Review

Biosorption: A new rise for elemental solid phase extraction methods

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ABSTRACT

Biosorption is a term that usually describes the removal of heavy metals from an aqueous solution through their passive binding to a biomass. Bacteria, yeast, algae and fungi are microorganisms that have been immobilized and employed as sorbents in biosorption processes. The binding characteristics of microorganisms are attributed to functional groups on the surface providing some features to the biosorption process like selectivity, specificity and easy release. These characteristics turn the biosorption into an ideal process to be introduced in solid phase extraction systems for analytical approaches. This review encompasses the research carried out since 2000, focused on the employment of biosorption processes as an analytical tool to improve instrumental analysis. Since aminoacids and peptides as synthetic analogues of natural metallothioneins, proteins present in the cell wall of microorganisms, have been also immobilized on solid supports (controlled pore glass, carbon nanotubes, silica gel polyurethane foam, etc.) and introduced into solid phase extraction systems; a survey attending this issue will be developed as well in this review.

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Contents

1. Introduction

Over the past years, efforts have been focused on the development of extraction techniques that allow efficient extraction of elements and species, high level of automation along with reduced solvent volumes and time. However, literature dealing with extraction procedures usually realm them in isolation of analytes from potentially interfering sample components getting these analytes into a form suitable for analysis. Nowadays, extraction techniques have expanded their boundaries by the introduction of new

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sorbents with higher retention capacity and selectivity [1–5]. The mechanism involving the removal of different element species through a passive binding to a biosorbent from an aqueous solution is defined as biosorption [6]. Biosorption has become an attractive tool for solid phase extraction (SPE) methods. This approach joins all the advantages of SPE in concordance with green chemistry statements. This survey reviews articles dealing with the specific application of microorganisms (MOs) and biomolecules applied specifically to SPE analytical approaches in flow injection (FI) and batch systems since 2000.

The search for novel sorbents and ion-exchange systems for analytical applications as well as remediation of metals from natural and industrial wastewaters has been, and still, is a challenge and has constituted in an important area of contemporary research [7,8]. In this context, new materials have incorporated to the wide list of traditional ones. It would be obviously impracticable to cite even briefly all the contributions on the uses of these materials that have enriched the relevant literature to date. Nonetheless, it was worth to mention some representative papers that have opened new trends.

A separation process removes, isolates, or separates a substance from a sample by the differences in the chemical properties of the substances involved [9]. When extracting liquid samples, the traditional liquid–liquid extraction faces several limitations namely, use of an extractant non-miscible with the sample, difficulty in extracting polar and ionic compounds from aqueous matrices, large organic solvent volumes resulting in a diluted extract, etc. As a technique derived from liquid chromatography, SPE starts with a conventional column cleanup and fractionation. The analytes are extracted by sorption, eluted with an appropriate reagent, and derivatized (if necessary) or directly detected. Through these procedures analytes can be preconcentrated as well, defining preconcentration as an operation (process) as a result of which the ratio of the concentration or the amount of microcomponents (trace constituents) and macrocomponents (matrix) increases [10]. Selective sorption or elution enables speciation analysis without the need of complex instrumentation for detection [11]. Speciation analysis is defined as the analytical activity of identifying and/or measuring the quantities of one or more individual chemical species in a sample, being chemical species defined as the specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure [12].

The state of the art of SPE indicates that there is a trend to develop green chemistry methods, miniaturizing, flow methodologies such as flow injection analysis (FIA), sequential injection analysis (SIA) and lab-on-a-valve (LOV) [13].

The desire for even more selective phases is the current driving force in SPE research. In order to achieve this goal more sophisticated sorbents are being developed. In recent years, one of the most attractive sorbents employed for elemental speciation have been microorganisms, or more specific biomolecules immobilized on different supports [14,15]. This tendency is attributed to their ability to retain metals and metalloids from solutions. One of the reasons appear to be the fact that more binding sites are available when microorganism are used for metal retention in comparison with those traditional chemical ion exchange chelating materials.

Diverse microorganisms have been immobilized and employed as sorbents in SPE for analytical purposes [14,16]. MOs are available and compatible with green chemistry methods, for instance many types of living microorganisms (such as marine, estuarine and freshwater algae) are environmentally ubiquitous and therefore easy to collect, while others such as green algae or yeast are easy to cultivate and harvest. Large quantities of dead biomass are available as byproducts of pharmaceutical and enzyme manufacturing or the fermentation industry. In order to be introduced in FI analysis, MOs must be immobilized on solid supports [17–21], which improve

their mechanical properties of strength, rigidity and porosity and reduce swelling compared to free suspended cells. On the other hand, biosorbents can be easily regenerated using the FI approach, and so they have found application in automated procedures. In these procedures, the biosorption processes take place due to a metabolism-independent mechanism on dead MOs cell walls being the major site of uptake. On the other hand, bioaccumulation does not occur under the mentioned experimental conditions because it involves a metabolism-dependant mechanism with MOs live cells, being the main difference with biosorption [22]. The solution containing the analyte ions are adsorbed on the surface through interactions with chemical functional groups found in the cell wall biopolymers. Potential binding sites on the cell surfaces include amines, amides, imidazoles, hydroxyls, carboxylates, phosphates, thiols, thioethers, and other functional groups [23]. Metabolism-independent biosorption is generally rapid and affected by pH and unaffected over a modest temperature range (4–30 °C) [23]. These characteristics of immobilized MOs enhance their suitability for analytes retention under controlled and normal laboratory conditions.

As mentioned previously, different functional groups on the surface of MOs are responsible for analytes sorption. Following this goal, different biomolecules have been immobilized on solid supports to be introduced into SPE systems, being amino acids and peptides the most employed [15,24–26]. These biomolecules possess N-, S-, and O-containing groups allowing a selective retention of different elemental species. The immobilization approach into solid supports, like controlled pore glass (CPG) [27–33], provides the opportunity of column packing and, under these conditions, amino acids acquire a specific binding orientation in the presence of a particular analyte. The high specific surface area of some solid supports like CPG enhances the number of immobilized molecules, providing an elevated metal retention with easy release and reusability [34]. Amino acids act as bidentate ligands with coordination involving the carboxyl oxygen and the nitrogen atom of amino group [35]. Furthermore, specific peptides can wrap around the analyte to provide a three-dimensional binding cavity [36]. Most of the bibliography discusses the application of immobilized amino acids to SPE system; however, other biomolecules had also been applied such as nucleic acids [37].

