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Biological substrates: Green alternatives in trace elemental preconcentration and speciation analysis

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ABSTRACT

Biological substrates have been introduced in Analytical Chemistry to encourage the development of environment-friendly methodologies. This kind of substrates offers advantages such as low cost, very simple production and biodegradability. Moreover, they are considered highly efficient materials for extraction and separation of elemental species.

The current article reviews the applications of biological substrates in elemental preconcentration and speciation analysis, with emphasis in the latest analytical methodologies developed in this field. Batch and on-line microextraction techniques, based on biological substrates are presented and discussed. The applications of immobilized biological substrates are also commented in this work. Special attention is given to the novel immobilization of these substrates on nanomaterials and nanoparticles to develop solid phase extraction techniques. A comparison of methods using biological substrates in terms of analytical performance is provided. Finally, future trends, developments and challenges related to the use of biological substrates for trace metal determination are discussed.

Contents

Abbreviations: AAS, Atomic absorption spectrometry; CPI-MIP-OES, Microwave induced plasma optical emission spectrometry with continuous powder introduction; CV-AAS, Cold vapor atomic absorption spectrometry; ETAAS, Electrothermal atomic absorption spectrometry; FAAS, Flame atomic absorption spectrometry; FIAS, Flow injection analysis system; GC, Gas chromatography; GO, Graphene oxide; HG-AAS, Hydride generation atomic absorption spectrometry; HPLC, High performance liquid chromatography; ICP-OES, Inductively coupled plasma-optical emission spectrometry; ICP-MS, Inductively coupled plasma-mass spectrometry; LLE, Liquid liquid extraction; LOD, Limit of detection; LOV, Lab-on valve; MWCNTs, Multiwalled carbon nanotubes; SEM, Scanning electron microscopy; SPE, Solid phase extraction; UV–Vis, Ultraviolet visible spectrophotometry.

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1. Introduction

In addition to natural sources, metals and metalloids can be originated from anthropogenic sources, such as industrial, agricultural and pharmaceutical activities [1]. Traffic is another anthropogenic source of metals and metalloids, especially in urban areas. It has been stated in the literature that some metal ions play an important role in biochemical processes, providing proteins functional flexibility, contributing with catalytic functions, among other aspects [2]. On the other hand, it is well-known that some metals manifest highly toxic effects in mammals [3]. Since their toxicity depends on their concentration and chemical species, it is essential to evaluate not only total concentration but also chemical species concentration in the samples under study.

Different methodologies and techniques have been used for metal speciation/determination, including high-performance liquid chromatography (HPLC) and gas chromatography (GC), coupled with atomic absorption spectrometry (AAS), inductively coupled plasma-optical emission spectrometry (ICP-OES) or inductively coupled plasma mass spectrometry (ICP-MS) [4–6]. However, the direct determination of metals at trace levels is generally limited because of their very low concentrations in environmental, biological and food samples. For this reason, a previous preconcentration step is required in most analytical methods. Among traditional techniques, solid-phase extraction (SPE) is the most widely used for preconcentration of metals because it offers advantages such as high enrichment factors, low consumption of organic solvent/acids, and allows the development of flow methodologies, minimizing time of analysis and risk of contamination [7].

Biological substrates include bacteria, fungi, algae, yeast, and plant-derivatives (Table 1). They offer several advantages, namely biodegradability, natural abundance, low cost, and simple production.

Table 1
Types of biological substrates used for elemental preconcentration and speciation analysis

Category	Subcategory	Examples
Bacteria	Gram-positive	*Bacillus sp., Streptococcus sp., Streptomyces sp.*
	Gram-negative	*Escherichia sp., Enterobacter sp., Klebsiella sp.*
Fungi	Microfungi	Filamentous fungi (*Aspergillus sp., Alternaria sp., Rhizopus sp.*), unicellular yeasts (*Saccharomyces sp., Yamadazyma sp.*)
	Macrofungi	*Pleurotus sp., Helvella sp., Coriolus sp., Agaricus sp.*
Plant-derivatives	Seeds	*Moringa oleifera*
	Peels	*Musa paradisiaca, Citrus reticulata*
	Husks	*Moringa oleifera. Glycine max*
	Other	*Zea mays, Sorghum bicolor*
Other substrates	-	Algae (*Chlorella sp.*), resting eggs, sea sponge, eggs shell membrane

Moreover, they show a high surface to volume ratio and diverse active binding sites at their surface [8]. It has been found that the numerous active sites of the biological substrates play an important role in the retention of metal ions [9]. These functional groups are represented mainly by carboxyl (–COOH), hydroxyl (–OH), amino ($–NH_2$), sulfate, and phosphate groups.

If we consider the last decade, different biological substrates have been applied in analytical chemistry as filling materials in columns for SPE methodologies [10,11]. Moreover, batch methods have been developed for preconcentration and determination of trace metal ions [12]. Researchers have invested their efforts in the optimization of the experimental conditions to obtain the highest retention efficiency of metals onto these solid phases.

In this review, a brief description of biological substrates mostly used in analytical chemistry for metal preconcentration/speciation is exposed. Recently developed batch and on-line microextraction techniques based on biological substrates are presented and discussed in detail. Advantages and limitations of the application of biological substrates for analytical methods development are also commented, as well as the use of free or immobilized biomass. In addition, possible mechanisms involved in metal retention on biological substrates are reported. Solid phase extraction sample preparation and instrumental techniques used for analyte detection are also considered as well as a comparison of different methods using biological substrates. Finally, future perspectives on the applications of biological substrates for metal speciation and determination are included.

2. Classes of biological substrates used for elemental determination

The main groups of biological substrates used in analytical chemistry for trace metal determination are commented in this section.

Bacteria are prokaryotic microorganisms without nucleus. The genetic information is contained in a single loop of DNA [13]. Some bacteria count with plasmids, which are described as a circular piece of DNA. The presence of plasmids in bacteria makes a difference over other common bacteria, due to plasmids offer advantages (e.g., they may contain a gene that make the bacterium resistant to a particular antibiotic) [14]. This topic acquires relevance in the analytical field, taking into account that some genera of bacteria containing plasmids could be potentially resistant to some toxic or non-toxic metals, which could improve the performance of biological substrates in elemental analysis. In fact, bacterial plasmids conferring resistance to several toxic heavy metal ions have been identified, including Ag^{+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Sb^{3+}, Zn^{2+}, AsO_4^{3-}, CrO_4^{2-}, and TeO_3^{2-} [15]. Regarding to the compounds present in bacterial cell wall, some differences in the chemical composition of Gram-positive and Gram-negative bacteria have been previously reported. The cell wall of Gram-negative bacteria consists in a single layer of peptidoglycan cross-linked by short chains of amino acids, and an outer membrane rich in lipopolysaccharides. Gram-positive

bacteria do not contain outer membrane but are surrounded by several layers of peptidoglycan. Long anionic polymers, named teichoic acids, are thread onto these layers of peptidoglycan [16,17]. Cyanobacteria cell wall presents a similar chemical composition to Gram-negative bacteria, being peptidoglycan the principal component. Archaea cell walls may contain pseudomurein, sulfonated polysaccharide and glycoproteins, offering anionic sites such as –COOH and sulphate groups [18], which could be involved in the mechanism for metal ions retention, and subsequently could be useful for elemental preconcentration. Differences in chemical composition between these groups of bacteria could affect the biosorption of metals, mainly due to the different affinities of the biological substrates for metal ions. These aspects are deeply discussed later in section 3.

Fungi have been also proposed and successfully used for elemental preconcentration and speciation analysis [12,19]. The cell wall of fungi is a microfibrillar structure containing polysaccharides as principal component, proteins, lipids and pigments as minority compounds. A structural component named chitin is also present in the cell wall of fungi [20]. Thus, the knowledge of the chemical composition of cell walls would allow understanding the potential of these microorganisms for metal ions retention.

Another class of biological substrates used in analytical chemistry for preconcentration and subsequent elemental determination are represented by algae [21]. It is an attractive group because the growth of algal cells is relatively simple and cost-effective [21]. The cell wall of algae is composed of polysaccharides as well as glycoproteins. Sometimes, chitin is present as an external thin layer [22]. Green algae cells contain neutral sugars, cellulose, uronic acid, proteins and glucosamine [23], while red algae cells are composed of agar, carrageenan, xylans, lectin, and cellulose [24]. Finally, the cell walls of brown algae exhibit alginic acid, fucoidan and cellulose [25]. It is well-known that the functional groups of cell walls of algae (e.g. –COOH, $–NH_2$ groups) can be implicated in metal binding [26]. It means that altering desorption or elution conditions, a separation of metal species could be possible. It is clear that the diversity and availability of natural algae offer the possibility to study the application of these substrates for preconcentration and speciation of different metal ions.

A great number of plant-derivatives have been used in analytical chemistry for elemental analysis, e.g. mandarin peel, beancoat, seeds, among others [27–29]. Previous articles have suggested some plant-derivatives as biological substrates, such as agricultural wastes. It is interesting to highlight that researchers have found applications of these wastes in analytical chemistry for metal determination, due to the fact that these substrates are extremely cheap and biodegradable, which is environmentally friendly [30,31]. Plant cell walls are composed of polysaccharides, proteins and glycoproteins [32]. Cellulose is the main polysaccharide found in these types of cell walls. Other components such as pectic polysaccharides and hemicellulosic polysaccharides are also presents in plant cell walls [33]. The variety of chemical surfaces in both, microorganisms and agricultural wastes, opens a wide range of possibilities for elemental biosorption. Of course, the choice of biosorbent will be conditioned by the analyte under study and the matrix.

