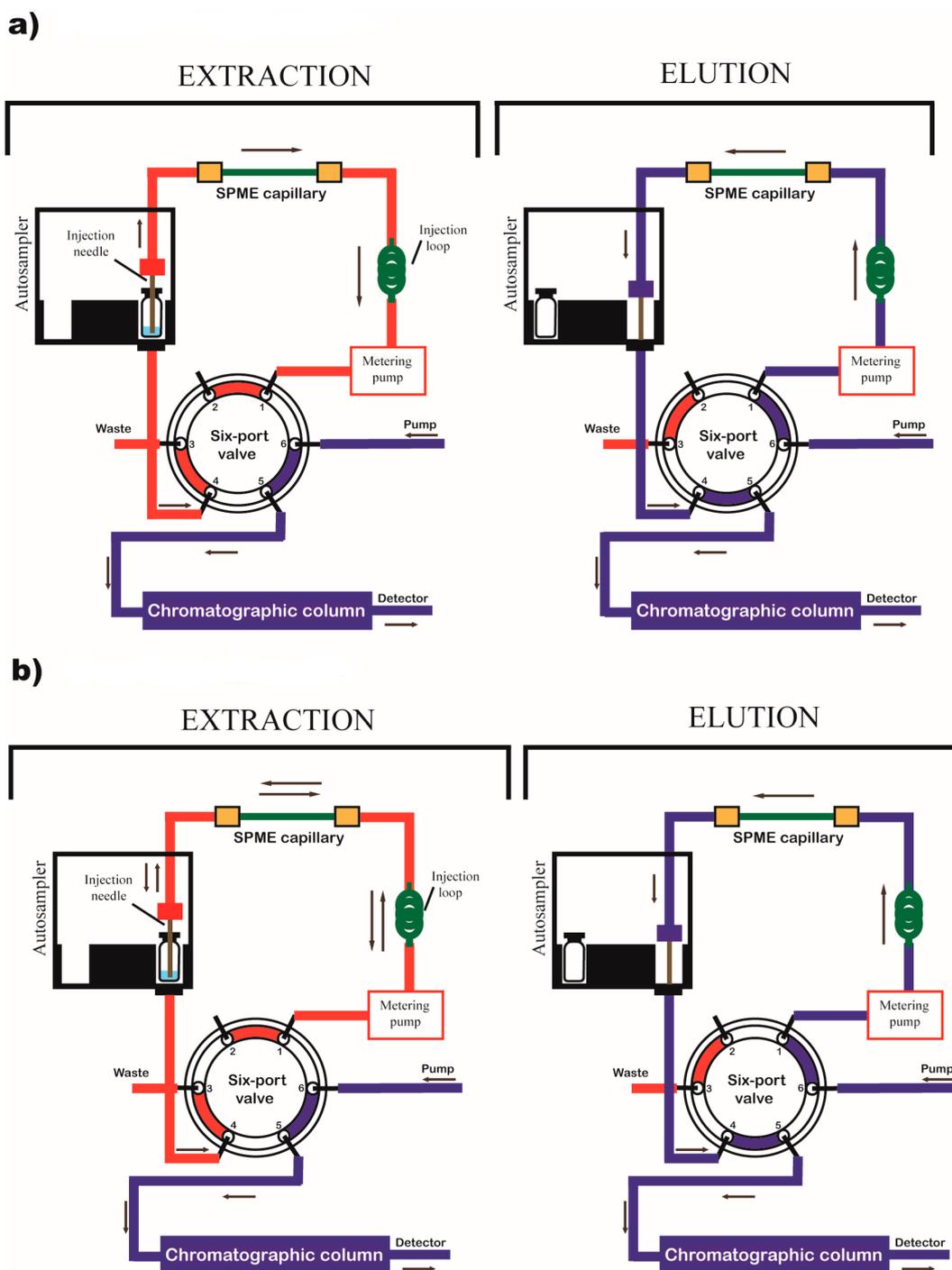


Figure 1. Graphic diagrams of in-tube SPME configurations. (a) Flow-through mode; (b) draw/eject mode adapted from reference [7].

In the draw/eject mode, the first in-tube SPME system to be proposed, the target analytes are extracted by repeated draw/eject cycles of the diluted sample (40–500 μL) through the capillary column (open fused-silica capillary column with crosslinked liquid stationary phase), under a programmable autosampler, until the sorption equilibrium is almost reached (25–30 draw/eject cycles). A capillary column is fixed between the injection loop and the injection needle of the HPLC autosampler or in the LC loop.

In the flow-through extraction system, the diluted sample (more often 0.5 to 3.0 mL) solution containing the target analytes is continuously percolated in one direction through an extracting capillary column until the analytes are adequately pre-concentrated. A

capillary column is fixed in the switching six-port valve, or it is sometimes placed in the loop (Figure 2). In general, extraction of the target analytes comprises four steps: conditioning of the capillary column, pre-concentration of the target analytes, washing, and desorption of the target analytes. A washing step with an appropriate mobile phase is included in the procedure to remove interferents (endogenous compounds of the matrix) before the target analytes are eluted to the chromatographic column. The mobile phase used in this step should be determined on the basis of the properties of the extractive phase used and the target analytes. The target analytes can be desorbed in the same flow direction as the extraction (straight flush—most used) or in the reverse direction (back flush). The systems are usually configured with two pumps, with one or two six-port valves.