2. Biosorption

Biosorption is a term that usually describes the removal of heavy metals through passive binding to biomass from an aqueous solution. It is mandatory to remark that only the biosorption process is responsible for metal concentration by non-living biomass owing to the absence of metabolic activity necessary for intracellular metal accumulation [3,38,39]. Biosorption is a very fast process involving dead MOs, in contrast with bioaccumulation that involves live MOs and parameters like temperature, metabolic energy, the presence of metabolic inhibitors, contact time and culture medium come into question. Other parameters are relevant and must be evaluated and optimized initially like the extraction pH, contact time between biomass and metal ions, the influence of other ions and sorption/elution flow rates when FI systems are involved, which should be also compatible with the selected determination system [1].

The cell wall constituents play a key role in metal sequestering [6]. Such compounds possess numerous functional groups, including carboxylate, hydroxide, amine, imidazole, sulfate and sulfhydryl, with various charge distributions and geometries, so they can selectively bind certain metal ions. Binding is attributed to ion exchange, adsorption, complexation, microprecipitation and crystallization processes occurring on the cell wall [8,40–43].
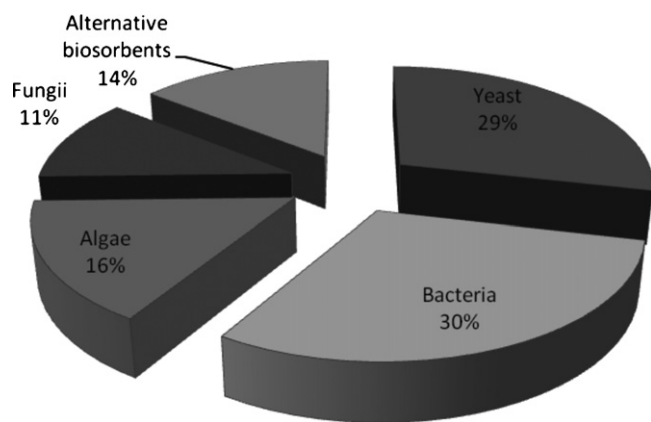
Fig. 1. Distribution of MOs employed as biosorbents for analytical purposes.

Both free and immobilized MOs could be used as biosorbents. Free MOs are generally used in batch procedures. The general batch procedure requires that the analytical solution be mixed with an exactly defined mass of a given microorganism. The concentration of metal ions continually decreases while the release of protons often increases, and the efficiency of biosorption can be altered due to unfavorable changes in solution parameters. Other disadvantages of such procedures are associated with solid/liquid separations, instability of the biomass over consecutive cycles, and the need for multiple extractions of metals that have low partition coefficients [1]. To overcome all these pointed out drawbacks, chemical transformation via the immobilization or "entrapment" of the biomaterial in an inert polymer has been employed. These procedures combine the high binding capacity of microorganisms with the advantages of on-line matrix isolation in flow-analysis, low resistance to fluid flow, self-supporting rigidity, excellent durability, easy regeneration and recovery of biosorbents and metals [23]. Covalent immobilization of algae, yeast, bacteria and fungi on controlled pore glass produces materials with excellent stability [44]. Thus, sepiolite [38], silica gel [45,46], and synthetic resins [47,48] had been also used as supports.

2.1. Biosorbents

2.1.1. MOs employed in biosorption processes

Different MOs have been employed into biosorption processes. Fig. 1 depicts a composite picture of the most studied sorbents in the covered period. It is clear that yeast, specifically *Saccharomyces cerevisiae*, is the representative microorganism of this group. Into this tendency, yeasts are followed by bacteria and algae. Other MOs like seaweed and fungus have been also studied but in less extension.

2.1.1.1. Yeast. The cell walls of yeast include a large number of complex organic compounds and their polymers, such as glucan (28%), mannan (31%), proteins (13%), lipids (8%), chitin and chitosan (2%) [49]. Different cell components allow different charge distributions and geometries, providing to yeast the possibility of binding different elements. The sequestration of metals has been the issue most studied as reflected by the number of publications reported. The research over the last ten years involving the use of immobilized yeast for SPE systems is detailed in Table 1. This table depicted the different yeast species employed for SPE, as well as the determination technique employed, the analyzed elements and the application of the SPE system to different samples. In order to compare the separation efficiencies of each system, the enrichment factor (EF) has been depicted. EF was chosen to compare since this is an important parameter in preconcentration process while others, such as adsorption capacity, are more important parameter in removal process. The EF achieved with yeast showed a good performance, with a maximum of 100-fold. The focus of the different analyses was put on metals and metalloids of relevant interest from the environmental and human health risk point of view as follows: Cd [14,50–54], Cu [50,51,53], Cr [16,48,50,51], Pb [50,51], Zn [50,51,53], Sn [55], Mo [56], Fe and Co [48], noble metals [45,57], Sb [58,59] and As [60,44]. Even more, Caldorin and Menegário [55] reported the sorption of metal complexes, like Cd-metallothionein, by *S. cerevisiae*.

As observed in Table 1, the most representative MO employed for biosorption from the yeast group is *S. cerevisiae*. In our opinion it is because this substrate is easy to obtain and cheap. Other species have been employed as well for SPE systems like *Debaryomyces hansenii* and *Candida tropicalis* [50]. Within the *Saccharomyces* genus, other species besides *cerevisiae* have been employed like *S. carlsbergensis* [48,53]. These MOs have been introduced in SPE systems in both batch and on-line modes. In the batch mode, solutions of yeast free cells in contact with the analyte were used [51,55,58,60]. Different approaches besides free yeast cells in solution have been employed in batch mode for SPE. Maqueira et al. [61] reported by first time the covalently immobilization of the yeast *S. cerevisiae* on CPG for analytical purposes. For Cd^{2+} preconcentration, Menegário et al. immobilized *S. cerevisiae* in agarose gel as a binding agent for diffusive gradients. Slurries were prepared from the agarose–yeast gel disks and directly introduced into an ICP OES [14]. Mapolelo et al. [51] employed a simpler configuration with a commercial preparation of baker's yeast, strains, with 90% of cell viability [62]. *S. cerevisiae*, *D. hansenii* and *C. tropicalis* strains were tested for Cd^{2+}, Cr^{3+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} preconcentration with FAAS as detection system [16]. On the other hand, the well-known advantages of on-line procedures for SPE systems made that most of the research was orientated to use cells immobilized on solid supports. To this end, other promising supports such as controlled pore glass [16,56,44], calcium alginate beds [57], sepiolite [38], amberlite XAD-4 [48], silica gel [45,53] and polyurethane foam [59] have been employed. As an example, Fig. 2 shows an electronic micrograph of cubes of polyurethane foam obtained by scanning electron microscopy at different magnifications.

