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Galactose-functionalized methacrylate polymers as affinity sorbents for extraction of food allergen lectins

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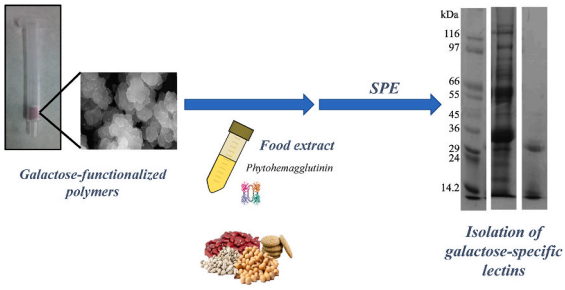
HIGHLIGHTS

- Galactose derivative-based polymeric materials were prepared as SPE sorbents.
- The developed material was used for isolation of phytohemagglutinins.
- High recovery efficiency and satisfactory reusability of sorbent was demonstrated.
- Selective separation of allergen lectins is achieved using the sugar-based sorbent.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, glycidyl methacrylate (GMA)-based materials functionalized with different galactose derivatives were prepared to be used as affinity sorbents for solid-phase extraction (SPE) of several food allergen lectins (such as phytohemagglutinin (PHA)). First, GMA-based polymers were synthesized and then galactose derivatives were immobilized onto the GMA surface using two different synthetic routes. In the first approach, the bare polymer was modified with ethylenediamine and glutaraldehyde, and subsequently two galactose derivatives were immobilized. In the second strategy, the starting polymer was modified with cystamine and gold nanoparticles (AuNPs), on which a thiolated galactose derivative was subsequently anchored. The resulting materials were characterized by scanning electron microscopy and used as SPE sorbents for the isolation of PHA (as probe protein) from food matrices. Different SPE parameters (sample pH, eluent solution composition, binding capacity, sample volume, selectivity and reusability) were evaluated. The material that provided the best PHA recovery (98%) was the one obtained in the second approach, being this material successfully applied to the selective extraction of PHA and other similar lectins from different foods (red and lima dried beans, fresh soybeans and biscuits containing soybean protein traces as indicated in their label). After SDS-PAGE of eluates, all samples only exhibited the characteristic PHA band around 30 kDa, suggesting the high potential of the developed material for application in food allergy field.

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1. Introduction

Lectins are glycoproteins, present in some parts of the plants, which selectively recognize and reversibly bind to carbohydrates and glycoconjugates through their binding sites [1]. Lectins are generally classified according to their structure, specificity for carbohydrates and species location [1]. In particular, lectins present in legumes, occupy a special position since they represent the largest family of proteins in these matrices [2]. They play an important role in different physiological processes, such as defense against pathogens [3,4], binding of symbiontic bacteria [3], stimulation of cell growth [3], and antifungal, antiviral, or antitumor activities [2], among others. In the last years, and due to the increasing interest in food allergy field, most of the studies related to lectins are focused on the relation between the ingestion of lectin-containing foods with several allergic responses. In fact, the most reported allergic symptoms manifested are naupathia, vomiting, diarrhoea or abdominal pain [2,5]. For this reason, to prevent the incidence of allergenic diseases and to assess consumers' safety, it is necessary to establish selective and sensitive methodologies for the extraction and determination of low lectin contents in foodstuff. To date, the most employed protocols to extract lectins from complex matrices require time-consuming steps (ammonium sulfate precipitation, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, among others) [6]. In fact, several commercial affinity sorbents commonly based on agarose support (such as Sepharose™) have been adopted for lectin purification [6,7]. However, these commercial phases show certain flaws such as their limited mechanical stability, the lack of precise information about the amount of immobilized ligand, and consequently, their binding capability, and their difficult coupling with on-line systems, among others [7]. Moreover, the direct lectins determination is sometimes considered a challenging task due to their low content and high matrix effect present in real samples. For this reason, a sample preparation process is crucial to isolate the target analyte and to remove interferences. In this sense, the synthesis of cost-effective home-made affinity materials for lectins analysis able to improve sorbent binding capacity and stability, and easily applicable to the analysis of real samples is advisable [7,8]. In this regard, several immunoassays using aptamers [9,10] or antibodies [11,12] as well as other affinity strategies based on hydrazine chemistry [13,14], boronic acid [15–17], hydrophilic interaction [18], or sugar-based ligands [19–21] have been reported, which have been mostly devoted to the isolation and enrichment of concanavalin A (Con A), as model lectin, in different matrices. In particular, these latter affinity reagents have been immobilized in polymeric matrices (such as monoliths, nanoparticles or cryogels) with enhanced mechanical and affinity properties [19–21]. Thus, Tetala et al. have been successfully purified Con A using mannose-affinity monolithic capillaries [19], whereas Gonçalves and coworkers [20,21] have synthesized functionalized cryogels with different aminated carbohydrates (such as N-acetyl-*d*-glucosamine) with large binding capacity towards Con A. The selection of these carbohydrates as affinity ligands is closely connected to the carbohydrate recognition of Con A lectin [22], which has been intensively investigated [23]. Taking into account the carbohydrate-binding specificity of lectins, the development of novel sorbents with dedicated affinity properties towards other lectins (known to have allergenic effects) is of utmost interest. In particular, legume lectins such as phytohemagglutinin (PHA) (present in several types of beans) and soybean agglutinins have demonstrated to show hemagglutinin activity giving rise to adverse effects in intestinal structure, the mucosal immune system, among others [24]. On the other hand, several studies have described that these lectins exhibit specific binding affinity for galactose residues [5,24–26], consequently, these oligosaccharides can be excellent candidates to prepare sugar affinity-based materials for these lectins.

