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Immunosorbents in microextraction

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Abstract

Trace analysis of target compounds from complex samples requires often a step of purification and of preconcentration before the chromatographic separation. Immunoaffinity sorbents functionalized with antibodies specific to the molecule(s) of interest appear as powerful tools for their selective extraction to obtain more reliable and sensitive quantitative analysis. Indeed, the high specificity and affinity of the antigen-antibody interactions allow an efficient and selective clean-up with high enrichment factors.

Considering the cost of antibodies, the miniaturization of these sorbents presents a large interest as it combines the advantages of the miniaturization such as the reduction of solvent consumption and the application of the devices to reduced sample volumes while keeping high enrichment factors with the high selectivity provided by the antibodies during the extraction process. The objective of this review is to present the developments proposed these last years in the field of microextraction methods involving antibodies.

Key-words: immunoaffinity; miniaturization; extraction; solid-phase microextraction; in-tube solid-phase extraction; dispersive extraction; monolith

Abbreviations

Ab: antibody ; AFP: α-fetoprotein; APMH: aminopropylmethacrylamide hydrochloride; APTES: (3-aminopropyl)-triethoxysilane; [BMIM]BF4: 1-butyl-3-methyl-imidazolium tetrafluoroborate; CE: capillary electrophoresis; COC: cyclic olefin copolymer; GMA: glycidyl methacrylate; GMM: glycerylmethacrylate; CSF: cerebrospinal fluids; DGS: diglycerylsilane; dSPE: dispersive solid-phase extraction; EDA: ethylenediamide; EDMA: ethylene dimethacrylate; EF : enrichment factor; ET: epitestosterone; Fabs: antibodies fragments; Fluo: fluorescence; GC: gas chromatography; GMA: glycidylmethacrylate; GMM: glycerylmethacrylate; GNP: gold nanoparticle; GTD: glutaraldehyde; HEMA: 2-hydroxyethyl methacrylate; IgG: immunoglobuline G; IA: immunoaffinity; IS: immunosorbent; IT: in-tube; IMS: ion mobility spectrometry; LIF: laser induced fluorescence; LC: liquid chromatography; LP: lipoprotein; LR: linear range; mAbs: monoclonal antibodies; MIP: molecularly imprinted polymer; MS: mass spectrometry; NHS: N-hydroxysuccinimide; NP: nanoparticle; OS: oligosorbent; pAbs: polyclonal antibodies; PBP2a: penicillin binding protein 2a; PBS: phosphate buffer saline; PEG: polyethyleneglycol; PEGDA: polyethyleneglycoldiacrylate;

PETIA: particle-enhanced turbidimetric assay; PMMA: polymethylmethacrylate; RE: recovery of extraction; RSD: relative standard deviation; SPE: solid-phase extraction; SPME: solid-phase microextraction; SSMCC: sulfosuccinimidyl-4-(N-maleimido-methyl) cyclohexane-1 – carboxylate; TEOS: tetraethyl orthosilicate; UHPLC: ultra-high performance LC; VDA: 2-vinyl-4, 4-dimethylazlactone

1- Introduction

These recent years, the evolution of the instrumentation in terms of separation and detection allowed a real improvement of sensitivity and analysis time. However, the analysis of target analytes in complex samples requires powerful specific analytical tools to provide reliable results. The specificity of an analytical method can be obtained by efficient separation methods but also by their hyphenation with a specific detection such as mass spectrometry (MS). Indeed, the specificity of the MS signal is particularly helpful to confirm the presence of a compound in a complex sample, particularly when the peak capacity of the separation is not sufficient, as it is often the case for complex samples. However, matrix components may interfere during the ionization process in the MS source thus affecting the reliability of the target analytes quantification. The removal of interfering compounds during sample pretreatment can be ensured by introducing a sample-clean-up step using sorbents providing a selective retention mechanism. For example, restricted access media, large particles sorbents or monoliths were proposed for the removal of macromolecules [1], and mixed-mode sorbents for the extraction of acidic or basic compounds from real samples such as biological fluids or environmental waters [2]. To go further in term of selectivity, sorbents providing a retention based on a molecular recognition mechanism were developed, such as molecularly imprinted polymers (MIPs) [3,4], aptamers-based sorbents, namely oligosorbents (OSs) [5] and antibodies-based sorbents, namely immunosorbents (ISs) [6,7].

If MIPs and especially OSs are quite new sorbents, immunosorbents (ISs) were developed since the pioneer work of Farjam [8] in the nineties and numerous ISs are commercially available mainly for the extraction of pesticides, toxins or drugs from foodstuff [6]. Indeed, their use for the trace analysis of mycotoxins in foodstuff is today widely implemented in control laboratory [7]. In most of the cases, antibodies (Abs) are covalently grafted onto activated Sepharose gel or activated silica to be packed between two frits into 1-6 mL disposable cartridges that can contain tens or even hundreds of milligrams of IS to be used as conventional solid-phase extraction (SPE) sorbents. Indeed, an immunoextraction sequence consists in the percolation of the sample through the IS cartridge after a conditioning step, washing of the IS to remove the residual interfering compounds, and elution of the target analyte(s) by disrupting the antigen-antibody interactions.

The easy automation of the on-line coupling of SPE with liquid chromatography (LC) makes this technique powerful by replacing conventional SPE sorbent with the IS. For this, tens milligrams of IS particles are packed in a small size-precolumn coupled on-line with the LC system [7]. This decrease in the size of the precolumn compared to that of the cartridges also allows reducing the cost of the ISs by decreasing the amount of immobilized antibodies while maintaining method performance.