Once yeast cells are immobilized on the solid supports, these sorbents usually are packed into minicolumns and introduced as part of on line systems [56]. As an example, the schematic diagram of the instrumental setup employed to preconcentrate Mo via *S. cerevisiae* immobilized on CPG and packed in a conical microcolumn is depicted in Fig. 3. In this study, USN-ICP OES was used as detection system. An aspect to consider when using on line approaches is the fact that the immobilization of biosorbents on solid supports results in low resistance to fluid flow. This issue is confirmed by analyzing the metal uptake flow rates. Menegário et al. [16] and Bağ et al. [38] have reported flow rates of 3.6 and 3.0 mL min^{-1}, respectively; for Cr analysis employing *S. cerevisiae* as biosorbent. The analysis of Mo with *S. cerevisiae* immobilized on CPG employed a sample loading flow rate of 5.0 mL min^{-1} reaching a sample throughput of 22 samples h^{-1} [56], it can be attributed to the high tolerance of biosorbents to high flow rates which is reflected in a higher sample throughput.

Many factors play an important role in the biosorption process. Since immobilized yeasts on solid supports are used as part of FI systems, variables like contact time between the sorbent and the analyte become irrelevant since this is minimal. Among the different systems, the pH is the most relevant factor optimized. Analytes' uptake was evaluated in a wide pH range, but the optimum pH value is generally in a narrow range: from 4.5 to 7.5 [38,56,44]. However, some exceptions can be found, Menegário et al. [59] reported a pH value of 9.0 for the Sb(III) uptake by *S. cerevisiae*. Working pH ranges values vary between species and elements and for this

Table 1
Comparison of solid phase extraction methodologies that use yeast as extractant agent.

Yeast	Analytes	Technique	Sample	Enrichment factor	Reference
Saccharomyces cerevisiae	Cr(III)/Cr(VI)	ICP OES	Water	5–12	[16]
Saccharomyces cerevisiae	Sb(III)/Sb(V)	ICP-MS	Natural waters	~9	[58]
Saccharomyces cerevisiae	Pd	GFAAS	Road dust	NI	[57]
Saccharomyces cerevisiae	Cd	ICP OES	River and sea water	12	[14]
Saccharomyces cerevisiae	Cd, Cr(III), Cr(VI), Cu, Pb, Zn	FAAS	Stream and dam water	1–100	[50]
Debaryomyces hansenii, Candida tropicalis, Saccharomyces cerevisiae	Cd, Cr(III), Cr(VI), Cu, Pb, Zn	FAAS	Stream and dam water, treated wastewater, metal effluent	1–98	[51]
Saccharomyces cerevisiae	Cr(III), Cr(VI)	FAAS	River water	75	[3]
Saccharomyces cerevisiae	Cd	ETAAS	River/lake water	38.6	[54]
Saccharomyces cerevisiae	As(III), As(V)	ICP OES	Natural waters	7	[60]
Saccharomyces cerevisiae	Mo	ICP OES	Water	48	[56]
Saccharomyces cerevisiae	As(III), As(V)	ICP OES	Oyster tissue, Montana soil, pesticide, herbicide, cigarette	NI	[44]
Saccharomyces cerevisiae	Cd(II) and Cd-metallothionein	ICP OES	Cytosols from mouse kidney crab hepatopancreas	1	[52]
Saccharomyces cerevisiae	Sn(II), Sn(IV)	ICP OES	River water, industrial waste water	NI	[55]
Saccharomyces carlsbergensis	Fe, Co, Cr	FAAS	Water	10–25	[48]
Saccharomyces carlsbergensis	Zn, Cu, Cd	FAAS	Green onion, parsley, dam water, lake water, tap water	50	[53]
Saccharomyces cerevisiae	Sb(III), Sb(V)	ICP OES	River water	2.5–8.7	[59]
Saccharomyces cerevisiae	Pt, Pd	GFAAS	Tap and waste water	2–12	[45]

reason, working pH values must be optimized in each case. This fact explains the different pH ranges reported in different studies. In most cases, the elution step is achieved with diluted inorganic acids such as hydrochloric or nitric acids [16,38,56,59,44]. Gil et al. [56] concluded that elemental elution from yeast occurs in a fast way when inorganic acids such as HCl are chosen as eluents in comparison with the study from Marcellino *et al.* [58], who employed thioglycolic acid as eluent to desorb Sb (III) from *S. cerevisiae* in 30 min. The selection of an appropriate eluent is reflected in the different preconcentration factors that can be reached (450-fold when HCl was employed in contrast with the 9-fold obtained with thioglycolic acid).

Regarding to elemental selectivity Zoe et al. [54] established through FT-IR that the functional groups responsible for Cd^{2+} retention by *S. cerevisiae* were hydroxyl, amide, and acetyl. In this context, species of Cr [16,38], Sb [58,59], As [60,44], and Sn [55] have been selectively retained by yeast biomass. Different strategies

have been developed for speciation analysis by the modification of some uptake parameter, mainly the pH. Marcellino et al. [58] reported the selective uptake of Sb (III) by *S. cerevisiae* at a pH value ranging from 5.0 to 6.0, while Sb (V) was not retained in this pH range. Other speciation strategies can be employed like selective elution, which has been reported for Cr speciation by *S. cerevisiae*. Since both inorganic species of Cr, Cr (III) and Cr (VI) were retained by the yeast biomass; the authors tested different concentrations of HNO_3 to elute the species separately [16].

2.1.1.2. Bacteria. Bacteria have a complex membrane that can act as a selective extractant by mimicking the best extraction conditions, usually showing high surface-to-volume ratios and containing abundant potentially active chemosorption sites in their walls. The cell surface hosts multiple functionally and structurally different proteins and they differ considerably from Gram-negative to Gram-positive bacteria [22].
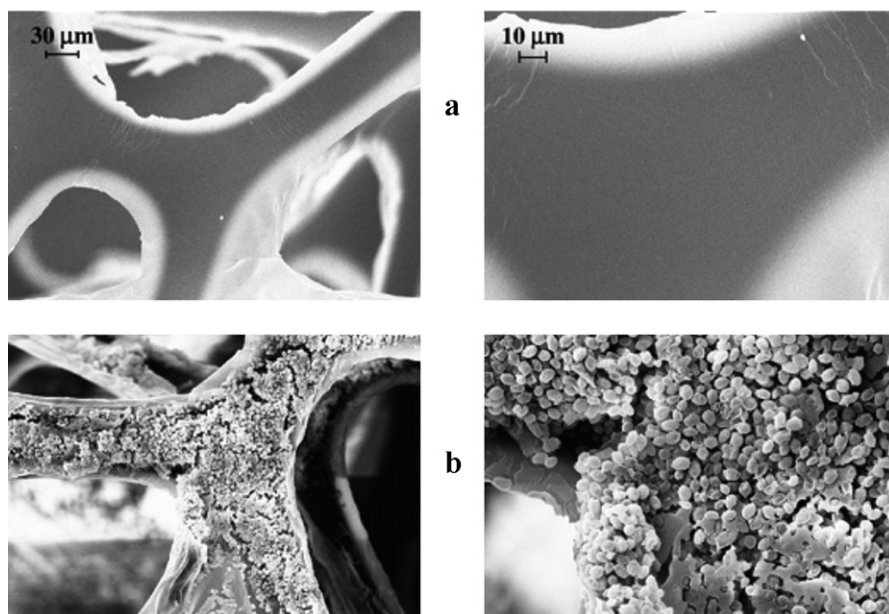
Fig. 2. Electron micrographs of cubes of polyurethane foam: (a) before immobilization; (b) after immobilization.

