6-Aptamer-based and immunosorbents

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Abstract

To face the analysis of compounds that may be present at very low levels of concentration in complex media, such as food, environmental and biological fluids, it is possible to exploit the affinity and specificity of antibodies for their antigen by immobilizing them on a solid support. The resulting immunosorbents allow an effective extraction of compounds of interest while eliminating the other components of the matrix. The objective is then to make the analysis more reliable in terms of quantifying target analytes by eliminating matrix effects. A similar potential can also be obtained using aptamers, i.e. DNA or RNA sequences with high specificity towards compounds. The objective of this chapter is to present the respective characteristics of immunosorbents and aptamer-based sorbents, also known as oligosorbents, and their potential for the selective extraction of targeted compounds for the treatment of real samples.

Key words: extraction; selectivity; antibodies; aptamers; complex samples; miniaturization; immunosorbents; oligosorbents

Abbreviation: AFP : α -fetoprotein; APTES : (3-aminopropyl)-triethoxysilane; BB: binding buffer; APTMS : aminopropyltrimethoxysilane; CEA: carcinoembryonic antigen; CNBr : cyanogen bromide ; DADPA ; diaminodipropylamine; dSPE : dispersive solid-phase extraction; EDC: ethyl-dimethylaminopropyl-carbodiimide; EDMA: ethylene dimethacrylate; FITC: fluorescein isothiocyanate GMA: glycidylmethacrylate; GMM: glycerylmethacrylate ;GTD: glutaraldehyde ; IS : immunosorbent; γ -MAPS : γ -methylmethacrylate trimethoxysilane; MOF : metal organic framework; MPTMS : mercaptopropyltrimethoxysilane; MTMS : methyltrimethoxysilane; NHS : N-hydroxysuccinimide; NP: nanoparticle ; OS : oligosorbents; PANCMA : poly(acrylonitrile-co-maleic acid); PBS : phosphate buffer saline; PDDA : poly(diallyldimethylammonium); PDITC; phenylene diisocyanate; PDMS : polydimethylsiloxane; PEGDA: polyethyleneglycoldiacrylate ; PETA: pentaerythritoltriacyrylate PMMA: polymethylmethacrylate; POSS: polyhedral oligomeric silsesquioxane ; PP: protein precipitation; SBSE: stir bar sorptive extraction; SPE: solid-phase extraction; SPME: solid-phase microextraction ; SSMCC: sulfosuccinimidyl-4-(N-maleimido-methyl) cyclohexane-1 -carboxylate ; TEG: triethyleneglycol TEOS: tetraethyl orthosilicate ; TFME : thin-film micro-extraction ; TMOS: tetratrimethoxysilane; VTMS: vinyltrimethoxysilane

1- Introduction

Despite technological advances leading to a gain in sensitivity of analytical instruments a sample processing step is generally required to extract and isolate compounds from environmental samples, biological fluids or food matrices. Solid phase extraction (SPE) is nowadays one of the most widely used methods for sample preparation. Conventional SPE sorbents are based on hydrophobic, hydrophilic or ionic interactions that allow to retain and extract the target compounds from liquid samples or extracts before their analysis by separation methods. However, while high extraction recoveries can be expected owing to the large variety of sorbents available in various formats, the co-extraction of compounds also present in the sample can lead to co-elution with analytes of interest during the separation step, which prevents their quantification. In order to overcome the lack of selectivity of

these sorbents, other sorbents involving antibodies or aptamers thus promoting a selective retention mechanism based on molecular recognition have been developed.

Antibodies are proteins produced mammalian immune system in response to the presence of a foreign substance (called antigen). Among the five major types of immunoglobulins (IgA, IgD, IgE, IgG and IgM), IgGs are the simplest ones with a molecular weight about 150 kDa. They contain two identical heavy chains and light chains (containing 450-650 and 214 amino acids respectively) that are covalently linked by disulfide bonds in an Y-shaped and forming two identical antigen binding sites located at upper ends of the Y structure (**Figure 1A**). The large number of interactions (hydrogen bonds, ionic, hydrophobic and Van der Waals forces) and an appropriate geometric adjustment between binding site of the antibody and the antigenic determinant provide an antigen-antibody complex that is formed with dissociation equilibrium constant from nM to pM.

Figure 1: Representation of the specific entrapment of a compound by (A) an immunosorbent (IS) or (B) an oligosorbent (OS), (C) selective SPE procedure on IS/OS and (D) expected extraction profiles by comparing the retention of the targeted analyte specifically retained on IS or OS this retention of compounds from the matrix sample

Aptamers are single-stranded oligonucleotides usually between 20 and 100 bases long and may have, as antibodies, a specificity towards a ligand (small organic molecules, peptides, nucleic acids, proteins, intact cells). They have a specific and complex 3-D shape, characterized by various elements such as stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes (1), that allows them to be bound to a wide variety of targets (**Figure 1B**). Indeed, they can be generated against various targets such as divalent metal ions, small organic molecules, proteins, and cells. They are selected from a random bank containing up to 10^{15} different sequences by an in vitro combinatorial selection method called "Systematic Evolution of Ligands by EXponential enrichment" (SELEX) according to their ability to recognize a target (2,3). Once the sequence is selected, aptamers are produced by a chemical way without requiring mammals. Some aptamers are characterized by dissociation equilibrium constants similar to those of antibodies as recently reported for different types of target molecules (4) and in particular for marine biotoxins (5).

While antibodies have been widely used for the development of bioassays such as the well-known ELISA methods or sensors, immunosorbents have already largely proven their effectiveness in

selectively retaining or extracting different types of molecules in terms of size and of physico-chemical properties. Indeed, many studies have described their potential in separation (chromatography, electrophoresis) (6,7) and solid-phase extraction (8–11). Numerous immunosorbents are now available on the market and their use has been validated for numerous applications, particularly for food analysis (12).