The aim of this work was the development of novel galactose derivative affinity polymeric sorbents for solid-phase extraction (SPE) of hemagglutinins like PHA and other similar lectins from different foodstuffs. For this purpose, a glycidyl methacrylate (GMA)-based sorbent was first synthesized, and three different galactose-derivatives were prepared by using two synthetic routes. The developed polymeric materials were morphologically characterized and evaluated as SPE sorbents being some important extraction parameters optimized. Then, the material that gave the best extraction efficiency was applied to the analysis of these legume lectins in real food samples.

2. Experimental

2.1. Chemicals and reagents

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), cyclohexanol, 1-dodecanol, 2,2-dimethoxy-2-phenylacetophenone (DMPA), cystamine dihydrochloride, ethylenediamine (EDA), glutaraldehyde, cysteamine, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), tetramethylethylenediamine (TEMED), ammonium persulfate, acrylamide, bisacrylamide, bovine serum albumin (BSA), Con A, D-mannitol, glycine, β-mercaptoethanol, sodium borohydride, sodium dodecyl sulfate (SDS), sodium hydroxide, tris(hydroxymethyl)-aminomethane (Tris), molecular-weight-size protein standard (6.5–200 kDa) and PHA were obtained from Sigma-Aldrich (Steinheim, Germany). Monosodium, disodium phosphate and orthophosphoric acid were from Merck (Darmstadt, Germany). N-acetylgalactosamine (GalNAc) and 1-thio-β-D-galactose sodium salt (GalSH) from Glycon (Luckenwalde, Germany). D-(+)-galactosamine hydrochloride (GalNH2) from TCI (Zwijndrecht, Belgium). Methanol (MeOH) and acetonitrile (ACN) were obtained from Labkem (Barcelona, Spain). Gold nanoparticles (AuNPs) suspension stabilized with sodium citrate (particle size, 20 nm, $6.54{\cdot}10^{11}$ particles mL^{-1}), and Coomassie Brilliant Blue G (CBB) were from Alfa Aesar (Lancashire, United Kingdom). Deionized water was prepared in Crystal B30 EDI Adrona deionizer (Riga, Latvia). Pierce BCA Protein Assay Kit was from Fisher Scientific SL (Kander, Germany).

Stock solutions of PHA (1 mg mL^{-1}) were prepared by dissolving an appropriate amount of protein in deionized water, then it was aliquoted and stored in the freezer. Before use, aliquots were defrosted and working standard solutions were prepared by dilution with deionized water. Phosphate buffer solutions (PBS) at different concentrations and pH values were prepared by mixing appropriate amounts of Na_2HPO_4, NaH_2PO_4 and H_3PO_4 according to the required content and pH.

2.2. Instrumentation

Photopolymerization of monoliths were carried out using a UV radiation chamber CL-1000 (UVP, Upland, Canada) equipped with five UV lamps (5 × 8 W, 365 nm). Scanning electron microscopy (SEM) images were collected by using a high resolution SCIOS 2 FIB-SEM microscope from Thermo Fisher Scientific. Energy dispersive X-ray (EDAX) analysis was also recorded using a large silicon-drift detector (Oxford Instruments Ultim Max 170 EDS). For these measurements, the monoliths were coated with a very thin-layer of conductive carbon instead of the more usual Au/Pd coating. Elemental analysis of synthesized material was performed using an EA 1110 CHNS elemental analyzer (CE Instruments, Milan, Italy). Attenuated total reflection (ATR) Fourier-transform infrared (FT-IR) spectra of materials were acquired using a Cary 630 FTIR (Agilent Technologies) interfaced with an ATR sampling accessory with a single bounce diamond crystal. Spectra were measured from 4000 cm^{-1} to 600 cm^{-1}, by accumulation of 64 scans at a spectral resolution of 4 cm^{-1}.

Protein concentration was determined by applying the BCA assay [27] measuring the absorbance at 562 nm with a NanoDrop™ spectrophotometer (DeNovix DS-11, Wilmington, USA). Colorimetric quantification of $GalNH_2$ and GalNAc was carried out by the Morgan-Elson assay, which was conducted by the published procedure [28]. Briefly, this method allows the determination of hexosamines after its conversion in N-acetyl derivates and reaction with

p-dimethylaminobenzaldehyde reagent and UV–vis detection at 585 nm. On the other hand, the evaluation of GalSH was done by Ellman method [29], which allowed to determine the content of thiol groups after reaction with Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid), being the product formed monitored at 412 nm. In all these assays, the corresponding absorbance was measured using a diode-array UV–vis spectrophotometer from Agilent Technologies (Waldbronn, Germany). The amount of immobilized carbohydrate was calculated by the difference between the sugar concentration in the initial solution of GalNH$_2$, GalNAc or GalSH, and the content of non-reacted sugar in the remaining reaction solution. SDS-PAGE experiments were performed using a vertical minigel Hoefer SE260 Mighty Small system (Hoefer, MA, USA).