a Load position
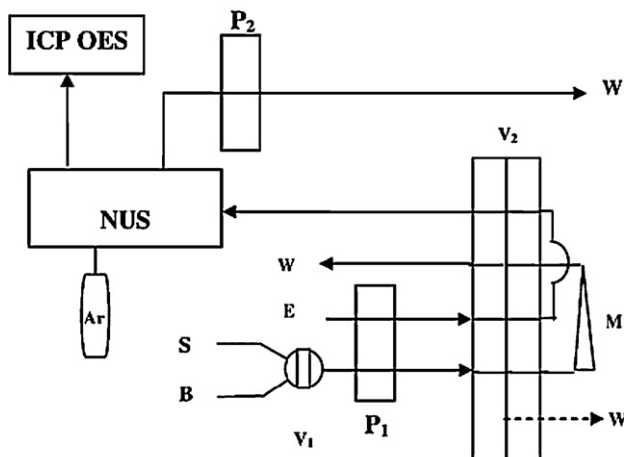
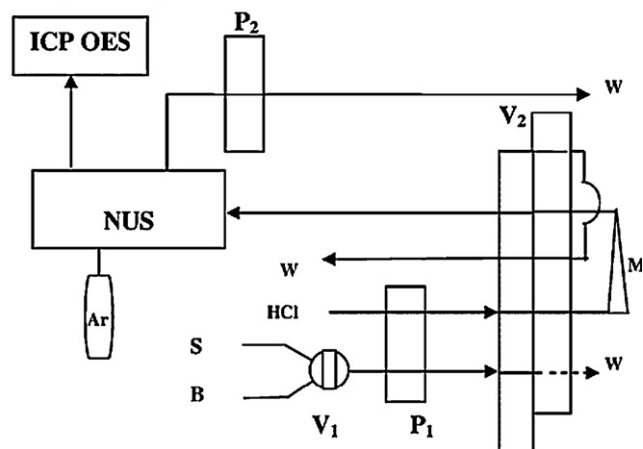
b Injection position

Fig. 3. Schematic diagram of the instrumental setup. B, conditioning line; S, sampling line; W, waste; V1, two way rotary valve, V2, load injection valve, M, conical minicolumn; NUS, ultrasonic nebulizer, Ar, argon gas supply either for plasma and for NUS.

Gram-negative bacteria have a cell envelope composed of a cytoplasmic membrane, a peptidoglycan layer, and an outer membrane with lipopolysaccharides (LPSs) [63], which act as a molecular sieve, while the hydrophilic LPS layer forms a carrier for lipophilic species. On the other hand, gram-positive bacteria lack the outer membrane. However, the cytoplasmic (or inner) membrane contains specific carrier proteins allowing the selective uptake of species [64]. Gram-positive bacteria walls also contain an external thick layer of peptidoglycan with two main components: a polymer of sugars (N-acetylglucosamine and N-acetylmuramic acid); and, crosslinks of short peptides. Other cell-envelope constituents of Gram-positive microorganisms are teichoic acids, which support a whole negative charge [65] and are based on polymers up to 30 units long of glycerol or ribitol joined to phosphate groups.

The use of living and no-living bacteria cells as solid phase extractants for speciation purposes has been reviewed by Aller and Castro [22]. As stated by these authors, the use of living bacteria has lead to some advantages: (i) that the extractant amount is reduced to a minimum; (ii) the selectivity is governed by specific interactions between analyte species and wall proteins; (iii) no-waste is generated; and (iv) it is usually a low-cost process. However, live bacteria used in batch procedures can operate only once per run, as

they are not re-usable, which is obviously a shortcoming. Particular care is required when handling live bacterial cells, as sterilization in all steps of the separation procedure is unavoidable. Alternatively, separation procedures followed using dead bacteria cells may incorporate continuous-flow systems, where immobilized cells are used as column filling [66,67]. In this type of configuration, the variables that need to be evaluated are quite different from those studied in the optimization process for a SPE including cell cultures (i.e. temperature, biomass amount, culture time, etc.). When using immobilized bacteria cells, special attention should be paid to the contact time (flowing rates of the solutions) and solution pH, being the most relevant one. Obviously, bacteria cells immobilized on solid supports have the advantage that they can be re-used in the same way as any other packing. For metal preconcentration purposes, lyophilized bacteria have been immobilized on different supports.

A survey of analytical methods designed for preconcentration purposes with bacteria cells immobilized on solid supports is resumed in Table 2. As it can be observed, most systems have been employed for metal separation [67–74] including some organometallic compounds like methylmercury [71]. The analyzed samples are mostly environmental [67–75], but food [67,69,72,74,75] and biological samples [71,72,75] have been also included proving to these kinds of biosorbents a broad variety of applications and versatility. Regarding separation efficiency, the EFs for the different type of immobilized bacteria are depicted in Table 2. An excellent behavior was reached for all the elements and samples analyzed employing bacteria a biosorbents with EFs varying from 25- [76] to 50-fold [70]. As expected, immobilized bacteria shows selectivity towards the elemental species they bind like the approach reported by Villadangos et al. [76]. In this case, As(V) retention was carried out in a batch mode, by exposing a living double-mutant of *Corynebacterium glutamicum* strain ArsC1-C2 to the sample for a retention time of 1–7 min, before the arsenic distribution equilibrium, between the sample solution and the extractant, was established. The amount of As(V) retained in the biomass was measured by inductively coupled plasma-mass spectrometry (ICP-MS) after a nitric acid treatment. A relationship between the retention time and the amount of analyte theoretically modeled, providing a feasible quantification of the retention process before steady-state, was reached. In this way, the agitation conditions and the retention time could be controlled. An analytical procedure for the retention/quantification of As(V) was thus developed; the detection limit was 0.1 ng As(V) mL^{-1} and the relative standard deviation 2.4–3.0%. The maximum effective retention capacity for As(V) was about 12.5 mg As (g biomass)$^{-1}$. The developed procedure was applied to the determination of total arsenic in coal fly ash, using a sample that underwent oxidative pre-treatment.