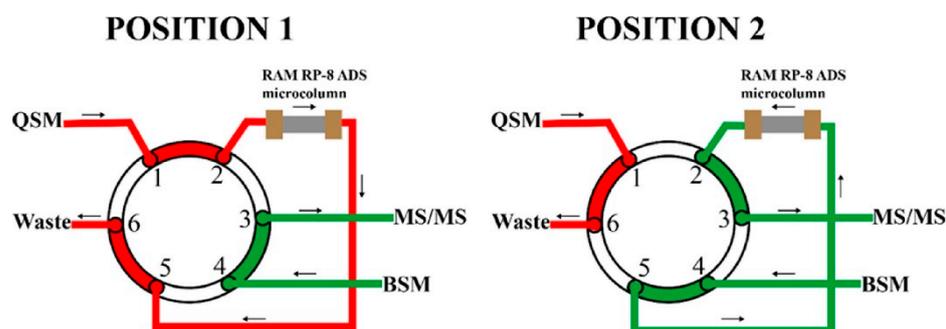


Figure 2. In-tube SPME-MS/MS system using the LC six-port valve from reference [20].

Using two binary pumps allows mobile phases with different compositions and flow rates to be employed during the extraction and chromatographic separation steps. In addition, chromatographic separation can be carried out simultaneously with the cleanup of the extraction capillary column.

By using the one-valve mode, which is more often employed, the extraction step (in both the draw/eject and flow-through modes) is performed with the six-port valve in the load position. After this step, the extracted analytes are directly desorbed from the capillary column by the mobile phase (dynamic desorption) after the six-port valve is switched (inject position). Finally, the desorbed analytes are eluted to the LC column, for chromatographic separation, and then to the selective detector. The in-tube SPME system has been combined with conventional LC with different detection modes (MS, MS/MS, DAD, UV, and FD) [21–34].

The advantages of the in-tube SPME system over other microextraction techniques include automation and online coupling to analytical instruments, which results in high-throughput performance. New couplings have also been evaluated on the basis of state-of-the-art LC instruments.

In-tube SPME has also been coupled to miniaturized LC techniques, such as capillary LC or nanoLC, to reduce solvent consumption and to enhance not only column efficiency (lower dispersion of the target analytes) but also mass sensitivity (better compatibility).

In-Tube SPME Directly Coupled to MS/MS System

Recently, in-tube SPME systems with selective bifunctional capillary columns have been directly coupled to MS/MS systems, without chromatographic separation, which offers several advantages, including high-throughput analysis.

Among the selective capillary columns developed for in-tube SPME-MS/MS, we emphasize restricted access molecularly imprinted polymers (RAMIPs) covered with bovine serum albumin (BSA) [13], monolithic capillary columns [35,36], organic–silica hybrid monolithic capillary columns with bifunctional groups (amino and cyano) [12], and restricted access material (RAM) with a hydrophilic diol external surface [20]. Directly coupling in-tube SPME to MS/MS systems is possible thanks to the selectivity of the capillary column coatings and MS/MS (SRM mode).

reduce the number of experiments. Both Nasrollahi et al. [29] and Sartore et al. [24] applied DoE to obtain the response surfaces and optimal in-tube SPME conditions. However, they chose different approaches for the initial screening experiments: the latter group used a fractional factorial design for screening the most influential variables, whereas the former group started assessing the variables by univariate experiments.

In-tube SPME optimization revolves around parameters that are directly related to sample pre-treatment, sorption and desorption of the target analytes, and geometrical characteristics of the capillary column. The next sections briefly discuss these variables. Table 1 summarizes the parameters discussed regarding the optimization of the in-tube SPME parameters.

Table 1. Optimization of the in-tube SPME parameters.

In-Tube SPME Steps	Parameters Evaluated
Sample pre-treatment	Sample pH Addition of complex agent Ionic strength Dilution
Sorption	Draw/eject mode: Sample volume (few microliters) Number of cycles and sampling flow = rate of each cycle
	Flow-through mode: Sample volume (few microliters to milliliters) Flow rate of mobile phase and pre-concentration time
Desorption	Composition and volume of desorption solvent Desorption time Flow rate of desorption solvent
Capillary column dimensions	Inner diameters and length

3.1. Sample Pre-Treatment

Depending on the nature of the sample, it may need to be pre-treated before in-tube SPME extraction is performed, mainly to obtain a cleaner sample and to prevent the capillary column and the LC tubulation from clogging. Pre-treatment also reduces the matrix effect or modifies the target analytes by means of derivatization or pH control, for example, to increase their interaction with the extractive phase. For matrixes of low complexity, such as water in environmental analysis, pre-treatment may consist of simple processes such as dilution and filtration [26,27,30]. As the complexity of the matrix increases, in some cases of food analysis and mostly in biological sample analysis, additional steps may be necessary, including homogenization [20,35], solvent extraction [21,22,28,30], or protein precipitation [12,20].

Sample pH is probably the most important parameter to assess before accomplishing in-tube SPME extraction of analytes bearing pH-dependent dissociable groups. By knowing the pKa values of the target analytes, the sample pH can be modified. When it comes to extractive phases with ion exchange capabilities, the sample pH can be modified with the aid of a buffer solution, to favor the dissociation equilibrium toward the formation of the ionized or unionized version of the molecule. In addition to that, the sample pH can alter the structure of the adsorbent, improving or reducing its extraction efficiency [7]. Extreme pH conditions can damage the capillary column coating, reducing its life span.

Other modifications can also be made in this step, such as adding a complexing agent during the analysis of free metal ions to form complexes and to improve the extraction efficiency or even detection [26,30]. In addition, the influence of the sample ionic strength can also be controlled due to the salting-out effect. Increasing the sample ionic strength by adding a salt or concentrated saline solution decreases the concentration of the target

analyte in the solution by the salting-out effect and may improve the extraction efficiency. However, excessive amounts of salt can block the capillary column [17].