In recent years, many researches were devoted to the development of miniaturized extraction devices with the objectives to limit the reagents consumption, to adapt the extraction devices and to reduced sample volume [9,10]. In this field, the use of a fiber as extraction device in

solid-phase microextraction (SPME) has proven its potential since more than 30 years [11,12]. Since this development, other formats have been proposed such as dispersive SPE based on micro- and nano-sorbents [13] including or not a magnetic core [14], in-tube SPME involving conventional sorbents such as polymers but also new types of sorbent or functionalization such as carbon nanotubes, metal organic frameworks, ionic liquids, surfactants or titanium dioxide [15]. The reduction of the size of the devices is particularly interesting when expensive reagents such as antibodies have to be used. This explains some recent developments related to the introduction of antibodies in such microextraction devices. In addition, in order to integrate those miniaturized immunoextraction devices within miniaturized analytical methods, such as capillary electrophoresis (CE) and nanoLC, new approaches were proposed that mainly consist in the synthesis of monoliths further grafted with antibodies. This also allows the integration of ISs into chips. The aim of this review is to present the different approaches reported for the development of miniaturized ISs (see Figure 1) and their potential for the extraction of target analytes from complex samples.

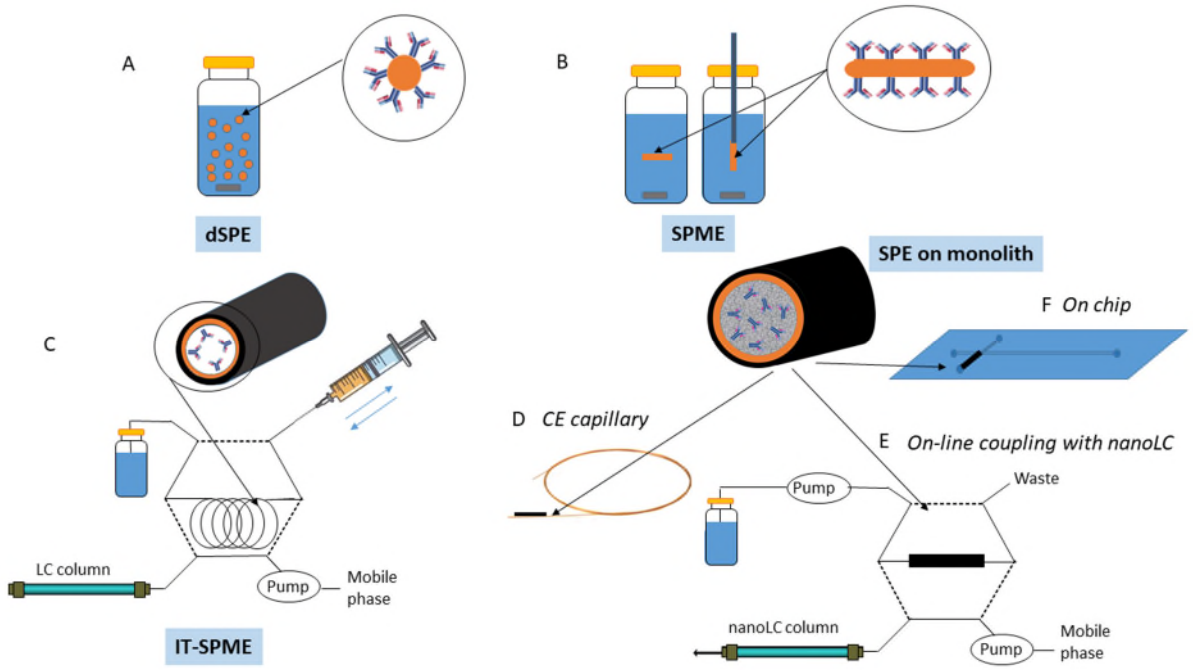

Figure 1: Different approaches reported in literature for the development of miniaturized ISs: (A) dispersive solid-phase extraction (dSPE); (B) solid-phase micro-extraction (SPME); (C) In-tube solid-phase micro-extraction (IT-SPME), monolith (D) integrated at the end of a capillary for its coupling with CE, (E) in a capillary coupled to nanoLC or (F) in the channel of a chip.

2- Dispersive solid phase extraction

In dispersive SPE (dSPE), the extraction is carried out by introducing the sorbent directly in the sample instead of percolating the sample through the sorbent packed in a cartridge. The use of nanoparticles (in the order of tens of nanometers instead of tens of micrometers) presents some advantages, such as the reduction of the amount of antibodies required, their application to reduced volume of samples and above all a large specific surface area and then high adsorption capacity thus favoring high enrichment factors [16]. Particles are first dispersed in the sample to interact with the analyte (Figure 1A). After a sufficient extraction

time under stirring, the particles are recovered mainly by centrifugation [17] or by a magnetic field (when using particles with a magnetic cores) [18–20] to be further introduced into a suitable desorption solvent. In the reported works, the extraction procedure takes from 20 min to overnight and some of the procedures include a washing step before the elution to ensure an optimal selectivity [17,18]. Therefore, if this approach allows to reduce sample volumes and antibodies consumption, extraction methods remain quite time consuming.

Different types of particles with immobilized antibodies have been already prepared to develop dSPE procedure. These studies are summarized in Table 1. The extraction particles were mainly prepared by the covalent immobilization of antibodies. When preparing such nanosorbents, as mentioned by Haller *et al* [17], antibodies surface coverage is strongly dependent on the size of the NPs, the immobilization strategy, reaction conditions, as well as the concentration of Abs in the reaction mixture. As a result, this group recently proposed a careful optimization of the immobilization conditions by exploring distinct random and oriented immobilization of Abs onto gold nanoparticles (GNPs), such as (i) direct adsorptive attachment on the NP surface, (ii) covalent bonding by amide coupling of Abs to carboxy-terminated-pegylated NPs, (iii) oriented immobilization via oxidized carbohydrate moiety of the Ab on hydrazide-derivatized NPs and (iv) cysteine-tagged protein A-bonded NPs [17]. It was observed that an oriented immobilization via Protein A was the most advantageous strategy in terms of colloidal stability, saturation capacity, and extraction recovery. This GNP-Ab conjugate was chemically very stable unlike the IS NPs resulting from the adsorption strategy. Moreover, it was also mentioned that no bleeding was observed for the conjugates with pegylated spacers which could have interfered with the mass spectrometry (MS) detection. To limit the saturation of the sorbent caused by steric hindrance due to the large antibody size, Qiu *et al.* proposed to immobilize half-antibodies, obtained by splitting in two the Abs using 2-aminoethanethiol that disrupts disulfide bonds between the two heavy chains [18]. The use of half antibodies with reduced SH moiety also allows an easy grafting of the antibodies to gold NPs via Au-S bonds. The grafting yield was then estimated to 1 mg of half Abs for 10 mg of NPs.