2.3. Preparation of GMA parent monolith

The parent poly(GMA-*co*-EDMA) monolithic material was prepared according to the procedure described by Mompó-Roselló et al. [16,30]. To synthesize this polymer, a polymerization mixture was prepared in a

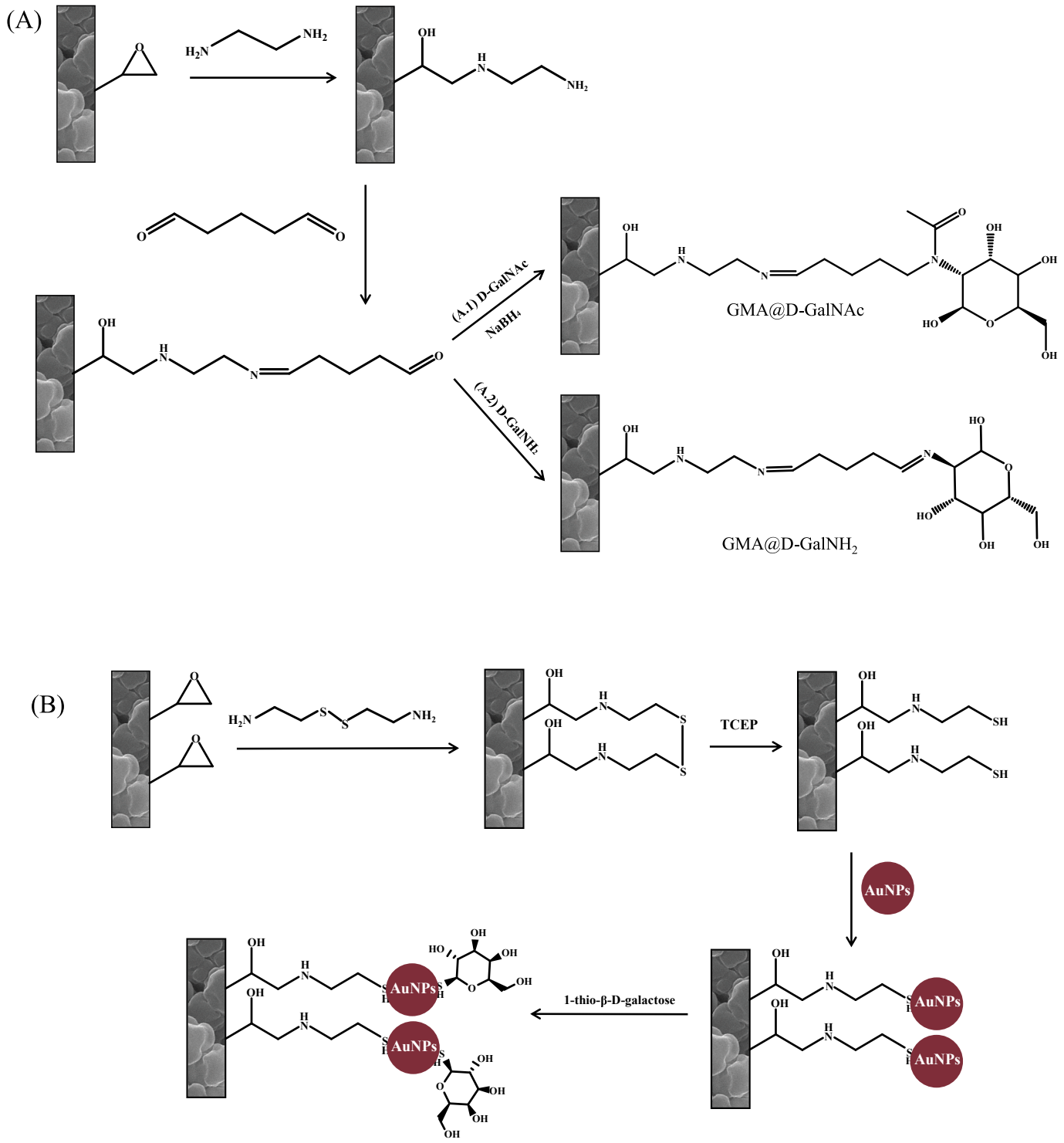

Fig. 1. Scheme of preparation of galactose-functionalized polymeric sorbents. Route A.1) GMA@GalNAc; route A.2) GMA@GalNH$_2$ and route B) GMA@AuNP-HSGal.

glass vial (without any previous modification of its inner walls) by weighing 40 wt% monomers (32 wt% GMA and 8 wt% EDMA) and 60 wt % porogens (56 wt% cyclohexanol and 4 wt% 1-dodecanol), in the presence of 0.1 wt % DMPA (out of the total weight of the monomers). This mixture was sonicated for 10 min and purged with nitrogen for 10 more min. After that, polymerization was carried out with UV light for 2 h at 1 J cm^{-2}. After polymerization, the vial was carefully broken to recover the monolith. Then, to remove possible unreacted monomers and rest of porogenic solvents, the polymeric material was transferred to a Büchner funnel and washed thoroughly with MeOH using a vacuum filtration system. The resulting bulk monolith was oven-dried at 60 °C overnight, and then it was ground with a mortar and sieved with a steel sieve (with sizes in the range 100–200 μm) and used for further immobilization of galactose derivatives.