It is important to note that the aforementioned biological substrates can be used for elemental preconcentration or separation of metal species, as both living and non-living cells. It has been previously stated that the employment of living bacteria in SPE techniques requires a minimal amount of bacteria to be used as extractant phase [34], contrarily to the use of classical chemicals such as complexing agents, which need to be added in an excess in order to favor equilibrium conditions. In our opinion, the use of a minimal amount of biomass is a great advantage in the development of these green analytical methods, avoiding possible drawbacks related to matrix effects in the detection stage by atomic detectors (e.g. ETAAS).

On the other hand, the use of died bacteria offers the possibility to develop continuous flow-systems using immobilized cells on different solid supports [35]. Immobilization of the cells on a solid support allows the reutilization of the biosorbent during successive cycles of preconcentration, which is really convenient for analytical methods development. Recently, researchers have intensively focused on the immobilization of biological substrates onto nanomaterials and nanoparticles due to their unique properties, including the wide number of atoms with high chemical activity and adsorption capacities to many ions found on the surface of the nanoparticles [36]. For example, heat inactivated *Fusarium verticillioides* has been immobilized on nano-silica for biosorption of Ca and Mg ions using SPE from aqueous solutions [37]. The procedure was developed by mixing 2.5 g of dried nano-silica, 2.5 g of powdered biomass and 5.0 mL of double distilled water for 15 min, and drying at 60°C for fully dryness. The mixing and drying steps were then repeated for five times in order to obtain a homogenous biosorbent. Dead coliform bacteria have been immobilized on TiO_2 nanoparticles for Pb preconcentration using a flow injection analysis system (FIAS) coupled to flame atomic absorption spectrometry (FAAS) [38]. These Gram-negative bacteria were grown, isolated, washed, centrifuged and lyophilized for obtaining a dry bacterial powder, which was mixed with TiO_2 nanoparticles. After two hours, a centrifugation and a dry step were performed. We consider that this preconcentration method shows the advantage of working with dead microorganisms, avoiding the risk of contamination with different species of bacteria or yeast which could grow on the substrate. Fig. 1 shows a Scanning Electron Microscopy (SEM) image of the coliform bacteria immobilized on TiO_2 nanoparticles.

3. Mechanism involved in elements extraction with biological substrates

Several mechanisms are involved in extraction of metal ions by biological substrates. Metals adsorption results of combining chemical or physical mechanisms [39]. Binding mechanisms consist on ion exchange, microprecipitation, complexation, and oxide-reduction processes (Fig. 2). Infrared spectroscopy has played an important role in the elucidation of the functional groups that are present on the surface of the biosorbents. FTIR spectra are generally used for determination of chemical changes generated on the nature of bonds when metals are retained on the biomass [40]. Another widely used tool to elucidate the type of mechanism that is taking place on the surface of the biomass is the use of SEM, which offers topographic information of the surface and the potential for the biosorption of heavy metal atoms in different parts of the biological substrate.

3.1. Ion exchange

Ion exchange is defined as the reversible interchange process between the ion present in a solution and the counterion attached to the surface of the biosorbent. The ion exchange phenomenon has been studied in detail and it is well established that bivalent metal ions exchange with counterions from active groups on biomass surface [41].

As mentioned earlier, several chemical groups exposed on biomass surface are the responsible for metals biosorption, e.g. –COOH, –OH, $–NH_2$, aromatic rings, and amide groups; given a negative charge to biomass surface [42,43]. In these groups the ion-exchange occurs between the metal ions and the hydrogen atoms of –COOH, –OH, $–NH_2$ and amide groups of the biomass. For this reason, the pH plays an important role in the biosorption efficiency of metals. Blázquez et al. have demonstrated that at low pH values, the high concentration of H^+ in the Cu solution leads to compete

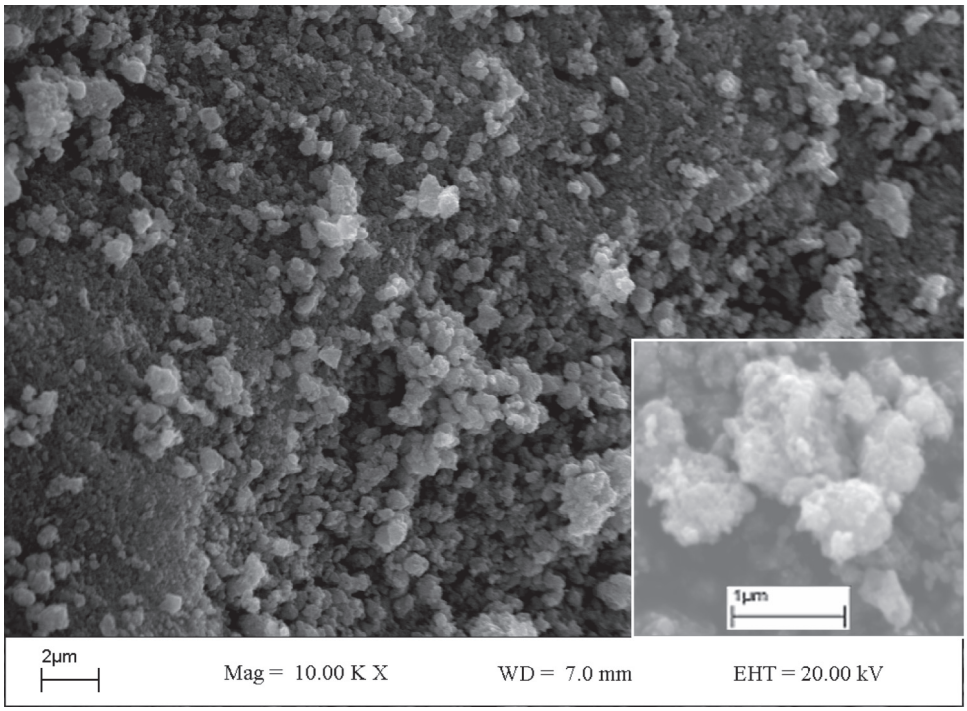

Fig. 1. SEM image of a coliform bacteria immobilized on TiO_2 nanoparticles (inset shows a high magnification image). Reproduced from [38] with kind permission of Springer Science and Business Media.

for the sorption sites of the biosorbent [44]. While pH increased, H^+ concentration decreased, leaving thus much more sites for retention of metals from the aqueous solution. In addition, most metal species have a differential behavior at the same pH, which opens the possibility to use this fact for speciation purposes.

3.2. Complexation

Complex formation is carried out by the combination of cations with molecules or anions with free electron pairs; and could be electrostatic or covalent in character [41,45]. Kotrba et al. reported that

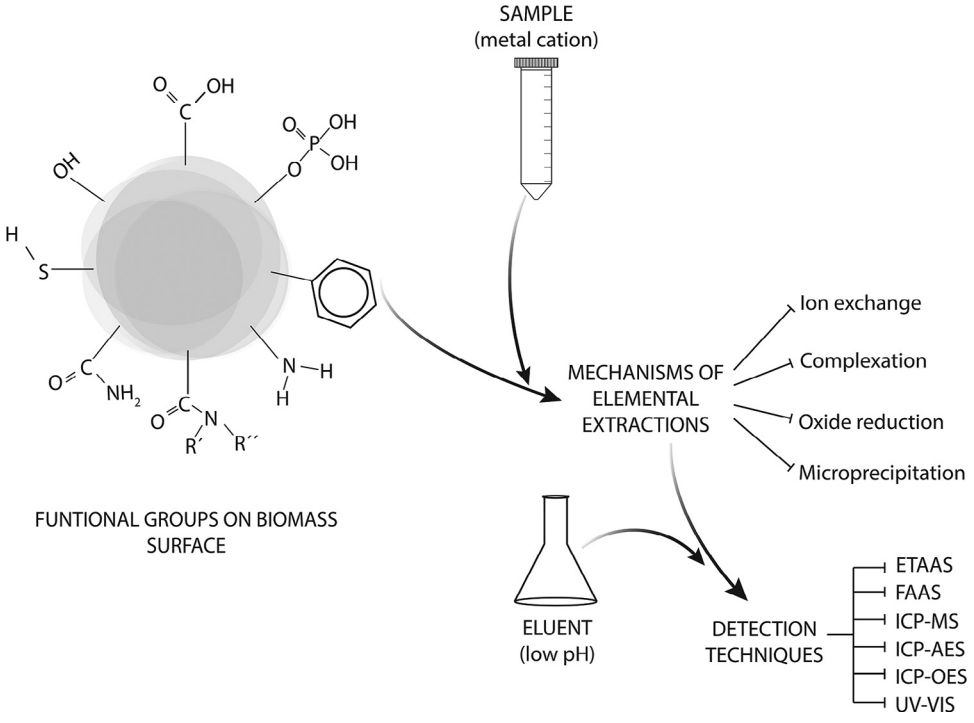

Fig. 2. Representative scheme of the mechanisms and detection techniques involved in the retention of metals onto biological substrates.

during the formation of complexes, the central atom is often the metal of interest and is distinguished from the anions or molecules with which it forms coordination compounds, the ligands. These ligands could be –OH, NH_2, –COOH, phosphate, thiol or other groups which have pairs of electrons to share with the metal. Chao et al. have reported that Cd, Pb, Cu and Ni formed complexes with -OH and -COOH groups, which are exposed on biomass surface of agricultural wastes [46]. Polak-Berecka and coworkers have studied the biosorption of Al and Cd ions by an exopolysaccharide from *Lactobacillus rhamnosus* and determined that -OH and -COOH groups were responsible for complex formation with the analytes [47]. It can be noted that the best biomass for heavy metals complexation is that showing chemical groups with a high number of oxygen atoms.