Among the FI approaches, Soylak and co-workers developed a number of methods including bacteria as substrate for solid phase extraction procedures. *Bacillus thuringiensis var. israelensis* immobilized on Chromosorb 101 was used for the preconcentration and separation of Cd(II), Co(II) Cr(III), Mn(II), Ni(II) and Pb(II) in environmental samples [75]. The retained metal ions were eluted with 8–10 mL of 1 mol L^{-1} HCl and analyzed by flame atomic absorption spectrometry (FAAS). This method was applied to the determination of the mentioned metals in water and urine samples. Another interesting approach [71] reported by this group was the utilization of *Streptococcus pyogenes* immobilized on Dowex Optipore SD-2 for mercury speciation. Selective and sequential elutions with 0.1 mol L^{-1} HCl (for methyl mercury) and 2 mol L^{-1} HCl (for mercury (II)) were performed at pH 8. The determination of mercury levels was carried out by cold vapor atomic absorption spectrometry (CVAAS). The detection limit for mercury (II) and methyl mercury was 2.1 and 1.5 ng L^{-1}, respectively. The procedure was

Table 2
Comparison of solid phase extraction methodologies that use bacteria as extractant agent.

Bacteria	Analytes	Technique	Sample	Enrichment factor	Reference
Bacillus subtilis	Cu(II), Cd(II)	AAS	River, well water	50	[70]
Streptococus pyogenes	Hg(II), CH$_3$Hg$^+$	CV-AAS	Dog fish muscle	25	[71]
Bacillus thurigesis israelensis	Cu(II), Fe (III), Zn(II)	FAAS	Multivitamin and multimineral tablet, dyalisis solution, natural waters, food	25–50	[72]
Bacillus sphaericus	Cr(VI), Cr(III)	AAS	Water	50	[73]
Geobacillus thermoleovorans subsp. stromboliensis	Cd(II), Ni(II)	FAAS	Natural water and food		[66]
Bacillus sphaericus	Ni(II), Ag(I)	AAS	Natural water, tea, tobacco, soil, sediments	25	[74]
Corynebacterium glutamicum	Arsenate	ICPMS	Solid samples	25	[76]
Escherichia coli	Cu, Zn, Fe, Ni and Cd	FAAS	Alloy	50	[67]
Escherichia coli	Fe, Co, Mn and Cr(III)	FAAS	Water and alloy	25	[68]
Agrobacterium tumefaciens	Fe, Co, Mn and Cr	FAAS	Water; alloy; infant food	25	[69]
Bacillus thurigesis israelensis	Cd(II), Pb (II), Mn(II), Cr(III), Ni(II), Co(II)	FAAS	Red wine, rice, canned fish, sea water, spring water, urine samples	31	[75]

successfully applied to the speciation of mercury (II) and methyl mercury in natural water and environmental samples.

2.1.1.3. Algae. The term "algae" refers to a large and diverse assemblage of organisms that contain chlorophyll and carry out oxygenic photosynthesis. Algae present some characteristics turning them into interesting options to be employed as biosorbents like growing in large quantities with relative easiness and simple handling [77] along with low cost production [78–80].

The cell walls of brown algae contain alginic acid (10–40%), fucoidan (5–20%), and cellulose (2–20%), being the carboxylic groups the most abundant acidic functional group [42]. Red algae cell walls' contain agar, carrageenan, xylans, lectin, and cellulose, while the cell walls of green algae contain mainly pectic substances and cellulose [42,81]. Zoe et al. established that the functional groups responsible of metal retention by *Chlorella vulgaris* were hydroxyl and ether [82]. The main species reported in bibliography employed for SPE systems have been *Ecklonia maxima* [83], *C. vulgaris* [77,42,84–87], *Lemma minor* [88], and *Pilayella litoralis* [89]. These species have been immobilized on two typical and well known supports such as silica gel [77,89] and the resin Celex T [84,85] the most employed. The algae *E. maxima* and *L. minor* were introduced directly without any immobilization process. *L. minor* was introduced in a solution containing Cr for metal retention. Then, the substrate was stirred, centrifuged, and analyzed as slurry by ETAAS. On the other hand, *E. maxima*, as seaweed, was introduced directly in a minicolumn, followed by the insertion of this minicolumn into a FI-system with ICP-MS detection. Elution with mineral acids as HCl is justified since they can cause a lowering in the pH, inducing changes in the tertiary structure of the cell wall, thus releasing the target element by proton interchange. This statement is in concordance with Bacteria and Fungii desorption mechanism. As regards elements, Romero-González et al. determined Cd^{2+}, Cr^{3+}, Cu^{2+}, Pb^{2+} along with the chromium species Cr^{3+} and CrO^{4-} [83].

If the retention mechanism of different elements by algae can be considered similar to the yeast mechanism based on functional groups on the cell wall, then pH becomes the most important factor to optimize. Researchers generally evaluate the elemental retention of algae at different pH values, and in contrast with yeast, they bind elements in a wider pH range. Romero-González et al. [83] reported that the higher retention percentages observed for Cd^{2+}, Cr^{3+}, Cu^{2+}, Pb^{2+} and Cr^{3+} were achieved at pH values from 6.0 to 10.0 with *E. maxima* seaweed. However, Godlewska-Żyłkiewicz et al. [84] studies showed that for Pt^{4+} the highest retention value by *C. vulgaris* was reached at a pH range between 1.5 and 1.8. In

this case, thiourea was used for elution. Zou et al. showed that the retention efficiency of Mn^{2+} and Cr^{3+} on *C. vulgaris* immobilized on silica beads improves at neutral pH values. In an effort to increase the retention efficiency of this system, the authors introduced an external magnetic field to the column to retard the movement of paramagnetic ions by creating an opposite drift velocity of the hydrated ions against the flow direction of the bulk of sample zone. This provided an extra contacting time for the paramagnetic ions with the cells on the micro-column and offered more chances for the ions to interact with the various functional groups on the cell surface, therefore resulted in a significant improvement of the cell-sorption efficiency (45–90%) [86]. In agreement with yeast performance, elution in algae is also characterized by fastness and instantaneousness, reaching elevated preconcentration factors. Tajes-Martínez et al. reported an enrichment factor of 75 when the mercury species, Hg^{2+} and CH$_3$Hg$^+$, where eluted from *C. vulgaris*. In this study, speciation was achieved by selective elution employing different HCl concentrations [77]. Using this system a detection limit of 0.5 µg L^{-1} for Hg^{2+} and 2.0 µg L^{-1} for CH$_3$Hg$^+$, was achieved when both species were determined in deionized, tap and filtered seawater samples.

Even when algae have been extensively studied, their selectivity properties towards elemental species have not been fully explored yet. Speciation studies employing algae have been focused mostly to Cr speciation [83,87,88] and in less extent to Hg speciation [77]. The introduction of different Algae species into SPE system should be extended to other elements in order to exploit the full capabilities of these biosorbent.