2.4. Preparation and functionalization of GMA-based materials with galactose derivatives

To obtain hemagglutinin-based affinity sorbents, the parent GMA polymer was functionalized with three different galactose derivatives (GalNac, $GalNH_2$ and GalSH) using two different synthesis approaches, as shown in Fig. 1. In the first approach (route A), adapted from Gonzalves et al. [20,21], 600 mg of ground GMA polymer was immersed in 30 mL of aqueous EDA 50% (v/v), being the suspension allowed to react 18 h at 60 °C. Then, the solution was discarded, and the residue was washed with water until neutral pH (to remove the free amine moieties). After that, 30 mL of aqueous glutaraldehyde 10% (v/v) was added to the residue and the mixture was stirred for 3 h at room temperature. Afterwards, the glutaraldehyde-modified GMA material was washed with water and functionalized with two different sugars: GalNAc (route A.1) and $GalNH_2$ (route A.2). In both routes, the glutaraldehyde-modified GMA material was treated with 30 mL of 2 mg mL^{-1} GalNac or $GalNH_2$ prepared in PBS 100 mM at pH 7.2. Then, the mixtures were stirred overnight at room temperature. After that, the polymeric materials were washed with water. In the case of GMA@GalNAc sorbent, 30 mL of $NaBH_4$ 2 mg mL^{-1} were added (to stabilize the carbonyl-sugar binding), and the mixture was stirred during 1 h and, finally, the resulting material was washed and dried for 16 h at 60 °C in an oven.

On the other hand, the sorbent designed as GMA@AuNP-HSGal (Fig. 1, route B), was prepared using a protocol adapted from previous works [30–33]. Briefly, the GMA ground material was treated with 1.0 mol L^{-1} cystamine dihydrochloride solution (prepared in 2.0 mol L^{-1} aqueous NaOH) during 2 h at 60 °C, and then it was washed until neutral pH. The remaining product was treated with a TCEP solution (0.25 mol L^{-1} in an equal concentration of NaOH solution) for 2 h at room temperature to achieve the cleavage of disulphide bonds to yield sulfhydryl groups, and then washed with water until neutral pH. Next, the ground polymer containing thiol functionalities (after TCEP treatment) (400 mg) was suspended in a commercial AuNPs dispersion (15 mL) and was allowed to react until saturation of surface of the polymeric materials, which was checked by monitoring the UV–vis spectra of the supernatants [34]. Next, the supernatant was removed, and the material was oven-dried 2 h at 60 °C. Finally, GalSH was immobilized onto the AuNPs-modified GMA powder by adding 20 mL of GalSH prepared in 25 mM in PBS at pH 5.0, being the resulting mixture stirred overnight at room temperature. The obtained material was finally washed and dried during 16 h at 60 °C in an oven.

2.5. SPE protocol

To prepare the SPE cartridges, 25 mg of the corresponding galactose derivative-modified material was packed into 1 mL empty propylene SPE cartridge using two frits. In all cases, the SPE sorbents were activated with 500 μL ACN and equilibrated with 500 μL PBS at pH 5.5. Then, 2 mL of standard PHA or food extracts, prepared in 10 mM PBS at pH 5.5, were loaded on the SPE material. The washing step was carried out with PBS (under the same pH conditions as the loading step), whereas the retained protein was eluted with 500 μL of 250 mM mannitol prepared in PBS at pH 7.2. In all steps, the fractions were collected and subjected to BCA assay to evaluate PHA retention efficiency onto the different galactose derivative-modified sorbents. Regeneration of sorbents was accomplished by passing throughout the SPE cartridges the elution buffer solution water during 30 min each.

2.6. PHA extraction from foodstuffs

The samples included in this work were purchased from a local market in Valencia (Spain). These samples were: red (*Phaseolus vulgaris*) and lima (*Phaseolus lunatus*) dried beans, fresh soybeans (*Glycine max*) as well as a type of biscuits in which label is indicated that could contain trace amounts of soy proteins. The protocol to extract proteins from the different matrices was slightly different depending on the nature of samples (dried or fresh). For dried samples (red and lima beans and biscuits), the extraction protocol was adapted from Ren et al. [35], which consisted of sample milling in a mixer grinder to obtain a fine powder. A proper amount of powdered sample was weighted and resuspended in 10 vol of 10 mM PBS at pH 5.5, and the resulting suspension was stirred overnight at 4 °C. Then, the mixture was centrifugated during 1 h at 5000 rpm at 4 °C, and the supernatant was collected and filtered using a PTFE 13 mm × 0,22 μm filter (Análisis Vínicos, Tomelloso, Spain). On the other hand, for fresh samples (soybeans), the protocol reported elsewhere [36,37] was followed. Briefly, a certain amount of milled sample was weighed and two volumes of aqueous NaCl (0.9%) were added. The suspension was stirred overnight at 4 °C, being later centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants (aliquots of 2 mL) were desalted using Amicon® Ultra-2 centrifugal filter units (MWCO, 10,000; Merck). The resulting protein solutions were mixed with 10 mM PBS at pH 5.5 up to a final volume of 2 mL, and then subjected to SPE analysis as described in Section 2.5 or were stored at 4 °C until use.