3.2. Sorption Conditions

The parameters related to the sorption conditions that need to be optimized depend on whether the draw/eject mode or the flow-through mode is used. A variable that is common to both in-tube SPME modes is sample volume. Enhanced extraction efficiency can be observed by using increasing volumes of the sample, up to a certain limit, which will depend on the sorption capacity of the capillary column coating. Consequently, this variable should be carefully optimized, so that adequate sensitivity can be achieved within the smallest volume possible. This reduces sample consumption, which can be a limiting factor, especially when biological samples are analyzed [8]. For the draw/eject mode, a few microliters of the sample tend to be used [21,22], while for the flow-through mode, the sample volume can vary from a few microliters [12,24,29,34] to larger volumes, in the order of milliliters [26,27,30,31].

Specifically for the draw/eject mode, the number of cycles through which the autosampler injection needle aspirates and dispenses the sample and the sampling rate of each cycle can be optimized. In this in-tube SPME mode, the capacity of the capillary column (internal volume) limits the sample volume [7]. Thus, multiple draw/eject cycles can be performed for the sample, so that the maximum sorption capacity of the capillary column coating can be reached. However, as the number of cycles increases, so does the extraction time, which may cause peak broadening and tailing [8]. The flow rate at which the sample is drawn by/ejected from the needle can affect the extraction as well because high flow rates prevent equilibrium from being reached, whereas low flow rates can promote desorption of the target analytes.

In the flow-through mode, the mobile phase that is used for continuously carrying the sample through the capillary column should be determined on the basis of the properties of the extractive phase and the target analytes. The mobile phase should consist of a weak solvent that can pre-concentrate the target analytes within the extractive phase without significant loss, or that can simply displace the sample through the whole extension of the capillary column when pre-concentration is not necessary. Apart from the constituents of the sorption mobile phase, the flow rate and the pre-concentration time should be optimized for maximum extraction efficiency to be achieved within the shortest time possible to improve the analytical frequency. The maximum flow rate is usually limited by the material from which the capillary column is made and the mechanical resistance and chemical stability of the extractive phase, because the backpressure of the system increases with the flow rate [17].

3.3. Desorption Conditions

The target analytes can be desorbed by static desorption—a fixed volume of the desorption solvent is introduced into the capillary column and maintained for a determined amount of time—or dynamic desorption, by flowing the mobile phase or an adequate desorption solvent through the capillary column. The first desorption mode is employed less frequently and is only necessary when the target analytes and the extractive phase interact too strongly. In this case, the volume of the desorption solvent and the time it remains in the capillary column must be optimized [17]. The second desorption mode is used more often, and the mobile phase employed during chromatographic separation is frequently the desorption solvent of choice. In this mode, the flow rate of the desorption solvent is the only related variable that should be optimized. When the mobile phase cannot elute the target analytes properly, a desorption solution with different composition should be evaluated.

3.4. Capillary Column Geometry

The geometric parameters of the capillary column, such as inner diameter and length, dictate the internal volume of the capillary column and the amount of sample that can be loaded onto it, directly impacting the sorption capacity of the capillary column and the extraction time of the target analytes. Therefore, these variables can be modified to obtain adequate extraction efficiency. Capillary columns with 0.32 mm inner diameter are the most used for in-tube SPME because they can maintain a good flow rate and internal pressure [8]. Although the sorption capacity can be increased by using longer capillary columns, the backpressure and extraction time also increase. In this sense, the length of the capillary column can be optimized to obtain a balance between adequate analytical response and short analysis time.

3.5. Parameters Optimization for In-Tube SPME Directly Coupled to MS/MS System

The in-tube SPME parameters that can be optimized for methods that use the directly coupling to MS/MS system are identical to those of conventional in-tube SPME-LC-MS/MS, the only difference being that in this case, after desorption, the analytes are directed to the MS/MS system. Since the chromatographic separation step is dismissed in the direct in-tube SPME-MS/MS mode, achieving adequate sensitivity and selectivity can be more problematic when compared with the conventional in-tube SPME; in that sense, the optimization of the extraction parameters is even more important to enable the direct coupling.

By the optimization of the sorption conditions, Miranda et al. [12] were able to eliminate the interfering endogenous compounds in the analysis of amino acids in plasma samples in the first 2 min using acetonitrile as mobile phase, while retaining the target molecules. After 10 min of pre-concentration, the analytes are desorbed from the SPME capillary, and direct MS/MS analysis was possible since the majority of the interfering molecules were excluded. Similar findings were reported by Santos and colleagues [13], where a decrease in the sensitivity of the method was observed when using extraction times of less than 2.5 min.

Wu et al. [36] observed that modifications in the desorption conditions, such as the water content and the percentage of formic acid in the desorption mobile phase, improved or decreased the MS intensity of the targeted alkaloids in the analysis of tobacco, demonstrating that in-tube SPME parameter optimization can directly affect the ionization efficiency of the analytes, which is critical in direct methods. The evaluation of the in-tube SPME parameters allowed Oliveira and coworkers [20] to directly analyze endocannabinoids in rat brain and achieve the lower limits of quantification required for these analytes, which are present in trace levels, without the need of chromatographic separation.

4. Innovative Stationary Phases

4.1. Commercial Porous-Layer Open-Tubular (PLOT) Columns

Although stationary phases with new physicochemical characteristics have been reported, commercial capillary GC columns still raise interest and continue to be explored for new in-tube SPME methods. Among commercial porous-layer open-tubular (PLOT) columns, Carboxen and Supel-Q are worth mentioning. These columns have larger adsorption surface areas and thicker film layers and can extract greater amounts of analytes than other liquid-phase columns [23].