Immuno-dSPE was applied to sample volumes ranging from 10 µl to 20 ml (i. e. 3 orders of magnitude), depending on the expected enrichment factor, with resulting extraction times between 15 min to overnight and recoveries higher than 80% in all cases. The immunoextraction was associated either to a separation method or directly to a detection mode such as fluorescence (Fluo) or ion mobility spectrometry (IMS) since the antibody selectivity allows the removal of interfering compounds, thus rendering the use of a separation method not necessary. This removal of interfering compounds is illustrated by the comparison of chromatograms reported in Figure 2 and obtained for a urine sample analyzed without (Figure 2a) or with the use of half antibodies grafted on magnetic NPs (MNPs) (Figure 2b) applied to the extraction of epitestosterone (ET), the absence of Abs on MNPs preventing the extraction of ET (Figure 2c) [18]. The contribution of non specific interactions mainly caused by the nature of the sorbent was not studied in detail in these works related to dSPE, but one study observed a low loading capacity of 10% of a structural analog compared to the binding capacity of the targeted lipoprotein, which was considered by the authors as a measure of the non specific events [17]. It is worthwhile to notice that the reusability of the IS particles was studied by some authors that reported the possibility to used them 5 [20] to 15 times [19] without observing any loss of performance.

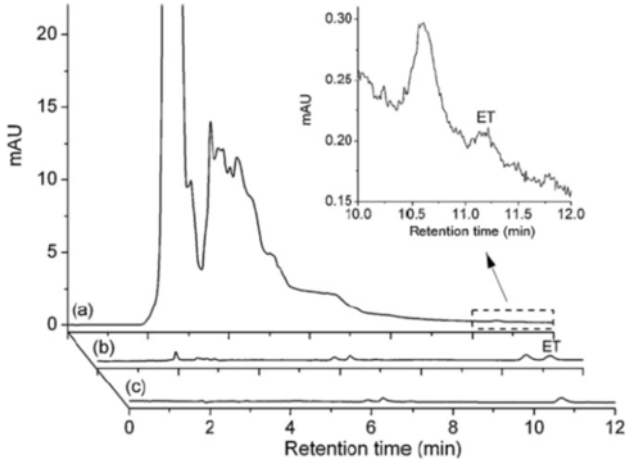

Figure 2: HPLC analysis of epitestosterone (ET) in a urine sample spiked with ET at 100 ng/ml (a) without immunoextraction-based dSPE, (b) with immunoextraction-based dSPE using Fe_3O_4@Au MNPs grafted with anti-ET Abs, and (c) dSPE using bare Fe_3O_4@Au MNPs. With the permission from Elsevier [18].

Table 1: Application of immunoaffinity-based dispersive SPE.

Target	**Sample**	**Extraction sorbent**	**Antibody grafting**	**Method**	**Analytical method**	**Method performance**	**REF**
Epitestosterone	Urine (20 ml)	Fe_3O_4-Au NPs (50 nm)	covalent grafting through Au-S bonds of half mAb (obtained by reduction of native disulfide bonds between the two heavy chains)	Incubation, washing (3x, water), desorption (3x, MeOH), 1 h for the whole procedure	HPLC-UV	RE: 92-103% (urine) EF: 100 LR: 20-200 ng/mL in PBS	[18]
Soy proteins	Soy milk (500 µl)	Fe_3O_4-Au NPs (12 nm) - 23 mg	grafting of oxidized pAbs on amino-functionalized Au-NPs (cysteamine)	Incubation (15 min, stirring), desorption (5 min, 10% MeOH)	Fluo	RE: 80-107.3% LR: 1-15 mg/L	[19]
Low density lipoprotein	Plasma (10µl - diluted 1/500)	Au-NPs (28 nm) - 10 µl, $9.91x10^9$ NPs	non-covalent grafting; covalent bonding via amide coupling of mAbs to carboxy terminated-pegylated NPs; oriented immobilization via oxidized carbohydrate moiety of mAb and hydrazide derivatized NP; immobilization via cysteine-tagged proteine A-NPs	Overnight incubation, washing (3 x Tris-HCl, centrifugation), desorption (MeOH + ultrasonication and centrifugation)	LC-MS	RE: 80% (pure media)	[17]

3- Solid phase micro-extraction (SPME) and related techniques

SPME is a miniaturized method that consists in the extraction of compounds from a sample onto a fused silica fiber (100 µm diameter) usually coated by an organic polymer (7-100 µm thickness). Initially developed for the extraction of the volatile compounds from environmental samples before their analysis by gas chromatography (GC), the applications of SPME has been extended to biological fluids with an off-line coupling with liquid chromatography (LC) in most of the cases [21]. Even if the whole automation is not as easy as for the SPME-GC coupling, SPME still provides many advantages by integrating sampling, extraction, and concentration in one-step with an easy transfer of the trapped compounds in an elution solution. The combination of this technique with the high antibodies selectivity that limits the co-extraction of interfering compounds present in complex samples such as biological fluids can constitute an interesting approach to improve the sensitivity of the method.