3. Results and discussion

3.1. Preparation and characterization of GMA-based materials with galactose derivatives

Fig. 1 depicts the preparation routes of the different galactose-derivative materials synthesized in this work. For this purpose, GMA-based polymers, described in previous contributions of our research group [30,34,38], were chosen as starting host substrate for the preparation of these galactose-based phases. Then, two different approaches were pursued to immobilize galactose derivatives onto the surface of methacrylate polymers. For this purpose, in the route A, GalNAc and $GalNH_2$ were selected as affinity ligands to be immobilized in aldehyde-activated polymers by nucleophilic substitution following the reaction conditions in Section 2.4. First, the resulting GMA-based sorbents were morphologically characterized by SEM (Fig. 2). As observed, similar morphological structure was found for the parent polymer (trace A) and for those materials containing immobilized GalNAc and $GalNH_2$, respectively (Fig. 2B and C). Moreover, FTIR spectra of the original and sugar-functionalized polymers were accomplished to confirm carbohydrate immobilization (Fig. S1). The host polymer showed the presence of epoxy functional groups at 845 and 907 cm^{-1}, which displayed a significant intensity reduction in the sugar-functionalized polymers. Besides, a characteristic broad band of D-galactose in the region of 3600–3100 cm^{-1} due to the presence of OH bonds was present [39]. These data support the conversion of epoxy moieties into active sites for galactose derivatives coupling. Then, the amount of galactose immobilized into these two substrates was quantified by the Morgan-Elson colorimetric assay, by measuring the absorbance of non-reacted sugar in the remaining reaction solution (see Section 2.2). The results revealed a content of 36.2 mg (0.164 mmol) of GalNac and 57.4 mg (0.267 mmol)

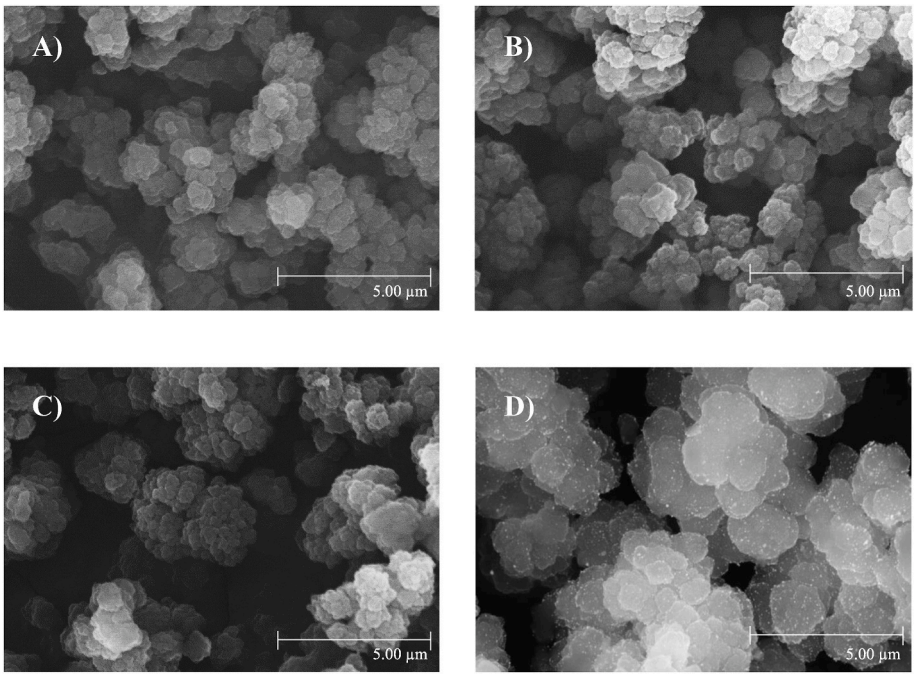

Fig. 2. SEM micrographs corresponding to the parent poly(GMA-*co*-EDMA) material (A), GMA@GalNAc (B); GMA@GalNH$_2$ (C) and GMA@AuNP-HSGal (D).

of GalNH$_2$ per g for GMA@GalNAc and GMA@GalNH$_2$ sorbents, respectively. The immobilized amount observed for the second material might be attributed to better electron-donating properties of amino groups of GalNH$_2$ as nucleophilic agent than N-acetyl radicals in GalNAc. In any case, the content of sugar immobilized was lower than those reported in literature [20,21] using supermacroporous cryogels for purifying Con A as lectin model. These differences could be attributed to the different properties of each sorbent (methacrylate-based monoliths compared to poly(acrylamide) cryogels [20,21]). Thus, these latter are commonly prepared by freezing a polymerization mixture by chemical initiation, whereas the organic polymer monoliths (synthesized in this study) were polymerized under UV irradiation. These differences in preparation conditions and polymerization mechanisms result in different polymer structure, porosity, swelling capacity, surface area, and accessible reactive functional (epoxy) groups for surface modification.