Carboxen 1006 is a carbon molecular sieve-based stationary phase with porous structure. The presence of large macropores and mesopores allows the target analytes to access the 7 Å micropores for rapid mass transfer. This column extracts small polar molecules such as nicotine and cotinine more efficiently [21]. Supel-Q contains divinylbenzene (DVB) polymer, which acts as a stationary phase. Supel-Q has been applied for continuously extracting and enriching nitrosamines [22] and sulfated steroid metabolites [23].

4.2. Monolithic Stationary Phase Columns

Monolithic capillary columns are promising for in-tube SPME due to their low column pressure (high flow through the macropores), fast mass transfer, and higher extraction capacity as compared with open-tubular capillary [7]. In these columns, the target analytes are preconcentrated in a continuous unitary mesoporous stationary phase prepared by in situ polymerization [35,36]. Normally, monolithic organic polymers are prepared by radical polymerization, while hybrid silica monoliths are synthesized via the sol-gel process by hydrolysis and polycondensation of alkoxysilanes [12].

In recent years, polyhedral oligomeric silsesquioxane (POSS) has gradually replaced the traditional alkoxysilane reagents in the preparation of organic-inorganic hybrid monoliths—POSS has good pH tolerance and excellent stability, and it is resistant to oxidation. POSSs are nanostructures with empirical formula $R\text{SiO}_{1.5}$, where R is a hydrogen atom or an organic functional group such as an alkyl, alkylene, acrylate, hydroxyl, or epoxide unit [38]. POSS feedstocks functionalized with various reactive organic groups can be incorporated into existing polymer systems through grafting, copolymerization, or blending. This incorporation reinforces the physical and chemical properties of the polymer. A hybrid monolithic column was synthesized with octa methacrylate-substituted polyhedral oligomeric silsesquioxane (POSS-MA) as crosslinker and N-butyl methacrylate (BMA) as functional monomer and applied for pre-concentrating phthalate esters in bottled water [31]. The column was prepared by using a (POSS-MA + BMA)/porogens (N-propanol + 1,4-butanediol) mixture at 30:70 (*w/w*) ratio. Zhao's group prepared a novel POSS hybrid monolith by using mono-methacrylate-functionalized POSS (mono-MA-POSS) and acrylamide (AM) as functional monomers to copolymerize with ethylene dimethacrylate (EDMA) [32]. The synthesized poly (POSS-co-AM-co-EDMA) monolith effectively extracted bisphenols from milk samples via π - π , hydrophobic, and hydrogen bond interactions.

The surface of monoliths can be functionalized or modified with different materials to obtain the desired chromatographic binding properties. Aptamers are short single-stranded oligonucleotides (DNA or RNA, typically 20–100 base pairs) whose sequence can specifically bind target molecule(s) with high affinity, equivalent to the affinity of antibodies [39]. Monolithic capillary columns were functionalized with aptamers to pre-concentrate ochratoxin A in beer [33,34] and red wine [33] samples. These aptamer-based affinity monoliths exhibited high recovery yields (>95%), specificity, and selectivity during ochratoxin A pre-concentration, even in the presence of high concentrations of ochratoxin B (analogous compound).

Organic monoliths doped with Fe_3O_4 particles or nanoparticles were reported for magnetism-assisted in-tube SPME. According to the magnetic microfluidic principle, an external magnetic field is applied during magnetic force to enhance the extraction efficiency. This approach was reported for chromium speciation studies [30]. Cr(III) and Cr(VI) were coordinated with ammonium pyrrolidinedithiocarbamate (APD) to form diamagnetic metal-organic complexes (Cr(III)/APD and Cr(VI)/APD), which could meet the requirements of chromatographic separation and diode array detection (DAD). The in-tube SPME capillary column was twined with a magnetic coil, which allowed changeable magnetic fields to be implemented during the total extraction procedure. The same technique was applied to trap and to pre-concentrate mercury species, which were coordinated with dithizone to form chelates (Figure 4) [26].

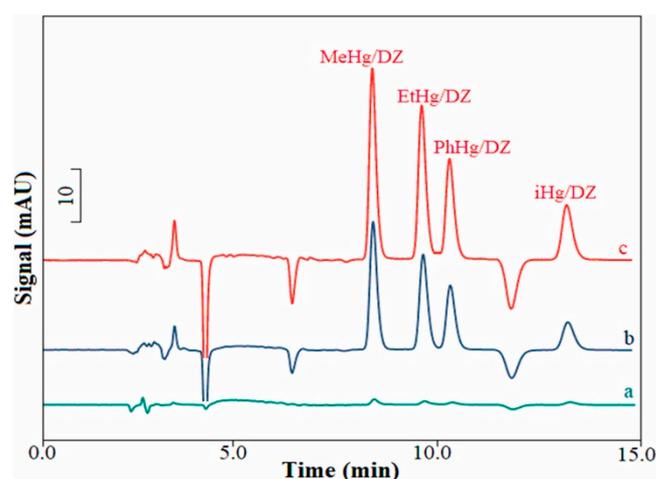


Figure 4. Chromatograms of studied mercury species before treatment with in-tube SPME (a), after treatment with in-tube SPME-HPLC-DAD (b) and treatment with magnetic-field-reinforced in-tube SPME-HPLC-DAD (c) from reference [26].

4.3. Particle-Based Stationary Phases

Silica particle-based stationary phases with different alkyl moieties, geometry, and internal diameter have been evaluated to determine cannabinoids and metabolites in human urine samples [24]. Although conventional simple reverse-phase silica particle-based sorbents exhibit good extraction efficiency, in-tube SPME capillary columns present short lifetimes due to adsorption of macromolecules from the matrix. More innovative stationary phases have been developing combining molecularly imprinted polymer (MIP) particles with a layer of bovine serum albumin (BSA) to obtain a restricted access molecularly imprinted polymer (RAMIPs) material [13] and octadecyl groups with RAM (C8-RAM) stationary phase [20].