2.1.1.4. Fungi. Despite the fact that the biosorption characteristics of fungi and its introduction in SPE systems for FI analysis have been studied in less extension than other biosorbents like bacteria or yeast, some studies have been reported in the literature (Fig. 1).

Most fungi have a cell wall consisting largely of chitin and other polysaccharides [90]. Fungus species employed as sorbent in SPE systems belong to the gender *Penicilium*, like *Penicilium digitatum* [91] and *Penicilium italicum* [92]; and to the gender *Aspergillus* [93] like *Aspergillus fumigatus* [39] and *Aspergillus niger* [2,94,95]. Like other biosorbents, these fungi species have been immobilized on solid supports and introduced in SPE systems. In this case, a wide variety of supports were assessed: pumice stone [91], sepabeds SP 70 [92], Daion HP-2MG [39], Silica gel 60 [2,94], sepiolite [95], and Cellex-T [93]. In this particular case, CPG has not been used.

In contrast to other biosorbents, Fungi immobilized on solid supports have only been employed for the retention of a rather discrete number of metals. Mendil et al. [92] reported the retention of Cu^{2+},

Cd^{2+}, Pb^{2+}, Mn^{2+}, Fe^{3+}, Ni^{2+} and Co^{2+} by *P. italicum* immobilized on Sepabeds SP 70. Other studies also involved the determination of these elements including Zn^{2+} [39,92,94] and Cr^{3+} [94]. In addition, Woińska and Godlewska-Żyłkiewicz detailed the retention of Pt^{4+} and Pd^{2+} [93]. In the mentioned studies, only FAAS was selected as detection system [39,91,95].

Different analytical parameters were optimized in order to reach the higher performance of these fungi-solid supports. In general terms, the higher retention occurs at neutral-alkaline pH range [39,91,92]. In this direction, Soylak et al. [39] established the maximum retention percentages for Cu^{2+}, Pb^{2+}, Zn^{2+}, Fe^{3+}, Ni^{2+} and Co^{2+} at a pH range of 8.0–10.0 when *A. fumigatus* were immobilized on Diaion HP-2MG. In FI systems load/elution flow rates are other important parameters to optimize since they control the time of analysis [96]. In agreement with the previously described biosorbents, immobilized fungi share with them a high tolerance to elevated flow rates with a constant maximum metal retention. Baytak et al. [91] reported flow rates between 6.0 and 4.0 mL min^{-1} for load and elution processes, respectively when *P. digitatum* immobilized on pumice stone was tested. This study also reported an enrichment factor of 50-fold for Cu^{2+}, Zn^{2+}, and Pb^{2+} determination.

Regarding selectivity of Fungii, only one study was found by the authors in literature and it was carried out by Bağ et al. [95] who developed the analysis of Fe species by the selective sorption of Fe^{2+} towards Fe^{3+} on *A. niger* immobilized on Sepiolite.

Summarizing and according to our revision, fungi along with yeast, bacteria, and algae constitute ideal sorbents to improve the analytical performance of instrumental techniques. We do not find a plausible explanation to the fact that only a few studies involving fungi as sorbent for SPE systems have been reported in the literature in the last decade taking into account that fungus encompasses selectivity as a key characteristic. The low cost, easy obtention, analytical properties and selectivity make fungi an excellent biosorbent candidate and open the opportunity to extend the studies towards this particular direction.

3. Biomolecules

Based on the properties of MOs, aminoacids and peptides were subject of many studies for metal retention as synthetic analogues of natural metallothioneins, which are proteins present in the cell wall of MOs [97].

3.1. Aminoacids and peptides

Amino acids and peptides have shown growing interest as new substrates for metal preconcentration and/or speciation analysis due to their metal binding capacity [15,24–26]. To be introduced in SPE systems amino acids and peptides require the immobilization on solid supports being CPG the most employed [27–33]. Many biological systems have demonstrated to provide selectivity, including metal-specific binding. As an example, a well-known class of metal binding proteins, the metallothioneins, are biomolecules that are characterized as having a high degree of metal binding specificity and have been isolated in a wide variety of organisms. For these reasons, metallothioneins have been employed in SPE studies for metal retention. However, the tertiary structure of the proteins, which is believed to contribute to this selectivity, is often lost when isolated from the unique chemical environment within the cell [36,98]. Recognizing that many metallothioneins have a large cysteine content and that sulfhydryl groups present on these residues are primarily responsible for metal binding [99,100], thus, one of the most studied peptides has been poly-L-cysteine [15,24,26,101,102]. Other amino acids and peptides have also been characterized for metal retention and preconcentration such as poly-L-aspartic

acid [32,103–105], poly-L-histidine [106] and poly-L-glutamic acid [105,107–109]. Despite the fact that many of the mentioned studies deal with metal retention by amino acids and peptides, the application boundaries between metal separation and preconcentration for analytical approaches and metal remediation seems to be diffuse. In addition, most of these studies involve the characterization of these amino acids/peptides-support systems with the lack of specific applications. This section will develop the specific application of amino acids/peptides-support systems in SPE systems to improve analytical techniques.

3.1.1. Amino acids and peptides immobilized on CPG

Since the pioneering studies by Holcombe et al. in the late 90s [15,24,26,36,102] and the beginning of the next decade [32,34,98,101,103,106] many studies related to the introduction of amino acids/peptides-support into SPE systems for analytical approaches have been reported. In the first studies, Holcombe and co-workers, characterized the system poly-L-cysteine–CPG as metal chelator [15,24,26], followed by other peptides like poly-L-aspartate [36,104]. The succesfull results obtained with these amino acids chains as metal chelators encouraged the research with new peptides like poly-L-glutamic [103] and poly-L-histidine [106]; and their combinations with the peptide glicine–cysteine–asparagine [101]. The road traced by Holcombe and co-workers was followed by other researchers. Since then, different amino acids have been immobilized on CPG and introduced in SPE systems like L-methionine [27,29–31,33] and L-proline [28].

The presence of diverse functional groups selectivity, strong binding capacity, and environmental innocuity [110–113] are some properties that convert amino acids into ideal molecules for trace element preconcentration. The high specific surface of CPG enhances the number of binding sites, providing a higher metal retention with easy release and reusability [34].

The higher retention capacity of amino acids immobilized on CPG has been demonstrated by Pacheco et al. [114]. This research group reported a retention capacity for Cd of $15.11 \pm 2.58\ \mu mol\,g^{-1}$ on L-methionine–CPG system (L-met–CPG). The amino acid immobilization onto the solid support elapses through its amino group with the carboxyl and the functional group available to interact with metals [114]. Since protonation and deprotonation of carboxyl and functional groups play an important role in metal binding, pH becomes an important factor to optimize in the elemental retention by this class of sorbents. The higher elemental retention occurring at neutral or alkaline pH ranges [27–31,114] is correlated with the deprotonation of the carboxyl group at alkaline pH values and making possible the retention of different metals. In addition, at alkaline pH range (8.0–9.0), it was shown that the system L-methionine–CPG presents both stronger and weaker binding sites for Cd retention [114].