In order to enhance the sugar coverage, alternatively, a second strategy (Fig. 1, route B) was proposed, where AuNPs were attached onto the GMA-based polymer surface (previously modified with cystamine and TCEP) [30,33,34,40]. This approach provides a smart way to increase the active sites for subsequent sugar immobilization. Before proceeding with AuNPs coverage and sugar immobilization, nitrogen and sulfur contents were established by elemental analysis (1.9 and 3.8 wt%, respectively), which corroborated the attachment of amine and thiol moieties on the polymeric matrix. The sulfur content found in the cystamine-modified polymer was comparable with previous reports (3.7 atomic % obtained by EDAX analysis) [33]. Unfortunately, the determination of nitrogen content by EDAX was not reported due to the overlap of nitrogen peak with peaks of carbon and oxygen.

Next, SEM image of GMA@AuNP-HSGal material was taken (Fig. 2D). As observed, AuNPs were evidenced on polymer surface as white spots randomly and homogeneously distributed. This is to be expected since the polymer (25 mg) was saturated with *ca.* 1.0 mL AuNP dispersion containing $6.54{\cdot}10^{11}$ particles (see Section 2.4), representing a coverage of *ca.* 26 wt% Au (see Fig. S2 for EDAX analysis); however, slight differences were found in the percentages of C and O in GMA@AuNP-HSGal material compared to AuNP-modified polymer. This Au content was similar to that observed by Connolly et al. [41], but lower than those reported by Svec's group [33]. These differences in terms of Au content could be explained taking into account several factors. On the one side, the format used here (glass vial) in the synthesis of bulk monoliths is rather different from that (fused-silica capillaries) reported in Ref. [33], which might affect the accessible number of reactive sites in the bare polymer [42]. On the other hand, other factor to be considered in the amount of attached gold found is related to the size of AuNPs used as suggested Lv et al. [33].

To confirm the sugar immobilization onto AuNP surfaces, FTIR analysis was performed (Fig. S1), where the absorption band of the –OH stretching observed at 3400 cm^{-1} suggests the attachment of GalSH to the AuNPs surface. Additionally, the amount of sugar immobilized onto AuNPs was evaluated using the Ellman method [26], giving a total of 86.9 mg GalSH (0.443 mmol) per g polymeric sorbent. This value can be favourably compared with other home-made materials [20,21] reported in the literature devoted to lectin isolation/purification, since the presence of commercially available extraction devices based on carbohydrate-functionalized substrates for this purpose is rather limited.

3.2. *Evaluation of GMA-based materials with galactose derivatives as SPE sorbents*

To further illustrate the performance of the developed galactose-based materials as affinity sorbents for lectins, the retention of PHA (as model lectin) was firstly evaluated. Lectin isolation using several carbohydrate affinity supports is commonly addressed at neutral pH [19–21,43], therefore, experiments were carried out by loading 500 µL of 100 µg mL^{-1} PHA in 10 mM phosphate buffer at pH 7.2. As observed in Fig. 3A, the GMA@AuNP-HSGal and GMA@GalNH$_2$ sorbents provided retentions close to 50%, whereas the GMA@GalNAc showed retention values around 10%. To increase the protein binding, the sample pH was adjusted close to the pI of this protein (pI 5.0–6.5) [34,35,38,44] in order to minimize possible electrostatic interactions (repulsions) between adjacent proteins retained onto the sorbent. As shown in Fig. 3A, an increase of the retention values of PHA for the tested sorbents was found, being especially remarkable for the GMA@AuNP-HSGal material. The superior performance found in this last sorbent could be attributed to the beneficial effect of AuNPs providing not only an increase of the total surface, but also acts as scaffolds for the attachment of thiolated galactose residues, thus

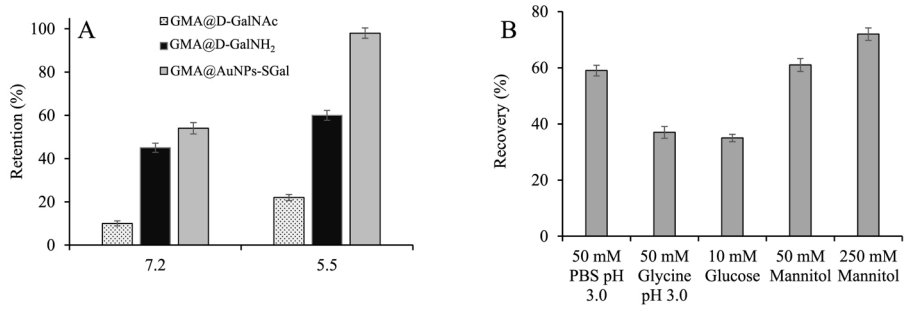

Fig. 3. Effect of sample pH solution on PHA retention (A) and eluent solution on the extraction recovery for PHA (B). Error bar = SD (n = 3). Other experimental conditions indicated in Section 2.5.

providing an enhancement of carbohydrate coverage, and consequently, an increase in the retention ability [45].