4.4. Fiber-Coated Stationary Phases (Fiber In-Tube SPME)

Fiber-in-tube SPME combines the fiber SPME and in-tube SPME features, providing advantages such as higher extraction efficiency, higher surface area/sorbent ratio, and reduced pressure drop. In this technique, a short capillary column is longitudinally packed with fine fibers (fine solvent-resistant synthetic polymer filaments), as extraction medium [5].

Stainless steel wires were coated with chitosan as a natural polymer and polyvinyl alcohol as a co-polymer by the electrospinning method. This polar sorbent exhibited hydrogen bonding, dipole interactions, and ion exchange properties during the pre-concentration step. By using a chemically coated nitinol with crosslinked zwitterionic polymeric ionic liquid stationary phase, Souza et al. [25] exploited the ability of this sorbent to establish ion-exchange and dispersive interactions for successfully pre-concentrating amyloid β -peptides. A PEEK tube was packed with fibers based on polyaniline/TiO₂ nanorods/carbon fibers for fiber-in-tube SPME. The combination of polyaniline and TiO₂ into one composite provided a great number of adsorptive sites on the TiO₂ nanorods and multiple interactions between polyaniline (PANI) and phthalate esters.

4.5. Stationary Phases for In-Tube SPME Directly Coupled to MS System

Methods developed considering the in-tube SPME directly coupled to MS system for the analysis of analytes in biological samples exhibit short analysis times but require selective sorbents. Therefore, the development of coatings for these methods is a challenging task. These sorbents must be able to effectively concentrate the analytes while excluding interferent compounds. Once chromatographic separation is not present in these methods, the requirements mentioned above are important to eliminate matrix effects and obtain high sensitivity and reproducibility in quantitative methods.

Hybrid stationary phases, which combine the bifunctionality of different materials, can be applied to improve the extraction efficiency of the sample preparation technique. Santos et al. [13] modified the surface of molecularly imprinted polymer (MIP) particles with a layer of bovine serum albumin (BSA) to obtain a RAMIP material. This stationary phase combined the characteristics of restricted access material (exclusion of endogenous components by the hydrophilic external layer, non-adsorptive network) and MIP to pre-concentrate tricyclic antidepressants in human plasma samples. Queiroz's group [20] reported a C8-RAM stationary phase where the target analytes were pre-concentrated by hydrophobic octyl moieties (inside the pores of the silica particle), while the hydrophilic diol external surface excluded the macromolecule. This extraction phase had an exclusion ability of approximately 95%.

An organic monolithic column was prepared by using a co-polymer of N-isopropylacrylamide (NIPAAm), divinylbenzene (DVB), and N, N'-methylenediacrylamide (MBAA). MBAA and DVB acted as mixed crosslinking agents, increasing the mechanical strength and hydrophobicity of the monolithic column. PEG20000 and methanol were employed as a mixed porogenic solvent. This column facilitated extraction and enrichment of tobacco alkaloids thanks to the hydrogen-bonding (with the $-NH-$ and $-C=O$ moieties of NIPAAm and MBAA), $\pi-\pi$ (with DVB), and hydrophobic (with the organic polymer backbone) interactions of the analytes [36]. Compared with the direct MS mode, the in-tube SPME pre-concentration increased the concentration of tobacco alkaloids by almost 400-fold, improving the signal-to-noise ratio by about two- to seven-fold. Wu et al. [35] used 3-acrylamidophenylboronic acid (APBA) instead of NIPAAm to prepare a boronated monolithic affinity column. This organic polymer backbone promoted $\pi-\pi$ interaction, B-N coordination, hydrogen bonding, and hydrophobic interaction with the target benzimidazoles (analytes).

As for hybrid silica monoliths, a dual-ligand sol-gel organic-silica hybrid monolithic capillary column was prepared [12]. To incorporate the bifunctional groups (cyano and amino) into the structure of the monolith, the authors used 3-aminopropyl triethoxysilane and 3-cyanopropyltriethoxysilane as alkoxide precursors together with tetraethylorthosilicate. The selectivity of the monolithic capillary (amino and cyano groups) and the MS/MS system allowed in-tube SPME to be directly coupled to the MS/MS system without the need for chromatographic separation. It also allowed amino acids and neurotransmitters to be determined in plasma samples from schizophrenic patients.

5. Applications

Table 2 illustrates the applications of in-tube SPME coupled to chromatographic separation for the analysis of food, biological and environmental samples. Table 3 lists the applications of in-tube SPME directly coupled to MS instruments.

Table 2. In-tube SPME-LC applications.