3.3. Oxide reduction

An electron donor and an electron acceptor are required for oxide reduction. Oxide reduction has been used not only to eliminate toxic metals present in industrial wastes, but also to recover precious metals [48]. However, another mechanism has been needed to finally retain metals on biological substrates. Huiping et al. worked on the reduction of Au(III) to Au(0) with a magnetotactic bacteria possessing functional groups like –OH on its surface. Nevertheless, electrostatic interactions occur before redox reaction for reduced distances between biomass surface and Au(III) [49]. On the other hand, Netzahuatl-Muñoz et al. have used an innovative substrate such as *Cupressus lusitanica* bark to retain Cr(III) species [50]. Some reducing organic compounds occurring on the biomass surface were useful for the chemical reduction of the highly toxic Cr(VI) species to the less toxic Cr(III) species. Albadarin et al. also described that organic groups (tannin and phenolic) were considered as electron-donor and may be involved in the reduction of Cr(VI) as the first step to retain Cr on the biomass [51].

3.4. Microprecipitation

Microprecipitation is a consequence of chemical interaction between a metal and the surface of a biological substrate and it does not depend on biomass metabolism [41]. Alidoust et al. have investigated the mechanism of Cd(II) removal from water samples by calcined oyster shells at different temperatures. The results showed the highest Cd retention when calcination exceeded 750°C [52]. They suggested that the involved mechanism was the precipitation of $Cd(OH)_2$ and $CdCO_3$, previous formation of $Ca(OH)_2$ and $CaCO_3$. The cation exchange between Ca(II) and Cd(II) ions was the first mechanism developed in this work, which confirms that various biosorption mechanisms can take place simultaneously.

4. Sample preparation and instrumental techniques combined with biological substrates

Sample preparation is a critical step in chemical analysis and it is useful for different purposes including the removal of potential interferences, the increase of analyte concentration, and the possibility of developing robust and reproducible methods [53]. As time went on, classical liquid-liquid extraction (LLE) techniques have been gradually replaced by SPE ones. They offer significant advantages over LLE, such as: i) higher recoveries; ii) higher enrichment factors, iii) improved selectivity, iv) specificity and reproducibility; v) less organic solvent and other reagents consumption (environmentally friendly); vi) shorter sample preparation time; vii) lower cost, easier operation and possibility of automation, viii) reusability of sorbents and ix) the possibility of coupling to different detection techniques [7,54].

The selection of a particular sorbent is a key point in SPE as it must fulfill the following conditions: (i) to have chemical affinity with the analyte, and (ii) to exhibit a good adsorption capacity. This choice depends not only on the analyte but also on the sample matrix, which could interact with the sorbent or the analyte [55]. Several sorbents, including silica gel, activated carbon, polymeric resins, activated alumina, zeolite, and nanomaterials, have been used for preconcentration of trace metals and metalloids from complex matrices [56–58]. Many reports can be found in the literature, where biological substrates implemented in SPE systems have been successfully applied for the extraction of metals [59–61]. For example, *Saccharomyces cerevisiae* cells have been used as an efficient substrate for the biosorption of Cr(III) and Cr(VI) species [62]. The biomass was immobilized on controlled pore glass, packed in a minicolumn and incorporated in an on-line flow injection system. Although the biosorption process was not selective for one species in particular, speciation analysis was possible by using a selective elution agent. This step turns the analytical development into a valuable purpose, considering that toxicity of Cr species is markedly dependent of the chemical form of the metal. Likewise, bacteria such as *Escherichia coli* have been applied for the preconcentration and trace determination of Cu, Zn, Fe, Ni and Cd [35]. In this work, a column containing the biomass immobilized on sepiolite was used. A preconcentration factor of 50 was reached and the column could be reused and was stable for 20 adsorption–elution cycles without significant decrease in analytes recovery. Regarding plant-derivatives, it has been reported the use of rice husk as biosorbent for preconcentration and separation of Bi and Cr [63] prior to their determination by FAAS, reaching detection limits (LODs) of 1.3 μg L^{-1} and 1.5 μg L^{-1} for Bi and Cr, respectively.

Several instrumental techniques have been applied for metal detection in biomass-enriched phases, including electrothermal atomic absorption spectroscopy (ETAAS), FAAS, ICP-MS, ICP-OES, and the widespread ultraviolet visible (UV-Vis) spectrophotometry. However, nature and complexity of the organic matrices provided by most biological substrates is a relevant aspect to be considered in the development of an analytical method. In this case, ETAAS might be the best choice due to the direct determination of the analyte is feasible even in the presence of a complex matrix. Thus, it is possible the direct introduction of the biomass loaded with the analyte into the graphite furnace. In order to avoid some drawbacks affecting the analytical reproducibility, a suitable solvent could be used before analyte injection into the instrument. The main challenge of using this detection technique is that the analyte reaches the atomization step after a complete elimination of the matrix in the pyrolysis step. Thus, in order to achieve the highest absorbance-to-background signal ratio, it is mandatory to carefully optimize both, the temperature ramps and duration of the pyrolysis step. Moreover, the injection of different chemical modifiers should be evaluated since they could improve the reduction of chemical interferences. For instance, a green microalgae biomass of *Chlorella vulgaris* has been directly introduced into a graphite furnace for Tl determination at very low concentrations [64]. The introduction of $Pd(NO_3)_2$ and $Mg(NO_3)_2$ as chemical modifiers promoted sharp and well-defined signals with a reduced background. Other alternative is the use of the slurry sampling technique that has demonstrated to be efficient for trace Au and Be determination [65,66]. In this case, bacteria were centrifuged and the resultant pellet was dispersed in 3.5 mol L^{-1} HNO_3 and introduced into the graphite furnace. Good LODs were obtained in both cases (0.004 and 0.05 μg $L^{-1}$for Au and Be, respectively). However, the method showed the disadvantage of being affected by interferences from alkaline earth and transition metals salts.

On the other hand, in on-line flow injection (FI) SPE systems, the challenge of the analyte determination in the presence of a complex matrix is avoided or minimized. Different reagents are used for the elution of the analyte. For instance, Tian et al. have used 70 μL of 1 mol L^{-1} HNO_3 for elution of Cd(II) from a mini-column packed with

bean coat prior to its analysis determination by ETAAS [27]. Since the use of a biological substrate as filling material in a mini-column allows the reutilization of the biomaterial several cycles of preconcentration/elution, the proposed methodology is really cheap and environmental friendly. Usually, the biosorbents need to be regenerated between cycle and cycle, but this step is still simple and economic because only a little volume of a common reagent (e.g. a diluted acid) is necessary.

Flame atomic absorption spectroscopy has been widely used to determine metals after preconcentration with biosorbents. Mineral acids such as HNO_3 and HCl are frequently employed to elute the analytes from the packed biological substrates. Hydrochloric acid has been proposed as elution agent of Pb from a column filled with fungal biomass immobilized on TiO_2 nanoparticles [19]. The volume necessary to ensure the complete elution of the analyte must be studied in detail in order to reduce the consumption of reagents and guarantee the highest preconcentration factor of the analytical method. In this work, a volume of 192 μL of 1 mol L^{-1} HCl was necessary for a complete elution of the analyte.

Regarding ICP-based detectors, two aspects can be mentioned. The first one is the direct introduction of an elution reagent containing the analyte. The second is related to a digestion of the biomass with the retained metal prior its introduction in the ICP [12,67]. In fact, Villadangos et al. have treated filters containing bacteria with 0.2 mL of 70% HNO_3 at 70°C for 120 min [67]. After an appropriate dilution of the digested material (filter and cells), the biomass was directly introduced into the ICP-MS. These previous steps ensured the accurate determination of As by ICP-MS without matrix effects. Nevertheless, these preliminary steps affect one of the main advantages that the ICP-MS technique offers: its rapidity, due to the introduction of a time consuming step, turning the proposed methodology into an unattractive alternative for routine analysis.

For methods using UV-Vis detection, sample introduction does not cause further problems. However, the need of derivatization has been reported for metal determination. It should be noted that the metal to be determined should be capable of reacting with a ligand to form a complex detectable by UV-Vis. For instance, U and Th biosorbed on bacterial biomass were first eluted with HCl followed by reaction with Arsenazo III to form a colored complex [68,69]. In this case, the time required by the analytical procedure is increased as result of the derivatization steps prior to the detection of the analyte. This extra-time will vary according to the kinetics of the complex formation involved in the procedure.