Different studies have demonstrated that a quantitative release from this type of sorbents can be achieved by simply lowering the pH of the solution [15,26,32,36,115]. It has been shown that acids can cause a reversible change in the tertiary structure of amino acids providing efficient and rapid release of metals from the binding cavity. It is of particular importance for analytical applications because the target metal can be easily released enhancing the preconcentration ratio. The analysis of reported enrichment factors showed remarkable values from 11- [28] to 110-fold [33]. This process can be easily observed by analyzing the elution profile of Cd retained on L-methionine–CPG in Fig. 4. The trailing edges of the strip peaks shows a very straighten increase attributed to the accessible binding sites of L-met–CPG to the eluent allowing a fast proton exchange. The transient signals lasted for less than 20 s, which corresponds to 0.6 mL of 10% HCl (v/v) solution for metal removal [114]. The minimal elution volume needed to reach a quantitative release encourages the introduction of these sorbents into
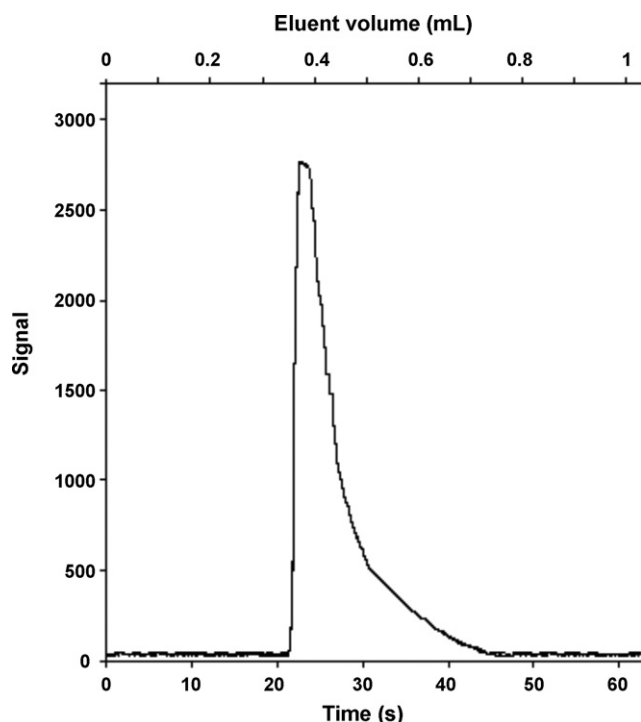
Eluent volume (mL)

Fig. 4. Transient absorbance signal observed when stripping Cd on-line with 10% HCl.

FI systems with ETAAS or ICP-MS as detection techniques. In this study, selenate was eluted with only 50 μL of 10% HCl (v/v) from L-met–CPG [31].

The fact that amino acids acquire a specific binding orientation in the presence of a complexing metal provides selectivity to amino acids and binding specific properties of elemental species towards others has been explored by several works [27–31,33]. Inorganic species of V [29], Sb [27,28], Se [30,31], and Cr [33] have been selectively separated by L-methionine and L-proline immobilized on CPG. The separation procedure was based on the selective retention of a species followed by the determination of the total elemental content and the calculation of the remained species concentration by difference. The selectivity was provided exclusively by the amino acid without the necessity of a complexing reagent. In addition, it was observed and demonstrated that the surface of CPG was not responsible for metal binding [114].

Selectivity and high enrichment factors allow amino acids–CPG systems the application to the analysis of a variety of samples. The analyzed samples have been biological matrices like urine [26,28,116], human hair, and saliva [115]; and environmental samples like river waters [28], soils, and waste waters [33]. Furthermore, sample throughput has been enhanced in FI approaches that include this type of sorbent. This is mostly due to the high load/elution flow that these sorbents tolerate. Load flow rates of up to 10 mL min^{-1} [29,31] and elution flows as high as 4.5 mL min^{-1} [27,30] have been successfully used. This fact favors the introduction of this type of sorbents into hydride generation systems where high sample loading rates are required.

4. Alternative biosorbents

Since the introduction of different MOs as sorbents into SPE systems as an alternative to traditional sorbents such as synthetic resins, polymer-based sorbents, bonded silica gel, zeolite, crown ethers, etc. they have proven to be effective in metal retention and speciation analysis. The most significant drawback associated with

typical ion exchangers is the lack of selectivity in metal retention or weak binding characteristics. On the contrary, several biological structures were verified for metal retention for their high selectivity. This section will discuss the introduction of these novel biological structures as biosorbents in SPE systems for FI analysis. An interesting alternative to MOs has been living cells. In the early 90s, Neidhart et al. [117] immobilized erythrocytes on Ca-alginate beads in order to selectively determine chromium species with ETAAS as detection system. Recently, Chen et al. employed Live HeLa cells immobilized on Sephadex G-50 beads for inorganic arsenic speciation. Researchers reported that both metabolism-independent capture on the cell surface and the metabolism-dependent active bioaccumulation/uptake occur [118].

Egg-shell membrane (ESM) is a biomaterial which consists of two-level tissues, i.e., a calcareous layer and an inner lamellar layer [119,120]. Owing to its unique structure with functional groups in the inner layer, as well as high surface area, ESM has been used as an adsorbent for the sorption of metal ions. Zou et al. reported the selective retention of Cr^{6+} on ESM for the speciation analysis of inorganic chromium. ESM was directly introduced in a minicolumn prior to FI analysis. In addition, Yang et al. [121,122] speciated inorganic selenium on ESM.

Botanically, mosses are bryophytes, or non-vascular plants. They differ from 'higher' plants by not having internal water-bearing vessels or veins, and no flowers and, therefore, no fruits, cones or seeds. They are small (a few centimeters tall) and herbaceous (nonwoody) and absorb water and nutrients through their leaves [123]. Krishna et al. [124] achieved chromium speciation with a minicolumn filled with moss (*Funaria hygrometrica*) immobilized on silica. Cr^{3+} was selectively retained on the moss vs. Cr^{6+}; the system achieved an enrichment factor of 20-fold employing ICP-MS for detection. On the other hand, it has been reported in the literature a couple of studies that employed plants derivates as biosorbents. Xiang et al. reported that peanut shell was chemically modified with phosphoric acid and used as a solid phase extraction material for the determination of trace amounts of Pb^{2+} and Cd^{2+} in food samples by FAAS. The biosorbent was applied to the analysis of tea, liquor, and milk powder, reaching an enrichment factor of 40-fold [125]. Similarly, Alves et al. employed *Moringa oleifera* seeds as a biosorbent for Cd determination in alcohol fuel in conjunction with FAAS. A flocculating protein from the seeds of *M. oleifera Lam.* was isolated and this particular characteristic was used for the treatment of turbid water. The amino acids detected in this protein were mostly glutamic acid, proline, methionine, and arginine [126]. The seeds were introduced directly into a minicolumn for SPE, tolerating sample flow rates of 6.0 mL min^{-1}. Suleiman et al. introduced used green tea leaves into a minicolumn for subsequent Cr determination. Adjusting chemical parameters, the authors reached Cr speciation by the selective retention of Cr^{3+} [127].