Matrix	Analytes	Sample Pre-Treatment	In-Tube SPME Procedure	Stationary Phase	Separation/Detection System	Linear Range (ng mL ⁻¹) Reproducibility (RSD, %) Durability (Extractions)	Ref.
Biological							
Hair (5 mg)	Tobacco-Specific Nitrosamines	Washing with 0.1% sodium dodecyl sulfate, water, and methanol. Stored in amber glass desiccator at room temperature until use. Addition of 0.1 mL of water and heated and extracted at 80 °C for 30 min. The extract was cooled to room temperature and filtered through a 45 µm hydrophilic PTFE syringe filter. Dilution of 1.0 mL of the filtrate with 0.5 mL of water.	Twenty-five repeated draw/eject cycles of 40 µL sample at a flow rate of 0.2 mL min ⁻¹	Supel-Q PLOT capillary (60 cm × 0.32 mm i.d.)	LC-MS/MS	0.0005–0.1 2.1–9.2% NM	Ishizaki and Kataoka, 2021 [22]
Hair (0.2 mg)	Nicotine and Cotinine	Washing with 3 mL of DCM by sonication to remove external nicotine and cotinine from the hair surface and stored in amber glass desiccator at room temperature until use. Addition of 1.0 mL of distilled water and extraction at 80 °C for 30 min. Dilution of 1.0 mL of the filtrate with 1.0 mL of distilled water.	Twenty repeated draw/eject cycles of 40 µL sample at a flow rate of 0.2 mL min ⁻¹	Carboxen 1006 PLOT capillary column (60 cm × 0.32 mm i.d., 17 µm film thickness)	LC-MS/MS	0.005–1 1.62–5.97% NM	Kataoka et al., 2021 [21]
Saliva (50 µL)	Sulfated Steroid Metabolites	Centrifugation at 2500 × g for 1 min followed by ultrafiltration using an Amicon Ultra 0.5 mL 3K regenerated cellulose 3000 molecular weight cutoff centrifugal filter device at 15,000 rpm for 20 min. Addition of 0.05 mL of 0.2 M potassium hydrogen phthalate-HCl buffer (pH 3). The total volume was made up to 1.0 mL of distilled water.	Twenty-five repeated draw/eject cycles of 40 µL sample at a flow rate of 0.2 mL min ⁻¹	Supel-Q PLOT capillary (60 cm × 0.32 mm i.d.)	LC-MS/MS	0.01–10 2.1–11.1% NM	Kataoka and Nakayama, 2022 [23]
Urine (250 µL)	Cannabinoids and metabolites	Dilution with ACN (1:2, v/v) and centrifugation	Loading flow rate: 0.25 mL min ⁻¹ Loading time: 2.5 min	LichroPrep RP-18 (508 µm i.d. ×50 mm)	LC-MS/MS	10–1000 NM 150	Sartore et al., 2022 [24]

Table 2. Cont.

Matrix	Analytes	Sample Pre-Treatment	In-Tube SPME Procedure	Stationary Phase	Separation/Detection System	Linear Range (ng mL ⁻¹) Reproducibility (RSD, %) Durability (Extractions)	Ref.
Plasma (300 µL) CSF (300 µL)	Amyloid beta peptides	Incubation process for 1 h at 37 °C with gentle agitation followed by dilution (1:1, v/v) with 1% formic acid aqueous solution	Wash and conditioning: 600 µL of methanol + 600 µL of formic acid aqueous solution 1% Sampling: 6 draw/eject cycles of sample. Clean up: 500 µL of: water/ACN (95:5, v/v) containing 1% of formic acid. Desorption: 100 µL of water/ACN (20:80, v/v) +1% of ammonium hydroxide. Sampling rate: 1.0 mL min ⁻¹	Crosslinked zwitterionic polymeric ionic liquid-functionalized nitinol wires (0.120 × 200 mm)	UHPLC-MS/MS	0.3–15 2.1–7.3% 90	Souza et al., 2022 [25]
Ambiental							
Sea, tap, river and lake waters (3.0 mL)	Mercury species (coordinated with dithizone)	Water samples were filtered, pH adjusted at 4.0. Dithizone was added to sample to form Hg complexes. EDTA was added to avoid the interference of co-existing ions.	Sampling (3.0 mL) at 0.10 mL min ⁻¹ , 20 Gs magnetic field with the identical direction of the flow of sample solution; Desorption: 80 µL MeOH at 40 µL min ⁻¹ , 30 Gs reverse magnetic field.	Task-specific monolith doped with Fe ₃ O ₄ (20 cm × 320 µm i.d.)	MFR/IT-SPME-HPLC-DAD	0.05–300 0.8–6.7% 60	Song et al., 2021 [26]
Sea, tap, river and lake waters (5 mL) and soil samples (5.0 g)	Cr(III) and Cr(VI) species (coordinate with APD)	Water samples were filtered, pH adjusted at 5.5. APD was added to sample to form Cr(III) and Cr(VI) complexes. Soil samples were crushed and dried to a constant weight at 80 °C. The samples were fully ground and passed through a 100-mesh sieve. The sample was extracted with ultrapure water. The pH of supernatant was adjusted (5.5) and filtered (0.22 µm).	Sampling (5.0 mL) at 0.14 mL min ⁻¹ , 30 Gs magnetic field with the identical direction of the flow of sample solution; Desorption: 60 µL ACN at 0.01 mL min ⁻¹ , 40 Gs reverse magnetic field.	Porous monolith doped with magnetic nanoparticles (20 cm × 320 µm i.d.)	MFR/IT-SPME-HPLC-DAD	0.010–100 1.4–11% 50	Pang et al., 2021 [30]
Plastic bottled water (18.0 mL)	Phthalate esters	NM	Sampling (18.0 mL) at 0.75 mL min ⁻¹ for enrichment. Desorption with 100% ACN at 0.2 mL min ⁻¹ for 5 min	POSS-co-BMA monolith (10 mm × 3 mm i.d.)	HPLC-UV	0.1–60 2.0–9.4% 60	Wei et al., 2022 [31]

Table 2. Cont.