As reported in Table 2, immunoaffinity SPME (IA-SPME) was developed for the selective extraction of a single target molecule, such as a small drug, *i.e.* theophylline [22], or protein, *i.e.* penicillin binding protein 2a (PBP2a) [23], or a group of structural analogs such as benzodiazepines [24,25], non-steroidal estrogens [26] or quinolones [27]. The extraction of a group of structural analogs takes advantage of the ability of an antibody to recognize the compounds having a strong structural analogy to the antigen used for its production. Since the SPME extraction is followed by the analysis of the extract with a separation method, the individual quantification of each compound is therefore possible.

The preparation of the extraction devices consisted either in immobilizing porous silica particles (5 µm) onto stainless steel devices, followed by the activation of the silica surface by an amino-organosilane ((3-aminopropyl)-triethoxysilane, APTES) and then the use of glutaraldehyde to covalently graft the antibodies by their amino-groups or by direct activation of silica-based material by the same reagents. The dimensions of the final sorbents, usually 1-5 mm diameter and 1.5-3 cm length, are larger than conventional SPME fibers (1cm length silica fiber - 110 µm diameter- coated, polymer - 7-100 µm thickness-) but still considered as miniaturized sorbent while considering the amount of immobilized antibodies. If most of the authors consider that they develop an immunoaffinity SPME method, one of them, that used the biggest device, described its method as immunoaffinity stir bar sorptive microextraction. Nevertheless, the dimensions of these different devices are not so different [27]. IA-SPME consists next in the direct immersion of the sorbent into the sample (Figure 1B) and different parameters can be optimized to extract as much as analytes as possible, such as the extraction time, the stirring speed, the nature and the volume of the desorption solution or the desorption time as for conventional SPME. The final conditions fixed in the different studies are described in Table 2.

For IA-SPME, the nature and amount of immobilized antibodies will also affect the extracted analyte amount. Indeed, it was shown that mAbs gave rise to higher extraction yield of oxazepam than pAbs (with or without a purification step of the pAbs on an oxazepam-based sorbent) and then to a larger linearity range (LR) [25]. However, the use of mAbs necessitates longer equilibrium time that has been explained by the higher affinity of the mAbs. Nevertheless, the amount of immobilized antibodies was not determined in this work [25]. In return, for purified pAbs, giving rise to higher binding capacity than non purified ones, binding

capacities from 0.8 [24] to 6.3 pmol [25] of antigen immobilized on 3.1 cm^2 of sorbent (i.e. 0.26 to 2 pmol/cm^2 thus corresponding to 59 to 150 ng of purified pAbs/cm^2) were reported while immobilizing directly the antibodies on the silica surface [24,25]. This binding capacity for the antigen was increased to 49.6 pmol/cm^2 while grafting mAbs onto silica particles previously immobilized on stainless still rods [26].

In most of the cases, the extraction and desorption times are about 30 and 20 min, respectively, as with conventional SPME sorbents. For the application of IA-SPME to the selective extraction of the PBP2a protein, the conventional desorption procedure using a solvent was replaced by the direct digestion of the protein by immersing the sorbent in a trypsin solution [23]. When they were reported, recoveries were between 13 to 67 % with associated RSD values lower than 14% showing an acceptable repeatability of the whole analytical procedure applied to trace analysis in complex samples. Concerning the reusability of the fiber, Yao *et al.* mentioned that it was evaluated once every 3 days for 45 days by column capacity determination showing a loss of the capacity by a factor 2 during this period [27]. This is in good agreement with the study of Wang *et al.* that mentioned that after 10 uses only 58% of the binding capacity remained [26]. They also demonstrated the repeatability of the preparation of the IA-SPME rods obtaining RSD values below 15% among 6 batches for each studied estrogen.

Table 2: Application of immunoaffinity SPME.

Target	Sample	Extraction sorbent	Antibody grafting	Method	Analytical method	Method performance	REF
Theophylline	Serum (diluted with PBS, 1/100, v/v)	silica fiber (1.8 mm, 2.3 cm), silanized with APTES	Covalent grafting of pAbs using GTD	Extraction (3 h), washing (x2)	Liquid scintillation	LR: 10-50 ng/ml	[22]
Benzodiazepines (3)	Urine	borosilicate glass rods (4 mm, 2.5 cm)silanized with APTES	Covalent grafting of pAbs, purified pAbs or mAbs using GTD	Extraction (30 min), desorption (500 µl, MeOH/water)	LC-MS/MS	LR: 0.02-0.5 ng/ml (purified pAbs)	[24,25]
PBP2a (penicillin binding protein 2a)	Standard solution	Stainless steel wires (0.061 in., 1.5 cm) coated with 5 µm-porous silica particles silanized with APTES	Covalent grafting of mAbs using GTD	Overnight extraction, several washing steps, trypsic digestion of PBP2a	LC-MS/MS	LOQ: 10 ng/ml	[23]

Non-steroidal estrogens (3)	Environmental waters (1 ml, dilution 1/2)	Stainless steel rods (2 x 18 mm) coated with 5 µm-porous silica particles further silanized with APTES	Covalent grafting of mAbs using GTD	Extraction (30 min), washing (5 s); desorption (1 ml, MeOH, 20 min)	UHPLC-MS/MS	RE:34.2-62.7%, RSD < 14%; LOQ: 0.5 ng/ml	[26]
Quinolones (11)	Bovine milk (centrifuged to remove fat)	Borosilicate glass bars (5 mm, 3 cm) silanized with APTES	Covalent grafting of mAbs using GTD	Extraction (30 min), washing, desorption (900 µl, MeOH/PBS 8/2, 20 min)	LC/Fluo	RE: 13.2-40% (milk), RSD < 12.5% LOQ: 0.2-0.3 ng/g (pure media)	[27]

4- In-tube solid-phase micro-extraction

Capillary microextraction, also called in-tube SPME (IT-SPME), shows several advantages compared to SPME such as an on-line coupling with separation methods (capillary electrophoresis (CE) and, more frequently, LC). Indeed, with this approach, the analytes are extracted and preconcentrated at the inner surface of a capillary before being desorbed and directly transferred to the separation device (Figure 1C) [15]. Applications of immunoaffinity IT-SPME are reported in Table 3. A method named “immunoaffinity capillary electrophoresis” was described by Phillips and Dickens in 1998 [28]. It consists in the covalent grafting of antibodies fragments (Fabs) at the inner surface of a 100 µm silica capillary . This coating was achieved on approximatively 6 cm, the remaining part of the 30 cm-capillary being used for the CE separation. This device was applied to the quantification of recombinant cytokines in human body fluids (urine, plasma, cerebrospinal fluids (CSF), and saliva). Cytokines were labeled directly in the biological fluids and a volume of 30 nl of the sample was next flushed through the capillary leading to the capture of the derivatized cytokines by the immobilized Fabs. A washing step was applied to remove the compounds that were non-specifically trapped such as the matrix interfering compounds and the free fluorophore used for the labelling. Finally, the cytokines were electrophoretically eluted with an acidic buffer.