5. Methods based on the use of biological substrates for elemental preconcentration and speciation analysis

Although the present review emphasizes on analytical developments based on the use of biological substrates from 2010 to date, it is important to highlight that the biological substrates including microorganism and plant-derivatives are being used in the SPE methods since many years. For instance, *Bacillus subtilis* with a modified membrane has been used for sorption of As species [70]. In this work, bacteria were incubated in presence of Fe(III) in order to create a ferrated bacteria able to bind with inorganic As species and selectively with As(V) at low pH values. Although Fe(III)-microbe systems have been studied previously, we consider the proposed work as a valuable contribution because an effort was made to modify the raw biological substrate for improving selectivity towards a particular elemental species. Moreover, *Penicillium italicum* have been loaded on Sepabeads SP 70 prior to preconcentration and determination of metal ions by FAAS [71]. This biosorbent is not selective and several metals can be retained at the same experimental conditions. Although it could be expected some drawbacks of interferences by other metal ions, this fact was not reported and the authors informed that the recovery of the analytes was not affected by the presence of concomitants ions. On the other hand, algae have been widely applied for preconcentration techniques. *Chlorella vulgaris* have been immobilized in a micro-column in order to retained paramagnetic metal ions in the presence of an external magnetic field [72]. The method showed a considerable decrease in the interfering effects related with other metal ions by the use of the magnetic field. *Chlorella vulgaris* and *Saccharomyces cerevisiae* cells have been mixed, immobilized on silica beads, packed in a micro-column and used for preconcentration and determination of Cd in water samples. The use of both biological species improved the analyte retention due to the increase of diverse binding sites in the surface of the new material. From our point of view, the use of hybrids biosorbents, resulting from the mix of two different microorganisms, is an excellent alternative to improve the retention capacity of an alone biological substrate toward metal ions. *Chlorella vulgaris* cells have been also used for elemental speciation. Thus, it has been reported its application in a dual mini-column sequential injection system for Cr species determination in water samples by ETAAS [73]. It is well known that *Chlorella vulgaris* is one of the most used algae in the development of green preconcentration/ speciation methods. Probably, new genera of algae should be deeply explored in order to expand the potential of these biological substrates. Other kind of biological substrates proposed are vermicompost, egg shell membranes, and HeLa cells [74–76].

Fig. 3(a) depicts the biological substrates most used from 2010 to date. It can be observed that plant-derivatives, bacteria, and fungi

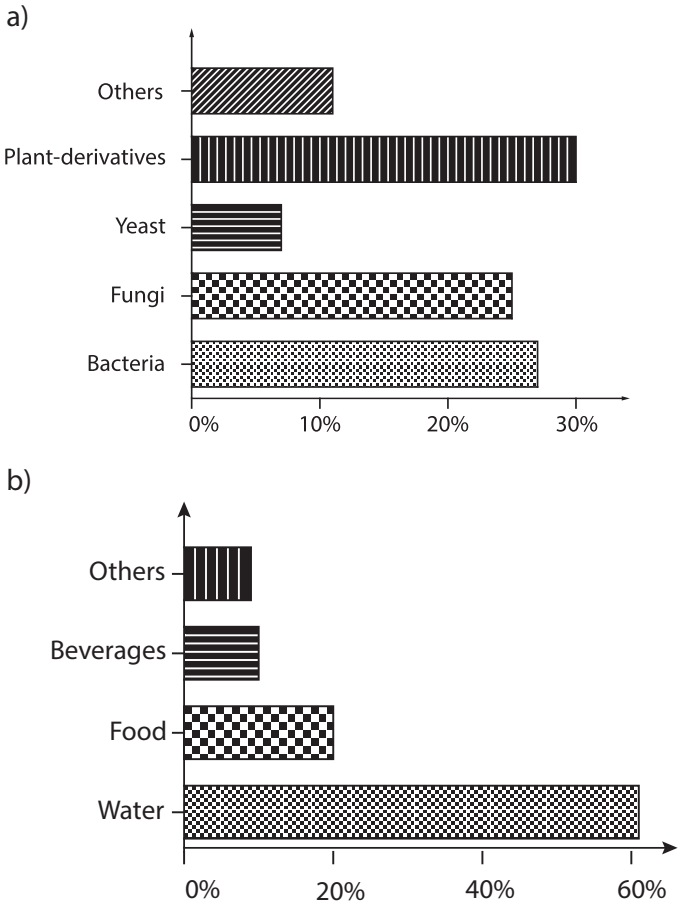

Fig. 3. a) A distribution of biological substrates used for elemental analysis; **(b)** Applications of methods developed in the covered period of the review.

are the most representative ones. On the other hand, yeasts have not been widely applied in the period covered by this review. It is important to mention that, ten years ago, yeasts such as *S. cerevisiae* were the most employed biological substrates for elemental preconcentration [10]. However, nowadays the tendency clearly changed, maybe due to the emergence of a wide variety of novel, cheap and eco-friendly biological substrates, such as agricultural wastes. Other substrates like egg-shell membranes have been also implemented in minor extension. Discussion regarding the implementation of biological substrates for chemical analysis will be given in detail in this section. Applications of biological substrates for preconcentration or speciation of trace metals are summarized in Table 2.

Fig. 3(b) exhibits the applications of the methods based on biosorption for elemental analysis. It can be observed that water samples, including tap, spring, lake, river, sea, snow and rain water, are the most analyzed samples. More complex matrices such as food samples (e.g. meats, eggs, vegetables, and milk) and beverages (e.g. wines, beers) are analyzed in a minor proportion. Minimal applications have been reported for other samples such as ore, gasoline, and coal fly ash samples. These latest contributions have used non living-biological substrates. It makes sense considering that the application of living cells could cause problems through their interaction with the components of a complex matrix, blocking hence some functional groups of the cell wall that could directly bind with the analyte.

The following sub-sections describe and discuss the most important groups of biological substrates used for the development of methods for elemental preconcentration and speciation analysis.

5.1. Bacteria

5.1.1. Gram-positive bacteria

Within Gram-positive bacteria, *Bacillus* genus has been widely employed as biosorbent for analytical purposes. Ozdemir et al. determined Th in a complex matrix such as ore using *Bacillus sp.* as a biosorbent for SPE technique [68]. The concentration of the analyte was evaluated with UV-Vis spectrophotometry. The reusability of the biological substrate was studied loading and eluting several times a Th solution on a column filled with *Bacillus sp* immobilized on Amberlite XAD-4 polymeric resin. It could be observed that 10 cycles of sorption did not change the recovery of the analyte. The method provided good enrichment factors (50–100) under optimal experimental conditions. Although no complexing agent was necessary for the preconcentration step, cost and time of analysis were significant as the eluate had to be collected, diluted with HCl and finally the analyte complexed with Arsenazo III before UV-Vis analysis. Güven et al. have proposed the preconcentration of Cu, Zn and Pb ions using SPE-ICP-OES and a thermophilic bacterium named *Geobacillus stearothermophilus* DSMZ 22 immobilized on silica gel [77]. Good recoveries were obtained for all analytes (97.3–103.1%), indicating little effects of the sample matrix. The method offers the advantage of the reusability of the column for at least 30 times. However, the capacity of the biosorbent for Zn was very low (1.63 mg g^{-1}), meanwhile sorption capacity for Cu and Pb could not be studied, due to their precipitation at high concentration levels at the optimized pH. Although the obtained enrichment factor for Pb was around 10, Cu and Zn showed more acceptable values (50 and 60, respectively). *Geobacillus thermoleovorans subsp stromboliensis* has been immobilized on Amberlite XAD-4 resin and used as a solid phase extractant for the preconcentration of U(VI) [69]. Several radionuclides, including Th(IV), La(III), and Ce(IV) were also evaluated. It could be observed that U(VI) was selectively retained by the biomass, while the rest of the elements remained in the solution. The column could be reused at least for 15 cycles (R > 94%). Under optimal experimental conditions, a 50-fold enrichment factor was obtained with only 5 mL of sample solution. *Geobacillus thermoleovorans subsp stromboliensis* has been also immobilized on Amberlite XAD-4 for preconcentration and determination of Cd and Ni by FAAS [78]. The proposed method showed recoveries between 97% and 100%, enrichment factors around 100 for 500 mL of sample and offered the possibility of using the column for at least 20 preconcentration-elution cycles. Although the authors stated that the biosorption capacity was higher in comparison to the use of chelating reagents, this parameter is low respect to other similar reported studies (4.19 and 3.27 mg g^{-1} for Cd and Ni, respectively).

Lactic bacteria have also found application in the analytical field. Tyburska et al. reported the application of living *Lactobacillus plantarum* immobilized on silica for preconcentration of Se using microwave induced plasma optical emission spectrometry with continuous powder introduction (CPI-MIP-OES) [79]. The authors emphasized on the advantages of silica, such as its chemical stability, noncompressibility, and availability in a variety of sizes and porosities. In order to keep the biological activity of bacteria and to simplify sample pretreatment, experiments were carried out at room temperature. The high enrichment factor obtained (1000) and the good sensitivity reached with helium plasma enabled the successful determination of Se at trace levels in drinking, mineral water, and beer samples. Despite the authors started the experiment with living bacteria, the immobilization on a solid support was developed with dead biomass. It is expected considering that it would be a huge challenge maintaining the cells alive during their use as biosorbents in successive preconcentration/ elution cycles.