Various analytical aptamer-based methods have been developed, including enzyme-linked oligonucleotide assay (ELONA or ALISA, variant of ELISA with aptamers instead of antibodies) (3) or biosensors (“aptasensors”)(5,13,14). The use of aptamers as SPE sorbents (i.e. of oligosorbents) is more recent than the use of antibodies recent but seems to be a very promising approach (4,15,16). This chapter describes the development, the characterization and the application of both sorbents to the selective extraction of target analytes from real samples. Thus, the principle, advantages, limitations and complementary of these sorbents will be presented. The introduction of these selective tools in miniaturized devices will also be discussed.

2- Grafting of antibodies and aptamers on solid support

When used as selective tools for the extraction of target molecules, antibodies and aptamers must be bound to solid supports (particles of different sizes –from large beads to nanoparticles, rods, fibers, internal capillary walls, chip channel surfaces ...) according to the method to be developed.

These supports must possess a chemical and biochemical inertness, good mechanical stability and homogeneity in terms of particle size or surface. They must be easily activated to allow effective binding of biomolecules and have large pore sizes, in particular to promote the accessibility of antibodies which are large molecules compared to aptamers (10-15 nm versus 1-2 nm). They must be hydrophilic in order to avoid any non-specific interactions. Finally, the immobilization procedure must preserve the affinity of antibodies/aptamers towards their target analyte.

If the trapping of antibodies by their immobilization in the pores of a hydrophilic glass matrix has been proposed, the covalent grafting of antibodies onto a sorbent by coupling accessible chemical functions of antibodies amino acids with reactive groups on the surface of the support is the most frequently described approach. This type of covalent immobilization on traditional supports, including silica, agarose, cellulose and synthetic polymers such as polymethacrylate derivatives, is often based on the reaction between the amino groups of lysine residues of antibodies. This leads to random immobilization of antibodies that can affect the accessibility of the antigen to their binding sites (9). Concerning aptamers, their chemical synthesis makes it possible to introduce modifications at the 5' or 3' end of the oligonucleotide sequence to facilitate their immobilization. This modification is chosen according to the nature of the bonding. Modification by an amino group has often been reported resulting in immobilization procedures similar to those applied for the random immobilization of antibodies with the advantage that the aptamer sequence is oriented directly towards the analyte. In addition, a spacer arm can be introduced to maintain the binding properties of the aptamer when it is bounded to a surface. It may be an n-alkyl chain in C6 or C12, an ethylene glycol derivative as illustrated by the data reported in **Table 1**. The aptamers have also been functionalized by thiol-groups thus favoring their binding to gold surface or, as often described for antibodies, to biotin for achieving a non-covalent grafting to streptavidin activated particles that are commercially available. Nevertheless, non-covalent binding procedure presents some drawbacks, in terms of reusability and sorbent life-time, particularly when using organic modifiers in the SPE procedures that affect the biotin-streptavidin affinity (17,18).

Several studies have attempted to optimize the antibody immobilization by oriented binding procedures by exploiting the carbohydrate moieties located on the heavy chains, at the lower ends of the Y structure (i.e. Fc region) after the mild oxidation of these moieties in aldehyde residues which may react with a sorbent containing a hydrazide or amine. The use of antibody fragments was also recently proposed to limit their size while facilitating their oriented grafting. This approach seems particularly interesting for the development of miniaturized extraction devices. It must provide greater antigen binding capacity because immobilization occurs far from the antigen binding sites and results

in a more oriented reaction. However, despite the theoretical advantages of the oriented immobilization procedure, the greater binding capacity generally observed using random immobilization that can result from a higher number of active groups greatly reduces its interest. This oriented immobilization can also be ensured by the use of a sorbent activated by proteins A or G which also bind the antibody in the Fc region. This non covalent immobilization strategy is based on the high affinity between the IgGs and these proteins but can be easily disrupted by lowering the pH or using an organic solvent, thus affecting the reusability of the resulting ISs as for streptavidin activated supports. A recent review on miniaturized immunoextraction based sorbents summarized in detail the different approaches proposed to improve antibodies immobilization based on this reduced-size extraction format (19).

3- Extraction procedure using IS and OS

During the extraction step on the immunosorbent (IS), the antibody-antigen complex is formed primarily by electrostatic forces, which attract and orient both entities. Then, this causes the formation of secondary hydrogen bonds, bringing the molecules closer together, and excluding water. Finally, Van der Waals forces are initiated to form a stable non-covalent bond. The establishment of these interactions is promoted in aqueous media and then well-adapted to aqueous samples (real waters, biological fluids). For other type of samples such as organic extracts of solid samples, dilution of the extracts with an aqueous buffer is recommended (8). Analytes are further recovered by the disruption of these complexes that occurs by applying conditions that may differ from the nature of the target. Indeed, the desorption of proteins is often carried out by chaotropic agents (chloride, iodide, perchlorate and thiocyanate ions at concentration between 1.5 and 8 mol/L), temperature increase, pH variations, and the use of denaturing agents such as water-organic solvent mixtures being applied to the desorption of small size targets (8,9).

For oligosorbents (OSs), effective trapping of the target analyte is promoted by a sample whose composition is close to the composition of the buffer used during the selection of aptamers (often called the binding buffer (BB)) to favor the interactions between both entities. The strength of the binding between an aptamer and its target, which is highly dependent on the conformation of the aptamer molecule, may be affected by a variety of cations. As an example, it is well known that some guanosine-rich aptamers can adopt inter- or intramolecular quadruplex structure that are stabilized by the presence of G-quartets (square arrangement of guanines) that are favored by the presence of K^+ as often reported for thrombin aptamers. This example shows the need to study the effect of different cations to determine the optimal conditions for a strong binding of the target on the OS during the extraction step. Some parameters such as temperature (20–24), ionic strength and pH (20) also strongly affect the conformation of aptamers. These parameters must be controlled and adapted to ensure high extraction recoveries or favor the elution (25). Different approaches are possible to induce elution: water-organic modifier mixtures and chaotropic agents (such as $NaClO_4$, denaturing agents such as urea or guanidine hydrochloride) as for antibodies, scavenging agents such as EDTA for aptamers sensitive to cations, temperature increase, pH variations. The choice of those conditions strongly depends on the nature of the analyte(s) and of the aptamers. The use of organic solvents never reported for antibodies such hexane (26), a hexane/ethylacetate mixture (27) or dichloromethane (28,29) was also reported without preventing the reusability of the OS. The addition of trypsin for the direct digestion of a target protein (30) or of recombinant DNase that damages the aptamers (31) have also been described.