Since this pioneering work, immunoaffinity IT-SPME was coupled on-line with LC-MS and LC-Fluo for the selective analysis of fluoxetine in serum [29] and of interferon α in plasma [30], respectively. For this, the inner surface of a silica capillary of 250 µm i.d. and 60-70 cm length was activated with APTES and grafted with antibodies. This capillary was placed at the loop position of a six-port switching valve and the immunoextraction was achieved by repeated aspirations (draw) and ejections of 50 or 150 µl samples through the capillary. After this, the capillary was eventually washed and the desorption was achieved by passing the LC mobile phase through the immunosorbent (by switching the valve) for the transfer of the analytes to the analytical column. The sample draw/ejection volume and the number of draw/ejection cycles were adapted to the capillary capacity (estimated to 0.92 pmol of target analyte, i.e.

fluoxetine) [29] and to the volume comprised between the injection needle and the capillary. Both parameters were optimized together with the flow-rate [29,30] and extraction times between 2.5 to 9 min were fixed.

As for other immunoextraction based methods, the maximal amount of analytes that can be extracted without overloading the immunoaffinity capillary strongly depends on the amount of immobilized antibodies. By measuring this maximal amount of antigen that can be retained on the capillary, i.e 0.92 pmol retained on 5.5 cm^2, a grafting density of 11 ng/cm^2 of active pAbs (i.e. about 0.08 $pmol/cm^2$) was estimated [29], which is a very low value. To improve this bonding density, the use of NP-coated capillary was proposed [31,32]. Indeed, polystyrene NPs functionalized with antibodies were immobilized onto the surface of a 250 µm i.d. x 60 cm silica capillary for the selective extraction of ß2-microglobin or cystatin C. The performances of this capillary were compared with those obtained by directly immobilizing the antibodies onto the inner wall of the capillary. If the reliability of both system was demonstrated with RSD values of recoveries lower than 5%, the use of NPs allowed to reach a bonding density of about 40 pmol/m antigen, increasing the sorbent capacity by a factor 5 [31]. The same group recently proposed to modify first the capillary surface by NPs further functionalized with antibodies that were immobilized with an orientated way (immobilization of the pAbs through the oxidized carbohydrate chain located on their Fc part). The orientation of the antibodies allowed to improve the capacity of the immunosorbent by a factor 3 compared to a random immobilization and the use of NPs by a factor 1.5, the highest capacity obtained with oriented immobilization of Abs on NPs being 39 nmol/m of antigen [32].

The coating of the surface of a chip channel was also proposed by the group of Woolley to couple IT-SPME on-line with microchip CE [33,34]. For this, a thin film of polymer was photopolymerized on a 0.6 mm-length channel of a chip to be used, after the antibodies coating, as an immunoextraction sorbent before the separation of the target analytes in another channel of the chip. This device was first developed for the trapping of a single molecule, α-fetoprotein (AFP), from human serum thanks to the immobilization of anti-AFP antibodies on the thin film of polymer [33]. Four antibodies specific of four different biomarkers were further simultaneously immobilized in the same way thus enabling the simultaneous extraction of these biomarkers from the same human serum sample, their separation being achieved after their transfer to the separation channel [34]. A low binding density of 0.1 $nmol/m^2$ of antibodies (i.e. about 0.01 $pmol/cm^2$) was reported that is close to the one reported for capillary without NPs. Nevertheless, it was considered as sufficient as it allows to reach a linear response up to 500 ng/ml of analyte.

Except for two studies reporting overnight extraction, the extraction times reported using capillaries are shorter than those reported in SPME, between 2 and 9 minutes. If the extraction time on chip was still quite long (10 min for the sample percolation and rinsing step on a 6 mm length channel), a short separation time of less than 3 min renders the whole analytical procedure quite fast.

Concerning the reusability of such devices, the use of a capillary for more than 200 analyses (spiked buffer) was reported without observing any loss of performance and with RSD < 5% for the same sample on 5 consecutive days [28] or up to 10 times and over 20 days with RSD values lower than 7% for recoveries in plasma sample [30]. The use of orientated immobilization of antibodies also gave rise to a higher stability of the sorbent compared to random immobilization [32]. At last, concerning the repeatability of the preparation of such a sorbent in chip, there was a 7.7 % and 10-30% variability in the amount of retained proteins between different batches of capillary (n=5) [32] or chip (n=3) [34], respectively.