Furthermore, Yildiz et al. have used *Streptomyces albus* immobilized on sepiolite for preconcentration and determination of Cd, Zn and Ni by FAAS [80]. Studies were also developed using only sepiolite as adsorbent, while optimizing several conditions (pH, adsorbent mass, etc.). Analytical recoveries were markedly lower than those obtained with immobilized sepiolite (57, 62 and 61% vs. 77, 93, and 97% for Ni, Cd, and Zn, respectively). Therefore, it is clear that bacterial cells were responsible for contributing with different functional groups to increase the retention of the analytes. The method was simple, sensitive, and had the advantage of preconcentrating the analytes without matrix interferences. In a different approach, a FI-SPE technique was applied for determination of As(III) and As(V) species in natural water and food samples using *Streptococcus pyogenes* immobilized on Sepabeads SP 70 followed by hydride generation atomic absorption spectrometry (HG-AAS) [81]. Under the experimental conditions, bacteria retained selectively As(III) species. The LOD, calculated under optimal experimental conditions, was 13 ng L^{-1}. The column filled with the biomass immobilized on the solid support could be reused at least 60 times; meanwhile similar studies have shown lower values between 10 and 20. Moderate biosorption capacity and good recoveries (7.3 mg g^{-1} and ~98%, respectively) were obtained by the proposed method.

It has been previously reported that living bacteria subjected to genetic manipulation improves both the biosorption capacity and selectivity towards target-metal ions [39]. Thus, a genetically modified coryneform bacteria named *Corynebacterium glutamicum strain ArsC1-C2* has been employed for preconcentration of As(V) in coal fly ash samples [67]. In this work, the biomass was directly mixed at room temperature with the sample solution at pH 7.3. The slurry was filtered with nitrocellulose filters and the retained cells were washed with a buffer solution. The filters, containing the retained cells, were digested with 0.2 mL of 70% nitric acid at 70°C for 120 min. After a dilution of the digested material, the analyte was directly determined by ICP-MS. The proposed method showed recoveries between 97.5% and 102%, an acceptable biosorption capacity (12.5 mg g^{-1}) and also offered additional advantages such as accuracy with no matrix effects. This and other examples available in the literature, show how the appropriate use of biotechnological tools could improve the performance of the process.

Table 2
Comparison on analytical performance of biological substrates-based methods for elemental preconcentration and speciation

Biological substrate	Supporting material	Analyte	Sample	Detection	LOD (μg/L)	Enrichment factor	Sorption capacity (mg g^{-1})	Ref.
				Bacteria				
Bacillus sp.	Amberlite XAD-4	Th	Ore	UV-Vis spectrophotometry	1.50	50	17.24	[68]
Geobacillus stearothermophilus	Silica gel	Zn(II)	Tap and river water	ICP-OES	4.48	60	1.63	[77]
		Pb(II)			3.11	10		
		Cu(II)			1.51	50		
Geobacillus thermoleovorans subsp. stromboliensis	Amberlite XAD-4	U(VI)	Lake water	UV-Vis spectrophotometry	2.70	50	11	[69]
Geobacillus thermoleovorans subsp stromboliensis	Amberlite XAD-4	Cd	Lake water	FAAS	0.24	100	4.18	[78]
		Ni			0.30		3.27	
Lactobacillus plantarum	Silica	Se	Drinking water and beer	CPI-MIP-OES	0.06	1000	0.70	[79]
Streptomyces albus	Sepiolite	Ni	Lake water	FAAS	81	25	5.48	[80]
		Cd			53		4.97	
		Zn			43		2.49	
Streptococcus pyogenes	Sepabeads SP 70	As(III) As(V)	Natural water and food	HG-AAS	0.013	36	7.3	[81]
Corynebacterium glutamicum	-	As	Coal fly ash	ICP-MS	0.10	25[a]	12.5	[67]
Agrobacterium tumefaciens	Amberlite XAD-2010	As(III)	Drinking water	UV-Vis spectrophotometry	60	n.r.	n.r.	[82]
Coliform	TiO_2 nanoparticles	Pb	River water, wine, and baby food	FAAS	0.90	1300	n.r.	[38]
Escherichia coli	Carbon nanotubes	Cd	Water	FAAS	3.1	20	4.16[b]/4.83[c]	[83]
		Co			6.2		1.89[b]/3.48[c]	
		Cu			3.0		1.84[b]/4.13[c]	
		Ni			3.0		2.00[b]/4.17[c]	
Synechococcus metallothionein	Graphene oxide nanosheets	Cd	Snow, rain, spring and sea water	ETAAS	0.001	14.6	7.70	[84]
				Fungi				
Filamentous fungi	TiO_2 nanoparticles	Pb (II)	Tap and sea water	FAAS	0.78	868	n.r.	[19]
Aspergillus niger	Activated Charcoal	As	Tap water	ETAAS	1.0	10[a]	n.r.	[87]
Aspergillus niger	-	Pb(II)	Aqueous solutions	FAAS	n.r	n.r	6.2	[88]
Aspergillus ustus	Silicon dioxide nano powder	Cd	Tap and sea water and sea sediment	FAAS	n.r	n.r	112.41	[36]
Rhizopus oryzae	Natural cellulose	Cu(II)	River water and food samples	FAAS	1.6	50	n.r.	[90]
		Fe(III)			1.8	50		
		Mn(II)			2.8	25		
		Zn(II)			1.2	50		
Aspergillus sp.	Cellex-T resin	Pt(IV)	Road dust	ETAAS	0.020	9.7[a]	0.47	[85]
		Pd(II)			0.012	11.5[a]	1.24	
Aspergillus sp.	Cellex-T resin	Pt(IV)	River water, road run-off, wastewater	ETAAS	0.02	2	n.r.	[86]
Fusarium verticillioides	Nano silica Inactivated *F. verticilloides*	Mg(II)	Sea water	FAAS	n.r.	n.r.	50.50	[37]
		Ca(II)			n.r.	n.r.	92.59	
		Mg(II)			n.r.	n.r.	45.66	
		Ca(II			n.r.	n.r.	103.09	
Alternaria solani	Diaion HP-2MG resin	As(III) As(V)	Tap, spring and sea water	HG-AAS	0.011	35	8.5	[89]
Saccharomyces cerevisiae	Cytopore® beads	Cd	Tap, spring, sea and rain water	ETAAS	0.001	30	n.r.	[91]
Baker's modified yeast	-	Sm(III)	River and waste water	ICP-OES	n.r.	5–20	7.24	[12]
Yamadazyma spartinae	TiO_2 nanoparticles	Cr	Tap and lake water	ICP-OES	0.17	250	n.r.	[61]
		Cu			0.45			
		Fe			0.25			
		Mn			0.15			
		Ni			0.33			
		Zn			0.10			
Pleurotus eryngii	Amberlite XAD-16	Cd	Vegetables	ICP-OES	0.67[d]	40	11.3	[60]
		Co			0.82[d]		9.8	
Helvella leucopus	Amberlite XAD-4	Sn	River water	ICP-OES	0.06	50	10.4	[92]
Coriolus versicolor	Amberlite XAD-4	La	Water	ICP-OES	0.27	50	16.5	[93]
Agaricus arvensis	Amberlite XAD-4	Pb	Vegetables	ICP-OES	0.25	40	31.2	[59]
		Al			0.07		45.7	
Agaricus bisporus	Amberlite XAD-4	Th	Ore	ICP-OES	0.10	100	18.28	[94]

(*continued on next page*)

Table 2 (*continued*)

Biological substrate	Supporting material	Analyte	Sample	Detection	LOD (μg/L)	Enrichment factor	Sorption capacity (mg g^{-1})	Ref.
				Plant derivatives				
Moringa oleifera	Seeds	Cd	Alcohol fuel	FAAS	5.50	n.r	n.r.	[95]
Moringa oleifera	Seeds	Zn	Alcohol fuel	FAAS	0.90	23	n.r.	[29]
Moringa oleifera	Seeds	Pb	Beer	FAAS	7.50	93	n.r.	[96]
Musa paradisiaca	Peel	Cd	n.r.	FAAS	2.40	10	1.70	[97]
		Co			27.0			
		Cr			49.4			
		Fe			31.1			
		Mn			6.70			
		Ni			29.6			
		Pb			46.2			
Musa paradisiaca	Peel	Cu	River water	AAS	n.r.	20	20.95	[98]
		Pb					41.4	
Citrus reticulata	Peel	Ni	Alcoholic beverages	FAAS	3.1	12	n.r.	[28]
Moringa oleifera	Husk	Cu	Gasoline	FAAS	0.75	14	n.r.	[99]
Moringa oleifera	Husk	Cr(III)	Water	FAAS	1.92	n.r.	n.r.	[30]
		Cr(VI)			2.45			
Vigna radiata	Coat	Cd	Snow water	ETAAS	0.001	19.8	n.r.	[27]
Glycine max	Hull	Cu	Dried sweet potato, lake water, milk powder	FAAS	0.8	18	18	[100]
Sorghum bicolor	Sorghum chemically modified	Cd	Tea and river water	FAAS	0.16	50	9.53	[101]
		Cu			0.21		11.77	
		Mn			0.33		11.64	
		Pb			1.21		15.01	
Zea mays	Corn silk chemically modified	Cu	Water	FAAS	0.45	39	2.20	[102]
Zea mays	Corn silk chemically modified	Cr(III) Cr(VI)	Wastewater	FAAS	0.85	30	35.21	[103]
				Other substrates				
Chlorella vulgaris	-	Tl(I) Tl(III)	Natural water	ETAAS	0.008	50	4.61	[64]
Egg shell membrane	-	As(III) As(V)	Snow, spring and river water	ETAAS	0.015	n.r.	0.79	[105]
Mercapto-grafted graphene oxide magnetic chitosan	-	Hg	Tap and sea water	CV-AAS	0.06	80	>400	[106]
Resting eggs	-	Cd(II)	Water, crab and seaweed	FAAS	2.4	67	34.3	[107]
		Co(II)			41.4		46.0	
		Mn(II)			3.0		27.2	
		Ni(II)			9.6		22.9	
		Cu(II)			4.2		32.9	
Sea sponge	-	Cu(II)	Tap and mineral water	FAAS	1.5–5.6	80	n.r.	[108]
		Fe(III)				40		
		Pb(II)				80		
		Ni(II)				60		
Eggs shell membrane	-	Se(IV)	Water	ETAAS	0.06	17.2	2.6	[104]
		Se(VI)					1.9	

[a] Enhancement factor.
[b] Non-living *E coli.*
[c] Living *E. coli.*
[d] Limit of quantitation.