Next, different metal ions (such as Ca^{2+} or Mn^{2+} at 1 mM) were introduced into the phosphate buffer at pH 5.5 taking into account their role in lectin/glycan binding [6]. However, no significant influence on recovery values of the target protein was evidenced. Based on these results, the GMA@AuNP-HSGal material was chosen and a loading buffer (10 mM phosphate at pH 5.5) was adopted for extraction of PHA for further studies.

Regarding to the elution step, two strategies were adopted. The first one was based on the use of drastic conditions by using extreme pHs or high ionic strengths. Indeed, several authors have reported the release of lectins using acidic conditions (such as phosphate buffers or 20–100 mM glycine-HCl at pH 2.0–3.0) [43,46]. Fig. 3B shows the recoveries achieved using a concentration of 50 mM phosphate or glycine at pH 3.0, giving the former values close to 60%. Higher concentrations of these buffers (<100 mM to avoid interferences in the BCA assay) did not yield a significant improvement in the recoveries. The second elution strategy adopted was the use of monosaccharides or sugar alcohols to release the lectin from the affinity sorbent by competitive elution. Thus, some authors have reported that several carbohydrates (D-glucose, L-arabinose, L-rhamnose, among others) and sugar alcohols (as mannitol and adonitol) exhibited a high carbohydrate affinity for this lectin or other galactose-specific lectins (such as soybean agglutinin) [43,47–49]. These reagents are commonly found through hemagglutinating inhibition tests for the lectins of interest, suggesting that they have an adequate binding ability to the lectin. Among these reagents, glucose and mannitol were selected taking into account considerations such as BCA assay compatibility, cost and availability. As shown in Fig. 3B, mannitol gave better results than glucose, and large concentrations of this sugar alcohol (up to 250 mM) were tried, giving quantitative recoveries (>85%). Therefore, these eluting conditions were taken for further studies.

Then, other important SPE parameters such as binding capacity, sample volume and reusability were estimated before the application of this material to real samples. Regarding binding capacity, aliquots of 500 μL containing different amounts of PHA (from 25 to 750 μg) were passed through the cartridge in the optimized SPE conditions. The maximum binding capacity of the GMA@AuNP-HSGal was 37 mg of PHA/g of sorbent. This capacity value was comparable to other sorbents based on carbohydrate-functionalized polyacrylamide cryogels reported in literature [20,21]. However, these studies are fully oriented to lectin purification by affinity chromatography, and information regarding recovery values after SPE process is not mentioned. This contrasts with the good extraction performances exhibited by the developed sorbent,

Additionally, the breakthrough volume was determined by passing through the SPE cartridge different sample volumes (0.5–5 mL) of PHA standard solutions with a constant amount of protein (500 μg). As shown in Fig. S3, satisfactory recoveries (>85%) were found with all tested volumes; thus allowing the use of reasonable volumes of samples to be analyzed with a sufficient sensitivity.

Next, sorbent reusability was evaluated by repeatedly analyzing aqueous standard solutions of PHA at 100 μg mL^{-1}. For this purpose, the SPE sorbent was repeatedly used by employing a regeneration protocol (with eluting solvent and water) as described above (see section 2.5). As shown in Fig. S4, a good performance with recoveries higher than 90% for PHA was observed after 10 re-cycles.

Next, the selectivity of the GMA@AuNP-HSGal was evaluated by separately loading solutions containing 100 μg mL^{-1} BSA or Con A lectin (a glucose and mannose-binding protein [50], which is present in other type of legumes). After BCA analysis of the loading fraction, no retention was observed for both BSA and Con A lectin, which demonstrated the selectivity of the synthesized material. To corroborate these results, the elution fractions obtained for both proteins (BSA and Con A), jointly with the elution fraction of PHA loaded in the same conditions, were subjected to SDS-PAGE (Fig. 4). As observed, only in the case of PHA, the elution showed a clear band of the protein (located at around 30 kDa [35,51]), whereas in the other experiments no bands due to the BSA or Con A proteins was evidenced.

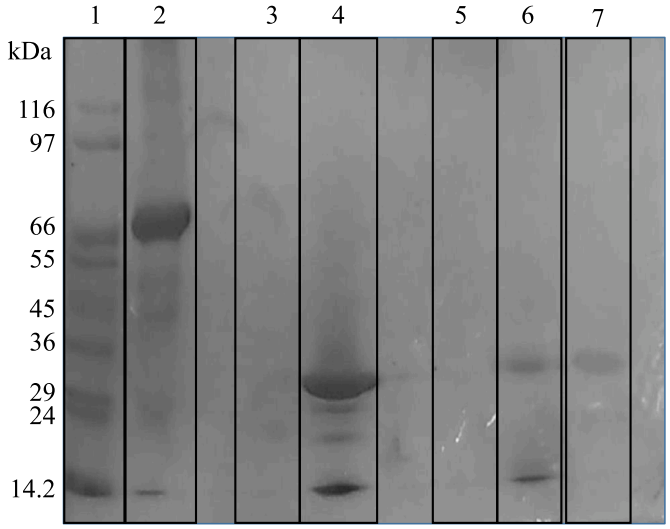

Fig. 4. SDS-PAGE analysis of eluted SPE fractions obtained for the selectivity study before (lanes 2, 4 and 6) and after subjection to the SPE protocol (lane 3, 5 and 7). Identification: lane 1, molecular weight standards (Mr in kDa); lane 2 and 3, a 100 μg mL^{-1} of BSA standard solution; lane 4 and 5, a 100 μg mL^{-1} of Con A standard solution; lane 6 and 7, 50 μg mL^{-1} of PHA standard solution. As evidenced, there are two blank lanes (between lanes 2 and 3, and between lanes 4 and 5) that were not loaded to avoid possible problems with lateral diffusion and consequently merging/distorted bands.