328
329 **Table 3:** Applications of immunoaffinity in-tube SPME
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Target	Sample	Extraction sorbent	Antibody grafting	Method	Analytical method	Method performance	REF
Cytokines	Urine, plasma, CSF, saliva (30 nl)	Activated (APTES) silica capillary (6 cm of a 30 cm capillary, 100 µm i.d.)	Fabs from mAbs immobilized using SSMCC	Extraction (2 min), washing, elution (acidic electrolyte)	CE/LIF	LOD: 5-20 ng/ml	[28]
Fluoxetine	Serum (20 x 50 µl)	Activated (APTES) silica capillary (70 cm, 250 µm i.d.)	Covalent grafting of pAbs using GTD	Extraction (20 x 50 µl, 400 µl/min, 2.5 min)	LC-MS	LR: 5-50 ng/ml LOD: 5 ng/ml,	[29]
Interferon α	Plasma (20 x 150 µl)	Activated (APTES) silica capillary (60 cm, 250 µm i.d.)	Covalent grafting of mAbs using GTD	Extraction (20 x 150 µl, 315 µl/min, 9 min)	LC-Fluo	LOQ: 6 IU/ml	[30]
ß2-microglobin or cystatin C	standard solution	Aldehyde activated-silica capillary (60 cm, 250 µm i.d.)	polystyrene NPs grafted with pAbs using EDC and sulfo-NHS and further immobilized on the silica inner wall	Overnight extraction	PETIA	-	[31]
ß2-microglobin or cystatin C	standard solution (1 ml inject in 30 min)	APTES-activated silica capillary (60 cm, 250 µm i.d.) grafted with poly(GMA) NPs immobilized via epoxy groups on silica inner wall	Grafting with pAbs on NPs : oriented immobilization via carbohydrate regions on Fc or random immobilization	Overnight extraction	PETIA	RE: 96.8-103.6% (SD < 8%, n=5) in pure media LOQ: 0.5-5 µg/l (random): 0.5-1 µg/l (oriented)	[32]
FITC-labeled α-fetoprotein (AFP), four labeled-biomarkers (proteins)	Labeled-human serum (10 µl)	Coating of the PMMA-chip channel with poly(GMA-co-PEGDA) monolith (3µm thickness, 6 mm)	Covalent grafting of Abs on poly(GMA-co-PEGDA) monolith	Extraction (5 min), washing (5 min), transfer to the separation channel (50-70 s)	CE/LIF on-chip	LR: up to 500 ng/ml; LOQ: ≈ ng/ml	[33] [34]

5- Solid phase extraction on monolith

To increase the amount of Abs immobilized in a reduced size device, the synthesis of a porous monolith followed by the grafting of the antibodies onto this porous support was proposed. This monolith must be hydrophilic to limit the contribution of non-specific hydrophobic interactions during the extraction of the target analytes and must have an accessible function for effective antibody grafting. Monoliths have already been proposed in large formats (50 x 4.6 mm columns or 12 x 3 mm discs) and are not included in this study. For the smaller devices, the monoliths were mainly *in situ* prepared in capillaries from 75 to 250 µm in diameter by polymerization of organic monomers [35–43]. The preparation of hybrid monoliths by hydrolysis and condensation of organosilanes and alkylethoxysilanes (by sol-gel process) has also been proposed [44,45]. These works are summarized in Table 4. These monoliths were synthesized at one end of a long CE capillary (Figure 1D), in a short capillary connected via a switching valve to nanoLC (Figure 1E) or in a short channel of a chip (Figure 1F).

Organic monoliths are considered the easiest to synthesize in a reproducible way and more stable than hybrid monoliths. Most of these monoliths were prepared using GMA as monomer and EGDMA as crosslinking agent. The hydrophilic properties of GMA, which possesses epoxy groups allowing antibody grafting, have often been advanced to justify its use in order to reduce the risk of non-specific interactions by limiting the hydrophobic effect. Nevertheless, for the immunoextraction of a serum protein, glycerylmethacrylate (GMM) was compared and then preferred to GMA to limit this risk [40]. Organosilane-based monoliths are considered to be even more hydrophilic, thus avoiding this type of non-specific interactions. To improve their stability and avoid gel shrinkage, a non-hydrolytic sol-gel process in the presence of ionic liquid at room temperature was reported [44].

The contribution of non-specific interactions in the retention process was particularly studied by groups preparing monoliths. This was certainly motivated by the largest surface of sorbent offered by this approach compared to other devices previously described that can increase this contribution. As an example, it was studied for an organic monolith by measuring the retention of compounds of different polarities [36,41,43] including the antigen [41,42,46] before [43] or after grafting the antibody [37,39,46]. Since antibodies can also develop non-specific interactions, the study of the retention of the target analyte on a monolith grafted with another antibody was also proposed [41,45] as also reported for in-tube SPME [32] and SPME [23]. This last approach appears also useful to optimize the washing step that is supposed to help in reducing non-specific interactions [45]. This contribution of non-specific interactions was studied in detail by Gunasena *et al*.. They reported the screening of different hydrophilic monomers for the development of an IS allowing the trapping of a target protein by limiting both the retention of this target with the monolith by non-specific interactions and the possible interactions of this target with other proteins in the conditions of use of the monolith, the final choice resulting from a compromise [40].

Some parameters such as the synthesis temperature or the quantity and nature of the porogen can affect the permeability of the monolith (which must be sufficient to allow the liquid to flow) and its specific surface area (which must be high to obtain a high number of potential grafting sites and therefore a large extraction capacity). However, promoting permeability which requires large pore size may induce a decrease in the specific surface area, and consequently the capacity. The determination of the capacity was performed by several groups. The entrapping of mAbs in a sol-gel gave rise to a grafting density of 0.1 mg (0.6 nmol)

active mAbs/g corresponding to 8% of the introduced antibodies (1.25 mg/g). For a poly-(GMA-EDMA) monolith, the amount of grafted mAbs was 18 mg/g. This sorbent was able to fixed 1.2 pg/cm (3 fmol/cm) of Ochratoxin A thus indicating to the authors that 39% of the randomly immobilized mAbs were active [41]. A higher capacity of 40 µg/cm (40.2 nmol/cm, 2.11 nmol/g) of microcystin-LR for a 100 µm i.d. capillary [45] was reported for a hybrid monolith based on APTES and TEOS. This difference is mainly due to the larger specific surface area of silica-based monoliths. This 40 µg/cm capacity of microcystin-LR corresponds to a binding density of 0.543 pmol/µl of active mAbs. This value is 40 times higher than the capacity obtained by direct coating of antibodies on the surface of a capillary [29].