5.1.2. Gram-negative bacteria

Recently, within Gram-negative bacteria, *Agrobacterium*, *Cyanobacterium*, and coliform bacteria including *Escherichia* genus have been used for elemental preconcentration and determination. A FI-SPE technique based on the application of *Agrobacterium tumefaciens* as immobilized cells on Amberlite XAD-2010 for As(III) preconcentration in water samples was reported [82]. A fixed volume of sample at pH 3 was loaded on the column. Then, the column was washed for removing free metal ions. Finally, an elution step with 1.0 mol L^{-1} HNO_3 was carried out. A sensitive spectrophotometric determination of the analyte was reached using IH-benzo[d]imidazole-2-thiol. The proposed method demonstrated to be inexpensive, simple and sensitive for the determination of As(III) in water samples. Recoveries from 97.75 to 99.35% and a LOD of 5.0 μg mL^{-1} were obtained. Regarding the reutilization of the column, it could be observed that after 15 preconcentration-elution cycles, the recoveries of As(III) slightly decreased (<95%).

Enterobacteriaceae, that is a large family of Gram negative bacteria, encompasses approximately 30 genera, which includes the coliform members such as *E. coli*, *Citrobacter*, *Enterobacter* and *Klebsiella* genera, among others. Bakircioglu et al. have used coliform bacteria physically immobilized on TiO_2 nanoparticles as a biosorbent for trace Pb determination in tap and sea water samples using a FIAS coupled to FAAS [38]. The proposed method reported good recoveries of the analyte (99–101%) and a very high preconcentration factor (~1300). Moreover, the biomass could be reused at least 150 cycles, which is markedly higher in comparison with other studies. The outstanding characteristics of this method could be attributed to the use of a bacterial consortium, which is directly related with an increase of functional groups available for metal retention. The present method demonstrated to be sensitive enough for trace Pb determination in river water, wine, and baby food, with good accuracy and precision. Furthermore, *E. coli* have been used for preconcentration of Cu, Co, Cd, and Ni prior to their determination by FAAS [83]. In this work, living and non-living bacteria cells were immobilized on multiwalled carbon nanotubes (MWCNTs). Initially, *E. coli* cells were mixed with MWCNTs in a 1:1 ratio, dried and placed in the tip of a 50-mL syringe mountable filter. The biosorbent was conditioned at the working pH and then, a volume of sample was passed through the syringe system. Finally, the analytes were eluted drawing and ejecting back with a volume of 0.5 mol L^{-1} HNO_3. The biosorption capacity of living *E. coli* was significantly higher than that of the non-living *E. coli* (4.13 and 1.84 mg Cu per gram of biosorbent for living and non-living bacteria, respectively). It could be due to the fact that living bacteria offer the possibility to bioaccumulate the analyte inside the bacterial cell through a biochemical mechanism; meanwhile the non-living bacteria are restricted in this way. On the other hand, the authors stated that the differences in biosorption capacities could be attributed to the variation in the amount of living bacterial cells because they still grow during and after the immobilization process. In our opinion, this is a real disadvantage because if the bacterial growth could not be controlled during and after immobilization process, the amount of substrate under study is not exactly known and hence it is not possible to estimate the reproducibility of the procedure.

In another work, Yang et al. developed a novel method based on the use of graphene oxide (GO) nanosheets decorated with a cysteine-rich metal-binding protein, cyanobacterium metallothionein (SmtA) [84]. The SmtA–GO composites were then assembled onto the surface of cytopore microbeads, used for selective Cd preconcentration, and incorporated in a sequential injection lab-on-valve system, with detection by ETAAS. Fig. 4 shows the steps for the fabrication of the biomaterial and its self assembly on microcarrier cytopore. It could be observed that the SmtA–GO loaded cytopore showed a higher biosorption capacity than the bare GO loaded cytopore (7.70 *vs.* 2.34 mg g^{-1}). The proposed method offers a novel and selective nanomaterial for trace Cd preconcentration and determination in water samples. However, under optimal experimental conditions, the method only provided a low enrichment factor of 14.6, thus limiting its application for trace element determinations. On the other hand, the proposed biosorbent is not affected by the presence of alkalis and alkaline metals, which turns the methodology into a convenient alternative to be used in elemental analysis of water samples with a high saline level.

5.2. Fungi

5.2.1. Microfungi

5.2.1.1. Filamentous fungi. Filamentous fungi are the most employed fungi for metal determination. Woińska et al. have used *Aspergillus sp.* strain immobilized on resin Cellex-T and packed it into a glass column to separate and preconcentrate Pt and Pd from a complex matrix of road dust [85]. The biosorption of Pd and Pt was carried out in an acidic medium. The selectivity of such biomass to retain these metals could be explained considering that this strain generally retains metals at neutral or slightly basic pH values. Very good LODs were obtained for both analytes (2×10^{-5} and 1.2×10^{-5} μg L^{-1} for Pt and Pd, respectively), even comparable with those obtained with ICP-MS detection. The contents of Pt and Pd in road dust sample were 166.9 ± 14.3 and 229.6 ± 17.8 ng g^{-1}, respectively. Moreover, *Aspergillus sp.* has been applied for preconcentration of Pt from river water, run-off road water and waste water samples [86]. A good LOD was also obtained under optimum condition (0.02 μg L^{-1}), but not as lower as the previous work. Also, a more specific strain *Aspergillus niger* has been used for preconcentration of different metals. For instance, preconcentration of As has been studied by Shahlaei et al. using dried biomass loaded on activated charcoal [87]. The pH of sample was buffered to 6, and the elution step was possible by exchanging metal cation by protons of the used acidic eluent. Finally, the eluate was directly injected into the graphite furnace simultaneously with $Pd(NO_3)_2$ and $Mg(NO_3)_2$ as chemical modifiers. Under the optimized conditions, an enrichment factor of only 10 was obtained. The pH in the culture medium is a parameter that would normally be taken into account when an analytical method is developed. Horrutiner et al. have determined the importance of pH on the growth of *Aspergillus niger O-5* grow, in a culture medium at pH values of 5 and 7 [88]. Punctually, the Pb sorption capacity of the biomass was compared at these pH values and the highest sorption was obtained when fungi were grown at pH 7. Furthermore, it has been reported that treatment of these strains with NaOH prior to contact with the sample, produced an increase in the biosorption efficiency of Pb. This last step causes the release of masked binding sites for proteins and lipids, and enzymes of putrefaction are eliminated thus increasing the likelihood of binding to metals [88].

Other strains of filamentous fungi have been used for metal preconcentration and determination. A consortium of filamentous fungi was collected from an oil treatment plant and used for the development of an on-line FI method coupled to FAAS for Pb determination in sea and tap water samples [19]. In this investigation, a polytetrafluoroethylene column was filled with the dried biomass loaded on TiO_2 nanoparticles. HCl and distilled water were employed for column regeneration after Pb elution with HCl. The column could be reused for 100 cycles without a measurable loss of analytical performance. Foreign ions did not interfere with Pb determination, and analyte recoveries were between 96 and 104%. Using the consortium of filamentous fungi loaded on nanoparticles, a high enrichment factor was reached (~800), which allowed the ultra trace determination of Pb. In a similar approach, a novel method was developed using a column packed with Diaion HP-2MG resin coated with *Alternaria solani*, for As speciation and determination by HG-AAS [89]. These filamentous fungus showed the capability