Matrix	Analytes	Sample Pre-Treatment	In-Tube SPME Procedure	Stationary Phase	Separation/Detection System	Linear Range (ng mL ⁻¹) Reproducibility (RSD, %) Durability (Extractions)	Ref.
Bottled water and water sample from Disposable lunch box (50 mL)	Phthalate esters	Samples were filtered (0.45 µm).	Sampling (50 mL) at 1.25 mL min ⁻¹ using 1.0% (v/v) MeOH containing 2.0% (w/v) of NaCl. Desorption with methanol-water (75:15, v/v) at 1.0 mL min ⁻¹ for 0.6 min.	Polyaniline/TiO ₂ nanorods functionalized carbon fibers (35 cm × 0.75 mm i.d.)	HPLC-DAD	0.03–30 3.5–13.9% 40	Sun et al., 2021 [27]
Food							
Fish feed (60 mL of filtrate)	Polar estrogens and non-polar PAHs	Fish feed was soaked in distilled water, and the solution was then filtered through a 0.45-µm membrane.	Injection rate: 2 mL min ⁻¹ . Desorption time: 2 min at a flow rate of 1 mL min ⁻¹	Brass wires modified with 2-naphthalenethiol (30 cm wires packed inside a stainless-steel tube of 0.75 i.d.)	HPLC-DAD	0.5–10.0 1.9–6.5% NM	Zhang et al., 2022 [28]
Milk (10 mL)	Bisphenols	A total of 10.0 mL milk sample was precipitated with 10.0 mL acetonitrile added (twice). The supernatant was concentrated to 1.0 mL and then redissolved with 100.0 mL ultrapure water, which was filtered (Nylon60 0.22 µm syringe).	Sampling at a flow rate of 1.0 mL min ⁻¹ . Desorption: 0.3% phosphoric acid–water:ACN (from 30.0–55.0% of ACN) for 2 min.	Poly (POSS-co-AM-co-EDMA) monolith (10 mm × 3 mm i.d.)	HPLC-UV	0.2–200.0 <2.7% 60	Liu et al., 2022 [32]
Beer and red wine (10 mL)	Ochratoxin A	Samples were degassed; pH adjusted to 8.50, filtration (0.22-µm filter membrane), and diluted with binding buffer solution at a ratio of 1:1 (v/v).	Sampling (20 µL) at flow rate of 0.1 mL min ⁻¹ ; Cleanup with binding buffer solution; Desorption: 20 µL ACN: Tris-HCl/EDTA buffer (40:60, v/v). Eluent was collected in a sample loop and injected to be measured with HPLC-FLD system.	Aptamer-based affinity monolith	HPLC-FLD	0.05–50.00 2.4–6.0% 30 days	Zhao et al., 2021 [33]
Beer (10 mL)	Ochratoxin A	Samples were degassed; pH adjusted to 8.50, filtration (0.22 µm filter membrane)	Sampling (20 µL) at flow rate of 0.1 mL min ⁻¹ ; Cleanup with binding buffer solution (40 µL); Desorption: 20 µL ACN: Tris-HCl/EDTA buffer (30:70, v/v).	Aptamer-based affinity monolith	HPLC-FLD	0.05–50.00 <2.8% 30 days	Zhao et al., 2021 [34]

Table 2. Cont.

Matrix	Analytes	Sample Pre-Treatment	In-Tube SPME Procedure	Stationary Phase	Separation/Detection System	Linear Range (ng mL ⁻¹) Reproducibility (RSD, %) Durability (Extractions)	Ref.
Pomegranate, red grape, and sour cherry juice (20 mL)	Acidic red dyes (Amaranth, Ponceau 4R, Allura red, Carmoisine, and Erythrosine)	Samples filtered (0.45 µm) and diluted (30 times for the pomegranate and red grape juices and 10 times for the sour cherry juice).	Sampling (20 mL) at 500 µg L ⁻¹ in a circulating path. Desorption (offline): 358.7 µL of ethanol (96%) with NaNO ₃ . Desorption solvent was dried and reconstituted in 50 µL.	Stainless steel wires coated with chitosan and polyvinyl alcohol	HPLC-UV	1.0–750.0 2.5–12.7% 85	Nasrollahi et al., 2022 [29]

AM: acrylamide; APD: ammonium pyrrolidinedithiocarbamate; BMA: N-butyl methacrylate; EDMA: ethylene dimethacrylate; FLD: fluorescence detector; CapLC-DAD: capillary liquid chromatography with diode array detection; DCM: dichloromethane; DES: deep eutectic solvent; LDH: layered double hydroxide; MIP: molecularly imprinted polymer; NPs: nanoparticles; PBS: phosphate buffer solution; PPy: polypyrrole; PTh: polythiophene; PDMS: Polydimethylsiloxane; CapLC-FD: capillary liquid chromatography with fluorimetric detection; Fmoc: fluorogenic reagent; GO: graphene oxide; PD: polydopamine; MWCNTs: multiwall carbon nanotubes; LC-PCD-MS: liquid chromatography–post-column derivatization–mass spectrometry; 3-OMD: 3-o-methylidopa; CME: capillary microextraction; MALDI-TOF-MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MFR: magnetic field-reinforced; UHPLC/Q-Orbitrap MS: Ultra-high-performance liquid chromatography and quadrupole high-resolution Orbitrap mass spectrometry; DTT: dithiothreitol; IAA: iodoacetamide; FA: formic acid; ACN: acetonitrile; VWD: variable wavelength detector; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; PEDOT: poly (3,4-ethylenedioxythiophene); PPy: polypyrrole; In: indole; Th: thiophene; DBS: dodecyl benzene sulfonate; CEC: capillary electrochromatography; MDMA: 3,4-methylenedioxyamphetamine; NM: not mentioned; POSS: polyhedral oligomeric silsesquioxane.

Table 3. In-tube SPME directly coupled to MS systems and its applications.