In the first studies, these monolithic ISs of 1 to 10 cm in length were applied to the extraction of analytes from standard solutions. A solvent plug was next pushed to desorb the analytes that were either directly detected by LIF through the capillary [35,36,39] or recovered at the end of the capillary and then analyzed by different methods (LC-MS/MS, SDS-PAGE, ICP-MS...) [40,44]. The coupling of the extraction to a separation in CE [41] or nanoLC [45,46] has also been reported more recently.

Concerning the introduction of antibodies on chips, their grafting at the inner surface of the injection port of a chip [47,48] and, later, on a glass fiber filter of 2 mm diameter then introduced in the chip port, was proposed by Phillips and Wellner. To improve the sensitivity of the method, the targets extracted from real samples by immunoextraction were further *in situ* labelled and analyzed by CE/LIF on chip, thus providing a lab-on-chip device that can be directly applied to real samples [49,50]. After coating the channel surface as previously mentioned, the group of Wooley achieved the *in situ* synthesis of a monolith next grafted with anti-FITC antibodies and used it for the selective extraction of FITC-labelled amino acids or proteins chosen as model molecules [37,38].

Despite the fact that monoliths are mainly coupled on-line with the separation or detection steps, the duration of the analyses is very variable, from 20 min to 3 h. In most of the cases, the limiting parameters are the loading mode and the volume of sample that varies from 100 pl injected on chip to more than 100 µl. The back-pressure generated by the monolith limits the flow-rate that can be used, the fastest method being obtained by combining on chip the injection of a 100-pl sample with a reduced-size monolith of 2 mm and a CE separation step [38]. However, the sensitivity of the device was poor.

Immunoaffinity monoliths (in chip or capillary) were mainly applied to standard solutions. The performances of these miniaturized devices for the analysis of a target molecule in real or complex samples were more rarely described. Among these applications, a 15-cm monolithic IS (in a 180-µm i.d. capillary) grafted with mAbs specific of a protein was applied to the selective trapping of some of its peptides present in a tryptic digest of this protein. The peptides selectively recognized by the mAbs were eluted and transferred to a trap column before being on-line desorbed and transferred to the nanoLC-MS/MS system [46]. This work is an illustration of what could be achieved by immobilizing anti-peptide antibodies as in the SISCAPA approach. In another study, a 100-µm i.d. capillary of a length of 4.5 cm containing a monolithic IS was placed on a 6-ports switching valve and, in this case, directly connected to a nanoLC/UV system[45] . After percolation of an algae extract and application of a washing solution to reduce the non-specific interactions to 8% while ensuring an extraction recovery of 71%, the target analyte, microcystin-LR, was transferred by the LC mobile phase to the reversed phase nanoLC column . Another fully integrated chip device was very recently applied to the simultaneous analysis of two proteins in a human plasma sample, the labeling of the proteins directly in plasma before its introduction on the chip allowing their detection at the

nM level [42]. The design and the photograph of the chip, the SEM of the monolith and the resulting electropherograms, showing again the contribution in terms of selectivity of the Abs, are reported in Figure 3.

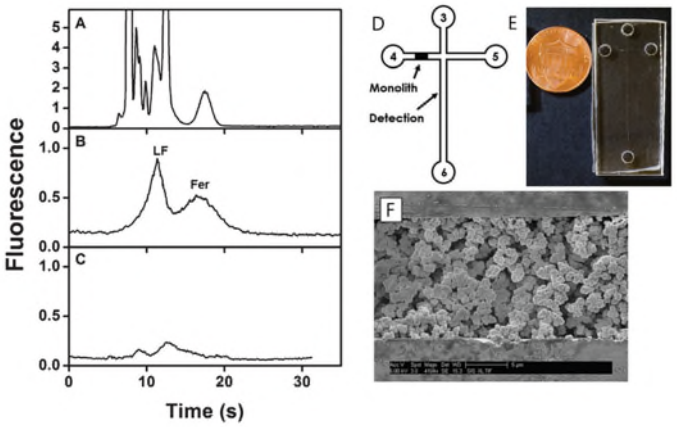

Figure 3: Integrated immunoaffinity extraction and CE in chip for the analysis of protein biomarkers in human blood serum. Electropherograms of labeled spiked human serum, (A) before and (B) after on-chip immunoaffinity extraction. Elution/injection times were: (A) 1 min and (B) 15 s. (C) Electropherogram of labeled unspiked human serum after on chip immunoaffinity extraction and CE. Elution time was 15 s. Design (D) and photograph (E) of the chip and SEM of a channel cross-section with GMA-EGDMA monolith polymerized inside (F). Adapted from [42] with permission of the Royal Society of Chemistry.

Concerning the reusability of the IS monoliths, it was reported that they can be reused up to 50 times without observing any loss of trapping efficiency [46], but some authors observed losses after 5 [35,41] or even 3 [38] uses. This loss of performance after 3 uses was observed on 6 monoliths whose performances were similar for the first uses, thus confirming this loss of performance but also the repeatability of the preparation of the monoliths as reported by other groups [45,46].

Table 4: Immunoaffinity monolith coupled to CE, nanoLC or integrated on-chip.