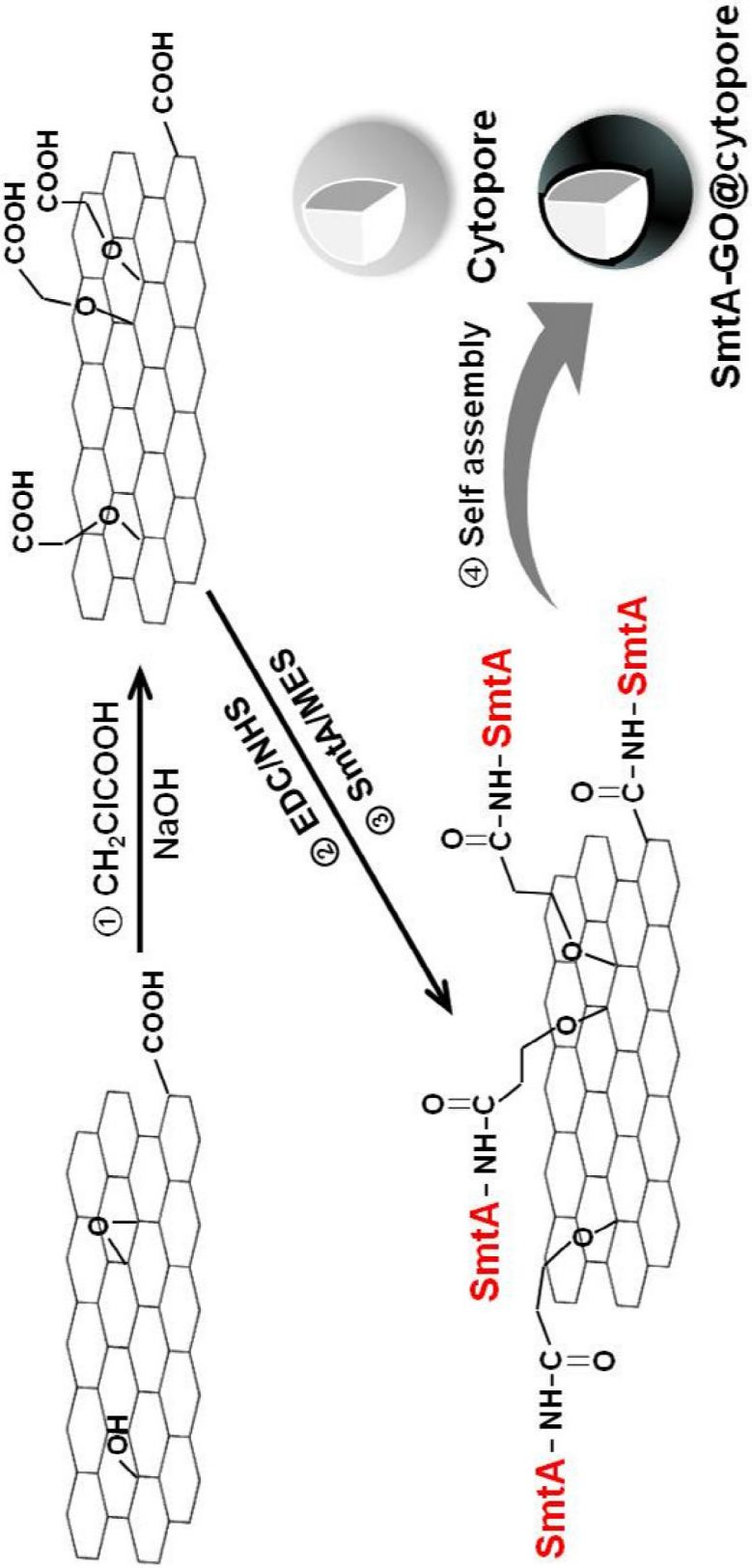

Fig. 4. Representative scheme of the fabrication of SmtA decorated GO and its self assembly on microcarrier cytopore. Reproduced from [84] with permission of The Royal Society of Chemistry.

of distinguish between different chemical species, being As(III) selectively retained on the surface of *A. solani* at pH 7 while As(V) species remained in the aqueous solution. Real samples were analyzed after microwave digestion, obtaining concentrations of total As around 0.15, 0.28, 0.13, and 0.26 mg g^{-1} for human hair, nails, black tea and cigarette, respectively. Despite the successful separation of As species, the method was applied for the speciation in natural water. The concentration of As(V) species was calculated by difference between the total concentration of As and As(III). In our opinion, the methodology presents limitations such the possibility of its application to the speciation analysis of analytes in highly complex matrices (e.g., food and biological samples). In a different study, Baytak et al. have developed a preconcentration method based on *Rhizopus oryzae* immobilized on natural cellulose as filling material of the column for Cu(II), Fe(III), Mn(II), and Zn(II) determination in river water, fish tissues and vegetables samples by FAAS [90]. Good recoveries were obtained for all analytes (80–85%), and a moderate capacity of reutilization of the column was reached (30–35 cycles of preconcentration), without expressing deterioration.

5.2.1.2. Unicellular yeasts. Microscopic fungi include yeast with spherical budding cells and molds with elongate filamentous hyphae in mycelia. *S. cerevisiae* is the most common unicellular fungus known, and widely used in different industries as bakery, wine and beer production. This species of yeast has been used with modification of the surface components by genetic engineering and without it. For instance, through the use of surface engineering procedures, a peptide has been exposed on the surface of a microorganism, and a high selectivity for Cd biosorption was provided by this short metal-binding peptide (*S. cerevisiae-CP2)* [91]. The modified cells have been immobilized on cytopore® microcarried beds and incorporated into a lab-on valve (LOV) system coupled to ETAAS for Cd determination in natural waters. It is an interesting work, since it invites genetic engineering to be part of the creation of a biological substrate with enhanced functionally properties. In this case, it was synthesized a material with a successful interaction with the analyte, with higher tolerance towards solutions with high ionic strength, with major tolerance to foreign metals, especially alkaline and alkaline earth. Another modification on the surface of *S. cerevisiae* has been made by Shakerin el at., in which an organic molecule (red HE-3B) has been biosorbed on the yeast surface for preconcentration and determination of Sm from complex matrices by ICP-OES [12]. Poor enrichment factors (5–10) and moderately sorption capacity (7.2 mg g^{-1}) were reported in this work. Moreover, it was observed that the presence of foreign ions such as Cu, Pb, Nd may affect Sm sorption by the modified yeast. Baytak and coworkers have used *Yamadazyma spartinae* immobilized on TiO_2 nanoparticles for preconcentration and determination of different elements in tap and lake water by ICP-OES [61]. The column was packed with the biomass-supported nanomaterial, preconditioned with HCl, HNO_3, and finally neutralized with water. Then, 20 mL of sample were passed through the column and the analyte was eluted with 2 mL of 5% HNO_3. The column proposed in the present method could be reused for 50–60 cycles without any change in the performance. Moreover, it was possible to achieve an outstanding enrichment factor (~250).

5.2.2. Macrofungi

This class of fungi has been studied as potential solid phases for preconcentration of metals. Özdemir et al. have used a column filled with *Pleurotus eryngi* immobilized on Amberlite XAD-16 resin for Cd and Co determination by ICP-OES [60]. An enrichment factor of 40 and a sorption capacity of 11.3 and 9.8 mg g^{-1} for Cd and Co, respectively were reported. The column could be reused for at least 20 cycles. *Helvella leucopus* immobilized on Amberlite XAD-4 resin has been used for preconcentrate Sn from river water at pH 6 [92]. An analyte recovery of 100% was reached, which exhibits an absence of interferences by other ions. A moderate enrichment factor and sorption capacity was obtained (50 and 10.4 mg g^{-1}, respectively). Moreover, *Coriolus versicolor* has been immobilized on Amberlite XAD-4 and used as filling material of a column for La determination in natural water samples [93]. The column shows an acceptable stability and potential of regeneration, since it could be reused up to 60-fold. The proposed method enhanced the sensitivity of ICP-OES by a factor of 46.8. The *Agaricus* genus has been used for preconcentration of different metals. For instance, *A. arvensis* has been reported for preconcentration and determination of Pb and Al [59], meanwhile *A. bisporus* has been applied for SPE of Th with a column reusability of 30 cycles without loss of the biosorption capacity [94].

5.3. Plant-derivatives

5.3.1. Seeds

Seeds have been applied as biosorbent for preconcentration and determination of several metal ions. Alves et al. have used *Moringa oleifera* seeds in an on-line system coupled to FAAS for Cd determination in alcohol fuel [95]. The seeds were treated with deionized water, dried at 25°C, crushed in a blender, passed through 850 μm-sieves, and used as filling material in a minicolumn. The optimization of flow rates and chemical variables was carried out by multivariate experimental designs. Characterization of the biosorbent showed a large number of functional groups (e.g. amide, carbonyl groups), which could be involved in analyte retention. The authors reported a LOD of 5.50 μg L^{-1} and recoveries from 97.5 to 100%. A similar work described the determination of Zn(II) in alcohol fuel by an on-line SPE system using *Moringa oleifera* seeds [29]. The method achieved an enrichment factor of 23. Although it was not reported the capacity of reutilization of the column, the authors stated that the minicolumn could be regenerated after each preconcentration cycle. *Moringa oleifera* seeds have also been used as biosorbent material for the determination of Pb at trace levels in beer samples using SPE with FIAS-FAAS detection [96]. An enrichment factor of 93 was obtained with the present method while similar works have shown markedly lower values. The recoveries were between 80% and 100%, which are considered as very good values.

5.3.2. Peels

Fruit peels have been proposed as economical biosorbents for preconcentration of several metal ions. Šabanović et al. have developed a SPE procedure using a column filled with pulverized banana peels for preconcentration of Cd, Co, Cr, Fe, Mn, Ni, and Pb with FAAS detection [97]. The proposed method demonstrated to be eco-friendly, inexpensive, and easily applied for preconcentration of different analytes in aqueous solutions. However, poor preconcentration factors and biosorption capacities were obtained. Additionally, Castro et al. have applied minced banana peel for preconcentration of Cu and Pb, prior to their determination in raw river water samples by FAAS [98]. A 20-fold enrichment factor was obtained and the column could be reused for at least 11 cycles without deterioration of its performance. Considering the results obtained in these works, it seems that banana peel is not the ideal sorbent to be implemented in analytical developments. Unfortunately, the authors didn't compare their research with other studies based on the use of similar agricultural wastes that would be useful to remark their achievements.