3.3. Application of the GMA@AuNP-HSGal sorbent to the analysis of PHA and other galactose-specific lectins in food samples

To demonstrate the applicability of the developed SPE procedure, the presence of PHA and other similar legume lectins in different food matrices was evaluated. These samples were red and lima dried beans, in these experiments, the loading and elution fractions were analyzed by SDS-PAGE to observe if the SPE methodology proposed in this study was able to isolate the target protein in presence of other high abundance proteins. Fig. 5 shows the protein profiles obtained before and after the SPE treatment. As observed in lane 4, band at 30 kDa corresponding to PHA was identified in the elution fraction after pass through the SPE sorbent, thus demonstrating an effective isolation of this protein from red kidney beans, Then, lectins from other Phaseolus varieties (Phaseolus lunatus L., commonly called lima bean) were subjected to the same protocol, giving a band around 32 kDa in the elution fraction (lane 6). These results agreed with those reported by Lacerda et al. [52], who indicated that the lectins from this species exhibited a molecular mass between 31 kDa and 33 kDa. Lately, lectin from glycine max (soybean agglutinin) from fresh soybeans as well as biscuits that containing traces of this protein (as indicated in the label) were analyzed. The eluted fractions for both samples displayed a band around 30 kDa, being ascribed to the corresponding soybean lectin [53]. All these results highlight the affinity of several galactose-specific lectins in different samples to the developed sorbent and the success of purification procedure.

4. Conclusions

Different GMA-based materials functionalized with galactose derivatives were prepared as affinity SPE sorbents for the analysis of the allergenic lectin PHA and other similar legume lectins present in food samples. For this purpose, two synthetic routes were conducted. The first approach consisted in the modification of the polymeric matrix via Schiff-base activation followed by the immobilization of two different sugar-derivatives, whereas the second one employed an AuNP-modified polymer as host support followed by the immobilization of GalSH. This last material (GMA@AuNP-HSGal) showed higher efficiency to retain PHA. It can be attributable to the increased affinity sites of the resulting material due to the beneficial effect of AuNPs, which can serve as excellent scaffolds to attach carbohydrate residues and thus enhancing the interaction with the target analytes.

After optimization of different SPE parameters, the selected sorbent showed satisfactory binding capacity, sample volume, suitable reusability, and good performances in selective isolation of PHA. Besides, the affinity sorbent was able to selectively extract PHA and other similar legume lectins (with high galactose specificity) from different foods, where high abundance proteins and other interferences were also present. The high potential of this type of affinity home-made sorbents to isolate and detect allergenic proteins was demonstrated; therefore, this methodology could be extended to the extraction of other allergenic sugar-binding proteins.

CRediT authorship contribution statement

María Vergara-Barberán: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **María Jesús Lerma-García:** Supervision, Writing – review & editing. **Ernesto Francisco Simó-Alfonso:** Supervision, Writing – review & editing, Funding acquisition. **José Manuel Herrero-Martínez:** Conceptualization, Supervision, Investigation, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

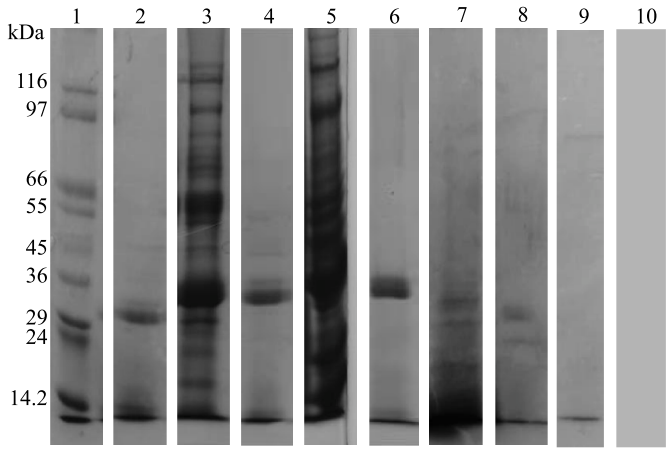

Fig. 5. SDS-PAGE analysis of the different food samples before (lanes 3, 5, 7 and 9) and after subjection to SPE protocol (lanes 4, 6, 8 and 10). Identification: lane 1, molecular weight standards (Mr in kDa); lane 2, a 100 μg mL^{-1} of PHA sample solution; lanes 3 and 4, red bean extract; lanes 5 and 6, lima bean extract; lanes 7 and 8, soy bean extract; and lanes 9 and 10, soy-containing biscuits. The original gels are shown in Fig. S5.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341142.

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