Target	Sample	Extraction sorbent	Antibody grafting	Method	Analytical method	Method performance	REF
Fluorescein	Standard solution (750 µl)	sol-gel (DGS/PEG) in a silica capillary (10 cm, 250 µm i.d.)	entrapping of mAbs (DGS/PEG)	Percolation (5 min), washing (10 min) and pulse elution (buffer with 20% MeOH, about 6 min)	Direct LIF detection		[35]

Ochratoxin A	Standard solution	poly(GMA-co-EDMA) monolith in a silica capillary (40 cm, 75 µm i.d.)	covalent grafting (epoxy) of purified pAbs on 8.5 cm	Percolation, washing and pulse elution (85 min)	Direct LIF detection		[36]
FITC-labelled amino acids	Standard solution (100-450 µL)	poly(GMA-co-EDMA) monolith on PMMA chip channel (20 µm x 50 µm x 0.5 cm)	activation with EDA/sulfoSMCC and grafting of reduced pAbs by thiol groups	Percolation (50-225 min), washing and CE elution (20 s)	CE/LIF on-chip	RE: 86% chip-to –chip variability of 3.1% (n=3)	[37]
FITC-labelled proteins	Standard solution (100 pl)	poly(GMA-co-EGDMA) monolith in a PMMA chip; 2mm of monolith in the separation channel	covalent grafting (epoxy) of pAbs	Percolation, elution under voltage in 1 min	CE/LIF on-chip	RE: 92%	[38]
Labeled-testosterone	Standard solution	poly(VDA-co-HEMA-co-EDMA) monolith in a silica capillary (1 cm, 100 µm i.d.)	covalent grafting (through VDA groups) of pAbs	percolation (0.5 min) washing (1 min) and pulse elution (80% MeOH, 1 min)	Direct LIF detection	LOQ: 0.02 mg/l (0.07 µM) RSD: 7.3% (n=4)	[39]
Human IgG	Diluted human serum with PBS (50 µl)	Hybrid monolith (APTES-co-TEOS, PEG, [BMIM]BF4) in a silica capillary (80 cm, 530 µm i.d.)	covalently grafted with pAbs using GTD on 10 cm of monolith	Percolation, incubation, washing and pulse elution (80% MeOH); whole procedure in 115 min	Off-line; (MCN)-ICP-MS	RSD on RE of 10.2% and 11.7% for one batch and inter-batch extraction efficiency respectively (n=7) LR: 0.2-10 µg/l LOD: 0.058 µg/l RE: 98 ± 5% (human serum)	[44]

Haptoglobin	Serum	monoliths of poly(GMM-co-PETA) (diol), poly(GMA-co EDMA) (epoxy), polyGMM-co-EDMA, aminopropylacrylamide hydrochloride-co-EDMA polymerized in 100 µm i.d. capillary	pAbs immobilized on diol or epoxy based sorbent after their conversion in aldehyde using sodium cyanoborohydride or pAbs and Fab fragments immobilized on amino group using GTD	Percolation, washing and pulse elution pH; whole procedure in 25 min	Off-line; SDS- PAGE or LC-MS/MS		[40]
Ochratoxin A	Standard solution (10 µl)	poly(GMA-co-EDMA) monolith in capillary (8.5 cm, 75 µm i.d.)	covalent grafting (epoxy) of mAbs on 5 cm of monolith	Percolation, washing, elution (short plug of organic solvent before CE separation) (33.5 cm capillary); whole procedure in 2h	CE/LIF	LR: 0.05-250 µg/l RE: 114 ± 11% (n=15)	[41]
Microcystin-LR	Algae extract (150 nl)	Hybrid monolith (APTES-co-TEOS, CTAB, ethanol) in capillary (100 µm i.d.)	Covalent grafting of mAbs on 4.5 cm of monolith using GTD	Percolation and washing (17min); elution by nanoLC mobile phase (10 min)	nanoLC/UV	RE: 70.4-77.6% (RSD: 2.51%, n=6, real samples)	[45]

Labeled - proteins (ferritin, lactoferrin)	Labeled human serum	poly(GMA-co-EGDMA) monolith polymerized in the channel (0.6 mm) of a a COC chip activated with PEGdiacrylate	covalent grafting (epoxy) of pAbs	Percolation, washing and elution steps under voltage in 21 min before CE separation on chip (< 1 min)	CE/LIF on chip	LOQ: ≈ nM	[42]
Labeled protein (ferritin)	Diluted (x5) human serum	poly(GMA-co-EGDMA) monolith (0.6 mm) prepared in a 3D printed chip (45 µm x 50 µm)	covalent grafting (epoxy) of pAbs	Percolation, washing and elution achieved under vacuum	LIF on chip		[43]
Labeled ProGRP digest	human serum digest (20 µl)	Poly(EDMA-co-VDM) monolith prepared in a capillary (15 cm, 180 µm i.d.)	covalent grafting (via VDM) of mAbs	loading of the sample (10 min), transfer of the peptides to trap column (5min), LC analysis (12 min)	on line coupling to nanoLC-MS/MS using a trap column	LOD: 520 pg/ml (plasma)	[46]

6- Conclusions

The use of antibodies in the field of analysis and more particularly as a masterpiece of extraction techniques to improve the selectivity of the sample treatment step has been developed for many years. With the miniaturization of the immunosorbents that replaces disposable cartridges or precolumns (packed with conventional particles) by capillaries, chip channels and nanoparticles containing lower amounts of antibodies, the cost of the analytical device should be reduced. However, this requires to develop new approaches for the Abs immobilization by choosing conditions that are efficient in terms of grafting rates but also of the accessibility of antibodies by the antigen. The use of purified polyclonal antibodies in place of monoclonal antibodies, which are more expensive to develop, or antibody fragments that can be grafted in an oriented manner, appears to be a high potential development pathway. Concerning the support for the grafting, the accessibility to large specific surface areas via the

use of nanoparticles in dSPE but also in SPME and IT-SPME proves to be an efficient and competitive approach compared to the use of monoliths whose synthesis conditions must ensure large specific surface areas while limiting non-specific interactions as illustrated in this review. Although many proofs of concept have already been proposed, it is nevertheless necessary to complete them for applications with real samples including their validation. At last, fast analysis were expected using miniaturized devices but, as reported in this review, they remain long even for fully integrated devices. This is due to (i) the low levels of concentration that have to be reached requiring large sample volumes to counterbalance the reduced sensitivity of optical detector often used in miniaturized device and (ii) the low permeability of monolith prepared in reduced size diameter devices that generate high back pressures that are difficulte to face with existing nanoflow pumping system and that are not always compatible with chip stability.

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