When not in use, both ISs and OSs are stored at 4°C in a buffer solution, a PBS buffer for ISs and the BB for OSs. Sodium azide, a bacteriostatic agent, is generally added in the storage buffer. This solution can be removed by pure water or by renewing BB solution during the conditioning step.

4- Various extraction format and methods

Immunoaffinity cartridge of 1-3mL is the most commonly used format for immunoextraction (8,9,12). Many oligosorbents were also developed in this format too. However, these last years IS and OS available in other formats and allowing the development of other extraction methods than SPE have been developed and are described below.

4-1- SPE in cartridge or column

Many ISs have been developed since the early 1990s in cartridges to carry out off-line SPE (8) such as the commercialized ISs dedicated to mycotoxins analysis, which constitute the most important ISs market (12). For application to real samples, 30 to 60 mg of ISs are generally packed between two frits into disposable cartridges or columns as conventional sorbent for SPE as shown in **Figure 1C**. The advantage of the cartridge format is that it is adapted to all the consumable and automate developed for conventional SPE sorbents. For OSs, their application in SPE to real samples is more recent. Those works are summarized in **Table 1**. As commercially available ISs, most of the OSs were prepared by immobilizing aptamers on activated Sepharose beads and by applying a similar extraction procedure. After conditioning them with a few ml of aqueous media that favors the interaction of the target analyte with the antibodies/aptamers, the sample is percolated. After a washing step that can be applied to remove some matrix components slightly retained during the sample percolation, the target analyte is then desorbed by a solution affecting the interactions with the antibodies/aptamers. As schematized on **Figure 1D**, matrix components, not recognized by antibodies/aptamers and therefore not retained on the IS/OS must be removed after the washing step thus giving rise to a final elution fraction that only contains the targeted analyte.

Table 1 : Applications of aptamers in extraction devices for the analysis of targets in real samples

Target analyte	Sample	Amount or dimension of the extraction device / V_{sample}	Sorbent and reagent used for the grafting	Aptamer modification /Spacer	Ref.
Off-line SPE					
Aflatoxin B2	Peanut extract (diluted)	60 mg / 5mL	CNBr-Sepharose	NH ₂ /C7	(34)
CEA	Serum	4μl (PDMS chip)/ 200 μL	Carboxylated-polystyrene beads (40- 50 μm), NHS	NH ₂ /-	(35)
Cocaine	Plasma (diluted x2)	35 mg / 200 μL	CNBr-Sepharose	NH ₂ /C6	(36)
	Post-mortem blood (after PP)	35 mg / 100 μl		NH ₂ /C12	(37)
Cocaine + Dichlofenac	Drinking water	50 x 8 mm i.d./ 0.2 to 1 L	CNBr-Sepharose	NH ₂ /C6	(32)
Ochratoxin A	Wheat extract	300 μL in cartridge / 10-12 mL	DADPA (non covalent)	-/-	(38) (39)
	Red wine	35 mg/1 mL	CNBr-Sepharose,	NH ₂ /C6	(17)
	Wheat extract (diluted x 10)			NH ₂ / C12	(40)

	Ginger powder (diluted x 10)	200 µL/3 mL	NHS-sepharose	NH ₂ /C6	(41)
Tetracycline	Plasma (after PP) and Urine (diluted x 3)	35 mg/200 µL	CNBr-Sepharose	NH ₂ /C6	(42)
Thrombin	Human serum	0.53 mm i. d. / 100 µL	Poly(TMOS-co-γ-MAPS) monolith	SH/C6	(43)
	Human serum (diluted x 50)	0.53 mm i. d. x 2 cm/1 mL	Poly(AEAPTES-co-TEOS) monolith modified with AuNP	SH/C6	(44)
On-line SPE/LC					
OH-deoxyguanosine	Urine (diluted x 5)	200 x 0.5 mm i. d. /2 mL	Amino-mNP, GTD	NH ₂ /-	(45)
Ochratoxin A	Beer (dilutedx2)	100 x 0.1 mm i.d. /100 µL	POSS-acrylate-based monolith (one-pot synthesis)	SH/-	(46)
	Beer, Wine (dilutedx9)	10 cm x 75 µm i. d./20 µL	Poly(TMOS-co-MPTMS) monolith modified with AuNPs (25 nm)	SH/C6	(47)
On-line SPE/nanoLC					
Ochratoxin A	Beer (dilutedx2)	70 x 100 µm i. d./ 250 nL	Poly(APTES-co-TEOS) monolith, GTD	NH ₂ / C12	(48)
In-line SPE/CE					
Ochratoxin A	Beer and white wine (dilutedx2)	15 mm x 75 µm i. d./2.65 µL	Poly(TMOS-co-MTMS) monolith, VTMS	SH/-	(49)
On-column concentration– direct injection of real samples					
Adenosine	Dialysated from rat cortex	5 cm x 150 µm i. d./4 µL	streptavidine activated - porous glass beads	Biotin/TEG	(50)
Cytochrom C	Serum (diluted x 10)	8.5 cm x 100 µm i. d./-	streptavidin-modified poly(GMA-co-TRIM) monolith	Biotin/-	(51)
Doxorubicin, Epirubicin	Serum, urine	10 cm x 75 µm i. d./ 20µL	poly(TMOS-co-γ-MAPS) monolith (one-pot synthesis)	SH/-	(52)
Lysozyme	Chicken egg white	10 x 4.6mm i. d./ 5µL	Poly(GMA-co-EDMA, ethylenediamine, GTD	NH ₂ /C6	(53)
L-selectin	CHO cell-conditioned medium	5 x 0.5 mm i. d. /-	Streptavidin-polyacrylamide resin	Biotin/-	(54)
Thrombin	Serum (diluted x 20)	250 µm i. d. /-	Poly(APTES-co-TEOS) monolith, GTD	NH ₂ /C6	(55)