Other biosorbent employed for Cd determination in alcohol fuel has been vermicompost, the final product of vermicomposting, a process that involves the degradation, biological stabilization, and neutralization of organic matter through its ingestion by earthworms [128]. Vermicompost was introduced into a minicolumn and directly coupled to a FI system. The sorbent showed mechanical stability tolerating sample flow rates of 4.5 mL min^{-1}. Elution was achieved with nitric acid [128].

Since the biosorption process is based on the elemental retention by functional groups on the surface of any biological structure, the possibilities of introducing radical materials as biosorbents reach no boundaries. Following this concept, an alternative material for metal retention, especially for remediation purposes, has been wool and/or fiber of animal origin since they present polar and ionizable groups on the side chain of amino acid residues and bind charged species such as metal ions [129,130]. Monasterio et al. introduced an innovative material such as llama (*Lama glama*) fibers

directly into a minicolumn to reach chromium speciation in drinking water. The system showed an enrichment factor of 32-fold and was applied to water analysis with FAAS as detection system reaching a limit of detection of 0.3 μg L^{-1} [131].

5. Actual and future trends: nanoparticles as solid support for biosorbents immobilization

Common supports such as resin beads, porous glasses, and carbon-based powders have diameters in the μm–mm range. If one considers "nanometer particles" in the range of 10–100 nm diameter and more conventional media in the 10–1000 μm range; for a given mass of media, the particle density increases with the nanoparticles falling between 10^6 and 10^{15} [132]. Ignoring interior sites, simply decreasing the size of the particles from micrometers to nanometers for a given mass of media, increases the amount of available metal binding surface area by 100–1000 times [133]. This fact clearly shows that the large surface areas of nanoparticles provide advantages *over* common supports becoming promising solid sorbents for SPE procedures.

In this context, in the last years MOs have been immobilized on different nanoparticles and introduced into SPE systems. In FI analysis, these materials are introduced into minicolumns. However, nanoparticles are mostly removed when passing through the column and/or the filter is clogged so that solution does not flow. These problems can be avoided by loading of different substrates on nano materials. The immobilization of various sorbents on TiO$_2$ and SiO$_2$ nanoparticles have been reported for the separation–preconcentration of metal ions in biological and environmental samples due their functional groups [134–136]. The support material is physically and/or chemically interacted with bacteria resulting in better permeability for sample solution. Moreover, the aggregates of support material and bacteria formed do not pass through the filter of the column. Bakircioglu et al. immobilized bacteria biomass on TiO$_2$ nanoparticles and introduced this sorbent in a minicolumn. The system was applied to Pb determination in water samples with FAAS as detector. The system showed a remarkable mechanical stability, tolerating sample flow rates of 9.1 mL min^{-1} [137]. In addition, Baytak et al. reported the immobilization of the yeast, *Yamadazyma spartinae*, on TiO$_2$ nanoparticles for the determination of Cr, Cu, Fe, Mn, Ni, and Zn in water samples by ICP OES [138]. The system reached a maximum enrichment factor of 250-fold.

Different carbonaceous materials such as activated carbon, graphitized carbon and fullerenes have been used in different approaches in analytical chemistry for metal retention [139]. Carbon nanotubes (CNTs) have been proposed as a novel solid phase extractant for various inorganic and organic compounds/elements at trace levels [140–143]. The hexagonal arrays of carbon atoms in graphite sheets of CNTs surface are ideal for strong interactions with other molecules. In addition, their large surface areas make them a promising solid sorbent for preconcentration procedures [144–146]. In this context, different biomolecules like amino acids have been immobilized on CNTs surface and introduced into SPE systems [147–149]. In order to create synergy between nanotubes and biomolecules, these must be connected to CNTs. The best stability, accessibility, and selectivity are achieved through covalent binding because of its capability to control the location of the biomolecules, improving the mentioned properties and reducing leaching [150]. Pacheco et al. have reported two studies employing L-tyrosine immobilized on CNTs for Co retention and Tl speciation, respectively. In the first case, the column reached a metal retention of 101.9 $\pm$ 6.94 μmol g^{-1} of sorbent, achieving a preconcentration factor of 160-fold. The system was applied to water sample analysis with FAAS as detector [147]. Tallium speciation was attained by the selective sorption of Tl^{3+}. Total Tl determinations were carried out by ETAAS. In this study, the system showed mechanical stability by tolerating sample flow rates up to 5 mL min^{-1} and thus obtaining an enrichment factor of 40-fold [147]. The combination of a sensitive technique such as ETAAS and a high enrichment factor due to the use of CNTs allowed reaching a detection limit as low as 3 ng L^{-1}. Recently, Parodi et al. reported the Cd adsorption on different CNTs configuration: CNTs, L-alanine immobilized on CNTs (L-ala–CNTs), and oxidized CNTs. Oxidized CNTs showed the best performance for Cd retention, being followed by L-ala–CNTs and, lastly, by CNTs. An important finding was achieved; the incorporation of specific functional groups in the surface enables differential analyte–adsorbent interaction [149].

6. Conclusion

From all the stated in this review, it is evident that biosorption has been consolidated as a valuable alternative into the solid-phase extraction methodologies. This approach is not only limited to separation, but also elemental preconcentration and speciation analysis are reached as well. From this point, the coupling of biosorption with atomic spectrometric detectors has expanded their boundaries in terms of sensibility and selectivity. Thus, the introduction of biosorbents, from complex living cells to simple amino acids, in synergy with different solid supports improves the retention capacity with easy release of the analytes, providing higher EFs and lower detection limits when compared with the use of traditional manufactured resins. Moreover, since these sorbents present mechanical stability, possibility of regeneration, and operation over a broad range of sample conditions (pH, ionic strength, temperature), they become ideal substrates to be introduced in FI systems. In addition, and due to their performance, application to the analysis of a great variety of matrices such as biological (urine, human hair, and saliva) and environmental samples (river waters, soils, and waste waters) has been achieved.

Recently, biosorption studies have been extended to the exploration of alternative materials like mosses, seeds, cells, egg shells and llama fibers; among others. The introduction of nanostructures (TiO$_2$, CNTs, etc.) as solid supports for biosorbents and biomolecules inmovilization is in the horizon of these SPE systems.

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