Matrix	Analytes	Sample Pre-Treatment	In-Tube SPME Procedure	Stationary Phase	Separation/Detection System	Linear range (ng mL ⁻¹) Reproducibility (RSD, %) Durability (Extractions)	Ref.
Human plasma (NM)	Tricyclic antidepressants	Dilution 1:4 with water, followed by filtration	Loading and reconditioning: water Desorption: 0.01% acetic acid aqueous solution: ACN (30:70, v/v)	RAMIP-BSA (10 cm × 4.6 mm i.d.)	MS/MS	15.0–500.0 4.1–13.6% NM	Santos et al., 2017 [13]
Human plasma (200 µL)	Amino acids and neurotransmitters	Protein precipitation (ACN, 400 µL), centrifugation (30 min, 9000 rpm), supernatant dried and reconstituted (50 µL ACN + 0.1% formic acid v/v).	Sample volume: 10 µL. Desorption: water at 0.1 mL min ⁻¹	Cyano-Aminopropyl (10 cm × 530 µm i.d.)	MS/MS	6–300 <15% 40	Miranda et al., 2019 [12]

Table 3. Cont.

Matrix	Analytes	Sample Pre-Treatment	In-Tube SPME Procedure	Stationary Phase	Separation/Detection System	Linear range (ng mL ⁻¹) Reproducibility (RSD, %) Durability (Extractions)	Ref.
Cigarette tobacco (2.0 g)	Tobacco alkaloids	The cigarette tobacco filing of cigarettes was placed into a centrifuge tube containing 2 mL of diethyl ether and 5 wt% aqueous NaOH (10 mL). The tube was shaken and vortexed for 15 min at 4000 rpm, and the layers were separated. The ethyl layer was collected and dried under stream of N ₂ to obtain a residue that was reconstituted in 1% of ACN solution (<i>v/v</i>) and filtered through a 0.45 μm nylon filter prior to analysis.	Pre-conditioning: 1% of ACN in water for 5 min Sampling solution: 300 μL at a flow rate of 30 μL min ⁻¹ for 10 min. Desorption: at a flow rate of 10 μL min ⁻¹ for 10 min	Poly (NIPAAm-co-DVB-co-MBAA) (15 cm)	MS	1–100 ** 0.34–1.22% 40	Wu et al., 2019 [36]
Rat brain (1.0 mg)	AEA and 2-AG	Homogenization with 4.5 μL of 0.1 mol L ⁻¹ formic acid solution. After homogenization, addition of 150 μL of ACN and 100 μL of 5.0 mol L ⁻¹ ammonium formate, followed by agitation at 3000 rpm for 30 s in a vortex mixer and centrifugation at 9000 rpm and 5 °C for 15 min for protein precipitation. The upper organic phase was collected (50 μL), dried, and reconstituted with 50 μL of water/ACN (30:70, <i>v/v</i>)	Sampling: water/ACN (80:20, <i>v/v</i>) at 0,2 mL min ⁻¹ . Desorption: 0.5% formic acid aqueous solution:ACN (30:70, <i>v/v</i>) at 0,2 mL min ⁻¹ . Washing: ACN at 0,2 mL min ⁻¹	Stainless steel tube packed with RAM phase (diol external surface and octyl inner surface) (25 mm × 4 mm × 25 μm)	MS/MS	6.0–30.0 1.9–15.7% NM	Oliveira et al., 2021 [20]
Animal products (2.0 g)	Benzimidazole	Homogenization with 5 mL of ACN + 1% formic acid followed by agitation for 3 min. Whereafter, 1.0 g of NaCl and 2.0 g of MgSO ₄ was added, followed by agitation for 2 min and centrifugation at 3000 rpm for 5 min. The supernatant was collected and dried under N ₂ stream at room temperature. Then, the dried residues were reconstituted with a solution containing water:ACN (99:1, <i>v/v</i>)	Equilibration: 2% of ACN in water for 5 min;	Poly (AAPBA-co-DVB-co-MBAA) (20 cm)	MS	514–1000 * <2.45% 60	Wu et al., 2021 [35]

AAPBA: 3-Acrylamidophenylboronic acid; DVB: divinylbenzene; MBAA: N,N'-methylenebisacrylamide; NIPAAm: N-iso-propylacrylamide; PA: polyacrylate; * μg mL⁻¹, ** ng g⁻¹.

6. Conclusions and Future Perspective

In-tube SPME is perhaps one of the most popular and spreading techniques that might be considered a multidimensional separation LC system. Due to the reduced amount of extraction phase required to prepare the miniaturized extraction column, in-tube SPME is frequently the technique of choice for testing new and innovative column formats. The in-tube SPME-LC techniques associate the advantages of online systems with the benefits of miniaturized systems. The coupling automation system not only improves accuracy and precision but also enhances high-throughput performance. Additionally, in-tube SPME can be directly coupled to newly developed methods in the mass spectrometry field.

The development of an in-tube SPME method initially requires selection of an appropriate capillary device and its extraction phase, which will depend on the sample and the analyte of interest. Capillary devices used for in-tube SPME can be categorized as surface-coated, particle-packed, fiber-coated, and monolithic. In addition, draw/eject cycles or the flow-through mode must be selected as the operating system, and the in-tube SPME parameters (capillary geometry, sample volume and pH, and sorption and desorption conditions) must be optimized.

Direct coupling of in-tube SPME to MS/MS systems, without chromatographic separation, favors high-throughput analysis. Direct in-tube SPME-MS/MS is possible thanks to the selectivity of capillary column coatings and MS/MS systems (SRM mode).

This review has discussed the use of different in-tube SPME methodologies coupled to chromatographic techniques or directly coupled to mass spectrometry systems for analysis in different fields of application (food, biological, and environmental fields).

Future challenges related to the in-tube SPME technique are associated with the development of new extraction phases with improved selectivity and extraction efficiency, as well as multifunctionality. Other future challenges refer to improving direct coupling of fiber-in-tube SPME capillary columns to MS/MS or NanoESI-MS systems to conduct high-throughput analysis.

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