An important parameter in this sequence, as in conventional SPE procedure, is the volume that can be percolated without loss of the analyte during this percolation step to ensure high recoveries. This volume highly dependent on the affinity of the antibodies/aptamers for the target analyte(s). It can be

drastically reduced by choosing a percolation medium that does not promote this affinity, as previously mentioned. To limit the risk of loss of affinity caused by matrix components, the dilution of real samples with a buffer is often performed.

If dissociation constants for antibodies and their antigens are in the nanomolar range, the K_d values for aptamers may fluctuate (2) and high K_d values limit the possibility to percolate high sample volumes and then to reach high enrichment factors. This was well illustrated by Hu *et al* who reported the possibility to percolate up to 2 L of drinking water containing dichlofenac without observing a decrease of recovery while the sample volume was limited to 0.5 L of water for the extraction of cocaine. Indeed, the breakthrough volume was lower for cocaine than for dichlofenac because of the higher K_d value of cocaine aptamer ($K_d = 5 \mu\text{M}$) compared to dichlofenac aptamer ($K_d = 47.2 \text{ nM}$) (32). In return, for antibodies, the K_d value can be low for its antigen but higher for a structural analog thus giving rise to a decrease of recovery for this molecule for too high percolated volume.

An example of evaluation of this breakthrough volume is given in **Figure 2 A** which illustrates the effect of the volume of sample percolated on an anti-ciprofloxacin (CIP) IS (2 mL gel) on the recovery of extraction of CIP. The affinity of the anti-CIP antibodies allows the percolation of up to 5.5 mL of sample while the recovery of quinine (QUIN), not recognized by these antibodies, decreases from 2 mL of sample (33). The optimization of an extraction procedure also consists in applying strong elution conditions to limit the volume of eluent, as illustrated by the elution of CIP with different ratios of methanol in water (**Figure 2 B**), thus favoring high enrichment factors without requiring in some cases additional evaporation steps of the final extract.

Figure 2: (a) Breakthrough curves of CIP and QUIN obtained in anti-CIP pAb immobilized column. (b) Elution profiles of CIP with different percent of methanol. Reproduced from (33) with permission from the Royal Society of Chemistry.

The selectivity brought by ISs and OSs is illustrated by comparing the chromatograms of elution fractions from an OS (**Figure 3a**), an IS (**Figure 3c**), and a hydrophobic support (C18 grafted silica, **Figure 3b**) during the purification of a cereal extract. The IS and OS allow the elimination of a large part of the interfering compounds and eliminate the visible co-elution at the OTA peak.

Figure 3 : HPLC chromatograms resulting from the analysis of elution fractions after the extraction of a red wine sample spiked at 2 $\mu\text{g/L}$ with Ochratoxin A (OTA) on the oligosorbent based on a covalent immobilization (a) on the conventional C18 silica cartridge (b) and on the immunosorbent (c). Reproduced from (17) with permission from Springer Nature.

To miniaturize these selective SPE devices, the immobilization of antibodies and aptamers onto monoliths in-situ synthesized into capillaries of 75-100 μm internal diameter or chip channel has been also proposed as illustrated in **Table 1** for aptamers and **Table 2** for antibodies. These miniaturized extraction devices can be directly coupled on(in)-line with CE, nanoLC or on-chip separations thus facilitating the automation of the whole analytical procedure while reducing the volume of sample and reagent consumption. This last point is particularly important when relatively expensive reagents such as antibodies are used.

Table 2: Immunoaffinity monolith coupled to CE, nanoLC or integrated on-chip and applied to real samples

Target	Sample	Amount or dimension of the	Sorbent and reagent used for the grafting	Antibody grafting	REF
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		extraction device / V_{sample}			
Haptoglobin	Serum	100 μm i.d. capillary/	poly (GMM-co-PETA) (diol), poly (GMA-co EDMA) (epoxy), poly GMM-co-EDMA, poly (aminopropylacrylamide hydrochloride-co-EDMA) monoliths	Reduced pAbs (aldehyde groups) grafted on diol or epoxy based sorbent or pAbs and Fabs (amino group) grafted using GTD	(56)
Microcystin-LR	Algae extract	4.5 cm x 100 μm i. d. capillary /150 nL	poly(APTES-co-TEOS) hybrid monolith	mAbs grafted using GTD	(57)
Labeled - proteins (ferritin, lactoferrin)	Labeled human serum	0.6 mm length/	poly(GMA-co-EGDMA) monolith polymerized in the COC chip activated with PEGdiacrylate	covalent grafting (epoxy) of pAbs	(58)
Labeled protein (ferritin)	human serum (Diluted x5)	0.6 mm length /	poly(GMA-co-EGDMA) monolith prepared in a 3D printed chip (45 μm x 50 μm)	covalent grafting (epoxy) of pAbs	(59)
Labeled ProGRP digest	human serum digest	15 cm x 180 μm i. d. capillary /20 μL	Poly(EDMA-co-VDM) monolith	covalent grafting (via VDM) of mAbs	(60)

At last, some applications of column containing grafted aptamers and directly connected to UV detection are listed in **Table 1**. In these works, if the concentration effect cannot be expected, limiting thus the sensitivity of the method, the aptamers are used to remove the matrix components at the beginning of the chromatogram before the increase in the eluent strength of the mobile phase allowing the elution of the target molecule. An example is provided in **Figure 4**. It shows the potential of this simple approach to differentiate serum of breast cancer patients treated with epirubicin and serum from a healthy control.