A SPE method has been developed for on-line preconcentration and determination of Ni in alcoholic beverages using mandarin peel as biosorbent and FAAS detection [28]. Although the enrichment factor was 12, the proposed method offered advantages such as good sample throughput (15 samples per h) and a column reutilization for 40 cycles of preconcentration without loss of stability. Moreover,

the method is low cost, needs minimal manual operation and avoids contamination risks by performing an on-line preconcentration, which opens up an interesting alternative in the area of automated preconcentration methodologies based on biosorbents.

5.3.3. Husks

Some recent analytical studies have proposed the used of husks as eco-friendly biosorbents. Husks of *Moringa oleifera* have been used as biosorbent in an on-line preconcentration system coupled to FAAS for the determination of Cu in gasoline samples [99]. It has to be pointed out that although the biosorption of metal ions by functional groups of the biomass is frequently dependent on pH, the effect of this variable was not significant in this study. In contrast, the mass of husks and sample flow rate had a significant influence on the biosorption of the analyte. It makes sense considering that the retention of an analyte could not be possible if the amount of biological substrate is insufficient, and hence the presence of binding sites potentially useful for retention of the analyte is low. Moreover, the sample flow rate is a significant parameter in the biosorption process due to it should reach the minimal value that allows the retention of the analyte into the biological substrate. Alves et al. have developed a SPE procedure using *Moringa oleifera* husks as biosorbent for the separation and direct determination of Cr(III) and Cr(VI) in water samples [30]. It was observed a selective retention of Cr(III) species at pHs 7–9 while Cr(VI) species was retained at pHs 1–2. The main advantage of the proposed method was the excellent capacity of reutilization of the biomaterial, since it could be reused for at least 100 preconcentration-elution cycles. A mungbeat-coat has been used to fill a minicolumn for an on-line adsorption and preconcentration of Cd from snow water samples with ETAAS detection [27]. An enrichment factor of 19.8 was obtained, and the column could be reused for 100 cycles of preconcentration with no deterioration. Finally, soybean hull was chemically modified with citric acid and used as a biosorbent for trace Cu determination in food samples by FAAS [100]. Under optimal experimental conditions, the biomaterial showed a very good adsorption capacity (18 mg g^{-1}), using the column up to 30 cycles with analytes recoveries around 90%. However, chemical modification of a biological substrate could be an expensive alternative in biosorption processes unless it shows to be highly selective for retention of a target analyte and/or of high economic value in the health or environmental field (e.g. toxic metal, precious metal).

5.3.4. Other agricultural wastes

Sorghum agricultural waste has been proposed as another SPE material for determination of Cd, Cu, Mn, and Pb in water and tea samples with FAAS detection [101]. Both, natural and chemically activated with phosphoric acid sorghum wastes were studied in detail. It could be observed that adsorption capacities were up to 4.5-fold higher when chemically activated with phosphoric acid sorghum was used, which is related to the presence of carboxylic acid groups originated from the chemical modification. This approach resulted fast, simple and inexpensive. Moreover, the method offered the advantages of high precision and accuracy. The LODs reached were in the range from 0.16 to 1.21 μg L^{-1}.

Chemical modification has also been proposed in corn silk using nitric acid for its use as a novel biosorbent for SPE of trace Cu in water samples with FAAS detection [102]. The modified biosorbent offered a new oxygen-containing functional group, –O–NO_2, and a large number of negative charges, which was favorable for Cu retention. A theoretical maximum adsorption capacity of 95.7 mg g^{-1} was reported. The column was reutilized for ca. 50 operating sorption-desorption cycles. Although a poor dynamic sorption capacity was reached (2.2 mg g^{-1}), the authors suggested that the chemical modification of the biosorbent is a simple and feasible process. Chemical modification has also been applied in corn silk for solid SPE of Cr(III) and Cr speciation [103]. An aliquot of 30 mL of sample, at pH 6, was loaded into a minicolumn filled with the biosorbent. Then, an air flow was introduced for cleaning the column and tubes. Finally, an elution step was carried out by passing through the column 1 mL of 1.5 mol L^{-1} HNO_3 in counter current. The biosorbent was selective for retention of Cr(III) species. Total Cr was determined after reduction of Cr(VI) species with hydroxylamine hydrochloride, and Cr(VI) concentration was determined by difference between total and Cr(III) concentrations. The proposed method showed a very good adsorption capacity (35.21 mg g^{-1}) and a LOD of 0.85 μg L^{-1}.

5.4. Other substrates

Even when less used, other substrates have demonstrated to be highly efficient for preconcentration of metals. Microalgae cells of *Chlorella vulgaris* have been used for the development of a simple and environmentally-friendly technique named cellular phase microextraction (Cell-PME) for Tl species determination in natural water samples [64]. In this work, Tl(III) species were selectively retained by the biomass, which could be attributed to a selective complexation between functional groups of microalgae cell walls and the trivalent Tl species. A challenge related with the direct introduction of the microalgae biomass into the graphite furnace of a spectrometer ETAAS has been successfully overcome in this work, leading to the sensitive and accurate determination of Tl even in the presence of a complex organic matrix. A biosorption efficiency of 65% and an enrichment factor of 50 were obtained with only 5.00 mL of sample. Egg shells has been used for inorganic Se speciation in water samples [104]. Initially, a thiol group was created on the egg shell membrane by reduction of disulphide linkage. The new biosorbent was useful to retain both Se(IV) and Se(VI) species. However, Se(VI) was selectively eluted with HNO_3. For this reason, $KMnO_4$ was used for oxidation of Se(IV) to Se(VI) and its subsequent determination by ETAAS. An enrichment factor of 17.2 was reached and the column could be reused almost 20 times. Chen and coworkers have employed egg shell membrane with a methyl esterification on the surface [105]. This chemical modification provided an improvement on biosorption efficiency of the analyte, and demonstrated a high selectivity toward arsenate ions over arsenite species. Other biological substrate, such as chitosan, has been modified for effective preconcentration of Hg(II) [106]. The biosorbent was synthesized by adding GO to a solution of chitosan-magnetic nanoparticles, following by the addition of 3-mercaptopropyltrimethoxysilane. These chemical modifications provided a magnetic separation, improving specific surface area and sulfur soft non-metal group, respectively. From our point of view, this magnetic separation allows not only the possibility of reutilization of the biological substrate but also helps with the reduction of reagents and wastes, thus promoting an analytical method based on the “Green Chemistry” concept. The proposed method reached a very good enrichment factor (~80), which was useful for success determination of trace Hg(II) in tap and sea water samples.

Biological substrates without surface modification have been explored by other authors. For instance, Saçmacı et al. used resting eggs of *Daphnia longisipina* for Cd, Co, Cu, Mn and Ni preconcentration and determination in seaweed, crab and different types of water [107]. This work reported an acceptable enrichment factor (67) and high sorption capacities for all the analytes (34.3, 46.0, 27.2, 22.9 and 32.9 for Cd, Co, Mn, Ni and Cu, respectively). Karatepe et al. have also used a biological substrate without chemical modification [108]. In this case, a sea sponge was chosen as biosorbent for preconcentration and determination of Cu(II), Pb(II), Ni(II), and Fe(III) in tap and mineral water. Enrichment factors of 80, 80, 60, and 40 were achieved for Cu(II), Pb(II),

Ni(II), and Fe(III), respectively. The LODs were lower than others reported values using similar methodologies (1.5–5.6 μg L^{-1}).

6. Future perspectives

At present, biological substrates have been successfully applied for elemental analysis, offering usually good enrichment factors and biosorption capacities. From our point of view, classical materials that are still being used as solid supports in SPE procedures will be progressively replaced by nanomaterials and nanoparticles mainly due to the larger surface area offered by these nanocompounds. It has to be mentioned that the main disadvantage in the use of classical biological substrates resides in the difficulty for the selective retention of metal species. For this reason, there is a trend towards the development of new and specific biological materials with high selectivity and good retention capacity. Although chemical modification can significantly improve the selectivity of a biological substrate for elemental speciation analysis, this treatment can be applied in a wide variety of substrates and is not restricted to biological ones. On the contrary, genetic engineering arises as a useful tool based on the manipulation of cells as novel substrates to improve sorption capacity and selectivity. For these reasons, it is expected that genetically modified biological substrates will continue replacing more and more the classic biological substrates in elemental preconcentration and speciation studies.

7. Conclusions

In this review, biological substrates have demonstrated to be efficient tools for improving limits of detection and sensitivity in elemental analysis through their implementation in SPE methods coupled to a variety of analytical techniques. Good recoveries and enrichment factors were obtained in most cases. Although the majority of the methods have found application in simple matrices, some complex matrices have been also considered. More research on their application to more complex matrices is necessary. The use of biological substrates turns the presented methodologies into safe, non expensive and environmental friendly alternatives, with consumption of organic solvents significantly diminished or even absent. Most of the research described in the precedent pages proposed methods that can be widely spread over routine-analysis laboratories for preconcentration and determination of metals and metalloids at trace and ultra-trace levels.

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