Figure 4: Chromatograms for the determination of epirubicin in the serum of breast cancer patients treated with epirubicin by intravenous injection. Facile one-pot synthesis of a aptamer-based organic–silica hybrid monolithic capillary column by “thiol–ene” click chemistry for detection of enantiomers of chemotherapeutic anthracyclines. Reproduced from (52) with permission from the Royal Society of Chemistry.

4-2- dispersive SPE

In dispersive mode, dSPE, the extraction is carried out by introducing a given amount of IS/OS into the sample. After a sufficient extraction time under stirring, the particles are recovered mainly by centrifugation or magnetic field (if magnetic core particles are used) and then introduced into a suitable desorption solvent after a possible washing step. Application of dSPE methods using antibodies and aptamers are summarized in **Table 3**. In these works, antibodies/aptamers were grafted on conventional beads, magnetic beads but also nanoparticles (in the order of tens nanometers instead of tens of micrometers). As with conventional dSPE sorbents, the key-parameters to be optimized are the sample volume/sorbent ratio, the extraction time, the stirring rate, the nature of the solvent and the time required for desorption (61).

Table 3 : Some applications of ISs and OSs in dSPE for the analysis of targets in real samples

Target analyte	Samples	Amount or dimension of the extraction device / V_{sample}	Sorbent and reagent used for the grafting	Grafting method	Ref.
Immunosorbent					
Anatoxin A	Water	10 μL /20 mL	NHS-sepharose magnetic beads (27 μm)	grafting of mAbs via amino groups	(63)
Epitestosterone	Urine	10 mg/20 mL	Fe_3O_4 -Au NPs (50 nm)	grafting through Au-S bonds of half mAb (reduction of S-S bond)	(64)
Human chorionic gonadotropin	Dried blood spot, serum, urine	20 μL /1 mL	Tosylactivated magnetic beads (2.8 μm)	Grafting of mAbs (previously stored at pH 2.5)	(65,66)
Low density lipoprotein	Plasma (diluted 1/500)	10 μL /10 μL	Au-NP (28 nm)	non-covalent grafting; covalent grafting of mAbs to carboxy terminated-pegylated NPs (amide), oriented immobilization of mAb via oxydized carbohydrate moiety on hydrazide derivatized NP, non-covalent via cysteine-tagged proteine A-NPs	(67)
Microcystins	Urine	5 μg /100 μL	Streptavidin - magnetic beads	biotinylated antibodies	(62)
Soy proteins	Soy milk (500 μL)	23 mg/500 μL	Fe_3O_4 -Au NPs (12 nm)	oxydized pAbs on amino-functionalized Au-NPs	(68)
Oligosorbent					
ATP	Deproteinized cell lysate	25 μL /250 μL	AuNP (13 nm)	SH- Apt	(69)

Adenosine	Urine	8 mg/200 μ L	Silanized mNPs, APTES, GTD	NH ₂ -C6- Apt	(70)
Aflatoxin M1	Milk extract (purified by PP and LLE)	16 mg/15 mL	Silanized mNPs, APTES, GTD	-	(28)
Aflatoxin B1, B2	Maize extract	1mL/1mL	Streptavidine-magnetic agarose beads (28 μ m)	Biotin-Apt	(18)
Amphenicol antibiotics	Milk (diluted X 100)	50 mg/100 mL	Silanized mNPs, APTES, EDC, Sulfo-NHS	NH ₂ - Apt	(20)
Arsenic	Ground water	2mg/1mL	Streptavidin-agarose beads	Biotin-Apt	(71)
Berberine	Herb extract	20 mg/1 mL	Avidin - activated amino-mNPs, GTD	Biotin-Apt	(27)
Bisphenol A	Human serum, urine (dilutedx2) treated with enzymes	5mg/1 mL	Avidin- mNPs	Biotin-Apt	(72)
17 β -estradiol	River water	50 beads/100 μ L	NCS-modified glass beads (250 μ m), APTMS, PDITC	NH ₂ - Apt	(25)
His ₆ -Tag protein	E Coli lysates	5mg/100 μ L	NH ₂ -magnetic beads, GTD	NH ₂ -C6 or C7-Apt	(73)
Histones	Cell lysate	-/-	Streptavidin agarose beads	Biotin-Apt	(74)
Ochratoxin A	Coffee extract (diluted x 2)	100 μ L/100 μ L	Carboxylated-mNPs (100-250 nm)+ EDC, NHS	NH ₂ -C6- Apt	(21)
	Corn, peanut extract diluted in BB	10mg/-	Streptavidin - magnetic MOF	Biotin-Apt	(75)
PCBs	Soil extract	30mg/100mL	mNP coated by amino-mMOF, GTD	NH ₂ -C6- Apt	(26)
OH-PCBs	Human serum	30 mg/40mL	mNP (18 nm), APTES	NH ₂ - Apt	(76)
Sulfanilamide	Milk	10 μ L/990 μ L	Magnetic carboxylated-silica particles	NH ₂ - Apt	(22)
Thrombin	Serum, blood	-/-	Magnetic beads coated with AuNP	SH-C6- Apt	(30)
	Plasma	100 μ L/100 μ L	Au-nanorods (77 x 17 nm)	SH-(EG) ₆ - Apt	(77)

Thyroid transcription factor 1 (TTF1)	Bacterial lysate	2mg/100µL	Streptavidin-magnetic beads	Biotin-Apt	(31)
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While some studies have reported the use of commercially available (strept)avidine-magnetic agarose beads that make it really easy to immobilize biotinylated antibodies/aptamers and apply them, numerous works reported the use of NPs.

The works reported in **Table 3** show that the quantities of supports used are variable, but the use of nanoparticles leads to a decrease in these quantities and therefore to a decrease in the quantities of aptamers and antibodies used thus decreasing the cost of the methods while reducing sample volumes. Immuno-dSPE, also known as immunocapture, was applied to sample volumes from 10 µL to 20 mL, depending on the expected enrichment factor, with extraction time between 5 min to overnight. For OS-based dSPE, ratios of 10 µL of beads for the extraction of 100 µL of sample to 50 mg of beads dispersed in 100 mL sample with extraction times in the range 3 min to 2 hours were reported. With both sorbents, recoveries higher than 80% were reported. The possibility of performing this extraction method in 96-well plates for easy automation has recently been reported (62).

4-3- SPME, SBSE and associated methods

Other methods based on the equilibrium of the target between the sample and the immobilized antibodies/aptamers were developed by replacing particles by other formats such as fibers or rods of different sizes up to thin films resulting in selective extraction methods named solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) or thin-film micro-extraction (THME) respectively. A quite exhaustive view of the applications related to the use of OS and IS in these formats is provided by **Table 4**.

Table 4: Applications of ISs and OSs in SPME, SBSE and associated

Method	Target	Samples	Amount or dimension of the extraction device / V_{sample}	Sorbent used for the grafting	Grafting method	REF
Immunosorbent						
SPME	Theophylline	Serum (diluted with PBS, 1/100, v/v)	fiber (1.8 mm, 2.3 cm)/-	silanized fiber	pAbs grafted with GTD	(79)
	Benzodiazepines (3)	Urine	rods (4 mm, 2.5 cm)/ -	silanized borosilicate glass rods	pAbs purified pAbs, mAbs grafted with GTD	(80,81)
	Non-steroidal estrogens (3)	Environmental waters (dilution 1/2)	stainless steel rods (2 x 18 mm) /1 mL	5 µm-porous silica particles coted on rods	mAbs grafted with GTD	(82)

SBSE	Quinolones (11)	Bovine milk (centrifuged to remove fat)	bars (5 mm, 3 cm) /-	silanized borosilicate glass bars	mAbs grafted with GTD	(83)
Oligosorbent						
SPME	Adenosine	Human plasma	1 cm length fiber/-	Wire (125 μ m diameter) coated with poly (TMOS-co-APTES)	COOH-Apt+ EDC, NHS	(84)
	Thrombin	Human plasma (diluted x20)	55 x 1.5 mm i.d./ 2 mL	stainless steel rod coated with PANCMA (electrospun microfiber)	NH ₂ -C6-Apt+ EDC, NHS	(85)
SBSE	PCBs	Purified (silica gel) fish extract	50 x 2 mm /-	Wire coated with MOF-5, PDPA	Non covalent	(29)
TFME	Codeine	Urine (diluted x 100)	-/10-15 mL	Oxidized cellulose paper	NH ₂ -C6-Apt. + NaCNBH ₃ ,	(23)
	Codeine, Acetamidrid	Pharmaceutic tablets, urine	triangle (14 mm x 4 mm)/15 mL		NH ₂ -C6-Apt. + NaIO ₄	(24)
	Methamphetamine	Saliva (diluted x10), precipitated plasma (diluted x20)	triangle (14 mm x 4 mm)/700 μ L	Cellulose treated by carbon dots	NH ₂ -C6-Apt. + EDC, NHS	(86)
	Thrombin	Plasma (diluted)	20 μ g/1 mL	Graphene oxide sheet	NH ₂ -C6-Apt. +EDC	(87)

Mostly developed for the extraction of the volatile compounds before their analysis by gas chromatography (GC), the applications of SPME have been extended to biological fluids with an off-line coupling with liquid chromatography (LC) (78). Therefore, its combination with the high antibodies or aptamers selectivity which limits the co-extraction of interfering compounds present in complex samples such as biological fluids can be an interesting approach to improve the sensitivity of the method.

SPME generally consists in the extraction of compounds by immersing a fused silica fiber (100 μ m diameter) usually coated by an organic polymer (7-100 μ m thickness) into a sample and different parameters can be optimized to extract high amounts of analytes, 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 same parameters have to be optimized in SBSE and THME that are only supposed to differ by the size devices. Nevertheless, the dimensions of these different devices are not so different as demonstrated by those reported in **Table 4**, the classification in this Table being in agreement with the name of the method provided by the authors of the reported works.

The preparation of the extraction devices mostly consists in the direct activation of silica-based material, cellulose paper and graphene oxide sheet allowing the covalent grafting with NH₂-functionalized aptamers or amino-groups of antibodies. The immobilization of activated particles or

the polymerization of a monolith onto rods were also proposed, those approaches being used to improve the sorbent capacity, as it will be discussed later on.

In most of the cases, the extraction times are about 30 and 60 min and desorption times between 20 and 40 min as for conventional SPME sorbent. Reported recoveries are between 13 to 67 % with associated RSD values lower than 14% for IS based methods and higher than 80% with RSD values lower than 10% for OS-based methods.

In addition to these methods, immunoaffinity-based capillary microextraction method, also called in-tube SPME (IT-SPME) was proposed. With this approach, the analytes are extracted by antibodies fixed at the inner surface of a capillary before being desorbed and transferred directly to the separation device. It presents several advantages compared to SPME such as an on-line coupling with separation methods (capillary electrophoresis (CE) and, more frequently, LC). Applications of immunoaffinity IT-SPME to real samples are reported in **Table 5**. To my knowledge, no similar developments were achieved using aptamers. In 1998 Phillips and Dickens proposed an “immunoaffinity capillary electrophoresis” methods that consists in the covalent grafting of antibodies fragments (Fabs) at the inner surface of approximately 6 cm of a 100 μm silica capillary, the remaining part of the 30 cm-capillary being used for the CE separation (89). Since this pioneer work, other methods were proposed. They can be based on the injection at a fixed flow-rate of a fixed volume of sample or by repeated aspirations (draw) and ejections of a fixed volume samples through the capillary. The sample draw/ejection volume and the number of draw/ejection cycles have to be adapted to the capillary capacity (90) and to the volume comprised between the injection needle and the capillary. All the extractions performed by this approach were achieved in less than 10 min, then in shortest time than using the previous methods.

Table 5: Applications of immunoaffinity in-tube SPME

Target analyte	Sample	Dimension of the extraction device/ V_{sample}	Extraction sorbent	Grafting method	REF
Cytokines	Urine, plasma, CSF, saliva	6 cm of a 30 cm capillary, 100 μm i. d. /30 nL	Activated (APTES) silica capillary	Grafting of Fabs from mAbs, SSMCC	(89)
Fluoxetine	Serum	70 cm, 250 μm i. d./20 x 50 μL	Activated (APTES) silica capillary	Covalent grafting of pAbs, GTD	(90)
Interferon α	Plasma	60 cm, 250 μm i. d./ 20 x 150 μL	Activated (APTES) silica capillary	Covalent grafting of mAbs, GTD	(93)
FITC-labeled AFP , four labeled-biomarkers (proteins)	Labeled-human serum	3 μm thickness, 6 mm/10 μL	PMMA-chip channel coated with poly(GMA-co-PEGDA) monolith film	Covalent grafting of Abs	(91,92)

In addition to the developments using capillaries, the coating of the surface of a chip channel was also proposed by polymerizing a thin film of a polymer on a 0.6 mm-length channel to couple IT-SPME on-line with a CE separation on chip (91,92). 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 (91). 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 individual identification being achieved after their transfer to the separation channel (92).

5- Capacity

The capacity is defined as the maximal amount of a molecule that can be retained by the sorbent during the percolation step. For these selective sorbents, it depends on the number of active immobilized antibodies/aptamers which is directly related to their bonding density, usually expressed in mg/ml of sorbent bed or mg/mg of sorbent.

This parameter strongly depends on the concentration of biomolecules introduced in the grafting solutions and of the specific surface area of the solid support accessible for the immobilization of the biomolecules (4,8). Supports with small pore sizes have a high surface area, but a low accessibility for the large biomolecules. On the other hand, supports with large pore sizes have a good accessibility but a small surface area. The bonding density can be calculated experimentally by measuring by UV spectrophotometry the concentration of the antibodies/aptamers that remains in the binding solution. Nevertheless, this approach only allows the determination of the amount of grafted biomolecules including also those that are not accessible for the target analyte.

The real capacity of ISs/OSs can be determined by measuring the amount of retained target analyte as a function of its concentration in the percolated sample after applying the optimized extraction procedure. By plotting the amount of target analyte retained by the sorbent (i.e. recovered in the elution fraction) as a function of its concentration in the percolated sample, a curve is obtained that is characterized by two different parts (17,34,36,37,40). First, for the lowest percolated amounts/concentration levels, a linear part is obtained and corresponds to a range of concentrations for which a constant recovery yield of extraction is obtained, this recovery yield being given by the slope of this linear part. Working in this range ensures the possibility to carry out quantitative analyses. In the second part of the curve, for higher percolated amount, the recovery yields decrease and the amounts of target analyte retained by the sorbent tend to reach a plateau. This decrease in recovery yield is caused by the overloading of the sorbent capacity, *i.e.* the saturation of the antibodies/aptamers binding sites. The capacity of the sorbent is given by the amount/concentration corresponding to the upper limit of the linear range. Knowing this amount, it is then possible to evaluate the part of active aptamers or antibodies among the total amount of immobilized ones thus allowing a better evaluation of the efficiency of grafting procedure(17,40).

Concerning immunosorbents, both polyclonal (pAbs) and monoclonal (mAbs) antibodies can be used for their preparation. The pAbs are cheaper to obtain, but their production suffers of a lack of reproducibility in terms of time of response of an animal, of quantity and even of specificity. In contrast the production of mAbs is costly but guarantees a long-term production of reproducible antibodies that does not require animals for further large-scale production. Some authors also proposed the use of purified pAbs allowing to increase the amount of specific immobilized antibodies (80,81), of Abs fragment (Fabs) (89) or of half-antibodies obtained by splitting them in two (disruption of disulfide bonds between the two heavy chain) (64), the objective being to increase the density of the recognition sites.

Concerning the OSs, the binding density of the aptamers must be facilitated by the possibility to introduce a chemical function during their synthesis according to the nature of the solid-phase selected for their immobilization. In addition to the introduction of this functionality, it was also shown that the length of the spacer affects the binding density. Indeed, when studying the immobilization of aptamers, specific to OTA, on CNBr-activated sepharose, a higher capacity was obtained using an aptamers linked with a C12 spacer arm to the amino-group than using a C6 spacer arm (40). In return, the immobilization of an aptamers through the 3'-end side and from the 5'-end side gave rise to similar capacities (40).

Values of capacity found in the literature for target molecule on OSs available as SPE cartridges are quite homogeneous and are in the range 0.26 to 69 nmoL/g of OS (4). Such capacity values are associated to binding density values in the range 19 to 37% on Sepharose beads, a binding density of

active aptamers of 68% being recently reported (34). Those capacity values for OSs are very close to those obtained with immunosorbents (from 4 to 93.6 nmol/g of sorbent, e.g., immunosorbents specific to pesticides) (94).

While it is sufficient to adapt the quantity of IS/OS introduced into an extraction cartridge to ensure a sufficient capacity, the miniaturization of extraction devices has led to the evaluation and comparison of new immobilization approaches. As an example, as the grafting of antibodies directly immobilized on the inner wall of a capillary was low, the use of NP-coated capillary was proposed and has allowed to reach a bonding density of to increase the sorbent capacity by a factor 5 (95). 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 (96). To improve the capacity of such miniaturized OSs, the introduction of NPs grafted by aptamers and further immobilized on monolith was also proposed (44,47).

6- Contribution in selectivity of the OS/IS and control of non-specific interactions

Non-specific interactions (NSIs) can occur between the analyte and the sorbent used for the immobilization of aptamers/antibodies and those macromolecules too. These NSIs can contribute to the non-expected retention of interfering compounds present in the sample thus affecting the selectivity of the procedure.

Agarose gel such as Sepharose and hydrophilic monoliths have been often select as solid-phase to reduce as much as possible NSIs, i.e. mainly hydrophobic and/or electrostatic interactions favored in aqueous media. The comparison of the retention of the target analyte on the IS/OS with the non-grafted solid-support, named control sorbent (17,21,34,36,37,40–42,44–47,70,71,73–76,84), or grafted with another antibody/oligonucleotide sequence, having no affinity for the target (18,21,25,34,36,37,42,47,48,54,55,70,84), is now often reported to evaluate the contribution of these NSIs. The use of these sorbents in parallel to IS/OS is particularly helpful for optimizing the washing step and then obtaining an optimal selectivity (48,57). At least the evaluation of the selectivity can be achieved by controlling the lack of retention of compounds often present in the studied matrices and not recognized by antibodies/aptamers (18,22,23,25–28,30,31,40,45–48,72,74,85).

7 - Specificity towards structural analogs

Oligosorbents and immunosorbents largely differ by their ability to retain target structural analogs. Indeed, antibodies may present a broad cross-reactivity, particularly when they have been produced for small molecules, thus allowing the trapping of a structural group of compounds. This is illustrated by the commercialization during the last decade of ISs for the clean-up of samples for the analysis of classes of mycotoxins (aflatoxins, ochratoxins, fumonisins...) in food, of class of veterinary drugs (clenbuterol and analogs) and of drugs of abuse (LSD and its metabolites...). The commercialization of ISs containing several antibodies specific of several toxins for their simultaneous trapping is now also proposed to expand this specific retention to compounds from different classes present in the same samples on a single cartridge. In return, the ability of aptamers to retain structural analogs can be poor. As an example, OTA aptamers present 100-fold less affinity for Ochratoxin B than for OTA, a difference of only 3-fold being observed for anti-OTA monoclonal antibodies (38). Moreover, a slight change in a sequence leads to loss of molecular recognition as it was showed by the loss of cocaine retention on an OS prepared with an DNA sequence similar to the DNA sequence of the cocaine aptamer but presenting only one mutation (37). Concerning theophylline aptamers, they have no affinity for caffeine despite the structural analogy of these molecules that only differ by a methyl group (97). However, in this last case, SELEX procedure was carried out in order to remove oligonucleotides

that could present an affinity for caffeine, using a counter selection process, thus showing that the affinity of an aptamer to recognize some structural analogs strongly depends on the SELEX process. Indeed, the SELEX procedure can also be adapted in order to select aptamers showing an affinity for different targets by using them alternatively during the selection. This shows the possibility offered by the SELEX technology when preparing aptamers to control their specificity that cannot be achieved with antibodies.

8 - Reusability, regeneration,

The reuse of ISs is not recommended by the suppliers of commercial immunoaffinity cartridges. However, many studies show that their regeneration is possible which is interesting considering their cost. Indeed, despite their susceptibility to irreversible denaturation in some conditions, numerous studies have shown the possibility to reuse ISs after their storage at 4°C in a PBS solution, which is close to physiological conditions and in the presence of an antimicrobial agent such as sodium azide. This possible regeneration was confirmed recently by a detailed study related to the reusability of commercial ISs dedicated to mycotoxin analysis (98). In the same way, despite their sensitivity to nuclease, OSs can be stored at 4°C in a buffer solution, whose composition is close to the binding buffer, and containing also sodium azide. Their reusability can be explained by their high stability to low and high temperature, broad pH range and salt, these different parameters affecting the binding of the target without causing irreversible denaturation. This can be explained by the stability of the phosphodiester bond and by the possibility to improve their stability by chemical modification (99). In addition to their less expensive preparation, aptamers have short renaturation times and can therefore be reused after a few minutes in a buffer a few minutes against 24 or 48 hours for antibodies. Thus, the reusability of OS-SPE cartridges has been demonstrated, as for IS, by their successive application, 5 times (39) and even 15 times (40), to the purification of cereal extracts.

The reusability also depends on the nature of the bonding, non-covalent bonding being less stable than covalent bonding as previously mentioned. It was also reported that the use of orientated immobilization of antibodies also gave rise to a higher stability of the IS compared to random immobilization (96). Of course, the stability of these sorbents depends on the number of applied real samples.

The reusability of OS and IS was also demonstrated for miniaturized devices. As an example, an OS-SPME fiber was applied up to 20 times to plasma samples (84). For IS-SPME, Yao *et al.* mentioned that the reusability 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 (83). This is in good agreement with another study mentioning that only 58% of the binding capacity remained after 10 uses (82). Similar results were reported for IS monoliths that were reused up to 50 times without observing any loss of trapping efficiency (60). In return, some authors observed losses after 5 (100,101) or even 3 (102) uses on pure samples. This loss of performance after 3 uses was observed on 6 monoliths (whose performance was similar for the first uses) confirming this loss of performance. The results of the first use nevertheless illustrate the repeatability of the monolith preparation also reported by other groups (57,60). Concerning this last point, it can be considered that the commercialization of Sepharose based-ISs illustrates the reproducibility of their preparation. Concerning other formats such as miniaturized ones, the reproducibility of the synthesis of monolith and of the grafting of antibodies or of aptamers was already demonstrated (48,57).

9- Conclusion

The potential of using immunosorbents for the selective extraction of molecules from complex samples is no longer demonstrated. In order to reduce the development cost of these selective supports, it seems interesting to replace antibodies by aptamers which allows to develop equally

varied extraction tools with a similar potential in terms of extraction yields and selectivity. However, it is important to note some properties that make them different, such as their ability to recognize different analogues, as a result of an immunization choice for some or as a result of selection criteria for others. The possibility of easily introducing certain chemical functions to assist in their grafting can increase the interest of aptamers. However, it is still important to continue work on identifying aptamers sequences of sufficient affinity, of the nanomolar order as is generally obtained with antibodies, to allow the development of supports with high retention potential.

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