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# Aptamer-based stir bar sorptive extraction combined with MALDI-TOF-MS for the analysis of milk protein allergen β-lactoglobulin in foods

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ABSTRACT

In this work, the preparation of an aptamer-based coating for stir bar sorptive extraction (SBSE) aimed at the selective clean-up and preconcentration of the allergenic milk protein β-lactoglobulin (β-LG) is described. To this end, a thiol-terminated aptamer was grafted onto a vinylized polytetrafluoroethylene stir bar via "thiol-ene" click chemistry. The aptamer affinity coating was characterized and several parameters that affect SBSE efficiency were evaluated. The optimized aptamer-based SBSE method combined with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) analysis provided suitable linearity (0.25–50 $\mu g \cdot mL^{-1}$), satisfactory limit of detection (LOD) (0.08 $\mu g \cdot mL^{-1}$ β-LG), and relative standard deviations below 5.2 % among the extraction units, which could be reused for 10 extraction cycles and were stable for at least one month. The applicability of the developed method was successfully demonstrated by analyzing low levels of β-LG in different dairy-free food samples (i.e. a 100 % cocoa dark chocolate, a hydrolyzed hypoallergenic infant formula, and a white bread) and in white breads with declared and undeclared contents of milk (84 %<recoveries < 93 %).

## 1. Introduction

Food allergy has triggered a considerable interest due to the high prevalence throughout the population of immune sensitization to certain food components [1–5]. The Food and Agriculture Organization (FAO) has reported a list of eight global priority food allergen categories, including milk and milk products [6]. Concretely, cow's milk and dairy products are causing most food allergies in infants, and the protein β-lactoglobulin (β-LG) is one of the most important allergenic proteins [3,6,7]. Even in small concentrations (typically lower than 20 mg/Kg [8]), which can vary among individuals, this protein can induce adverse food reactions with severe symptoms and eventually anaphylactic shock [7,8]. Therefore, it is crucial to establish accurate and sensitive analytical methods for detecting and quantifying these allergenic proteins in food, thereby ensuring the safety of allergic individuals. These methods must be able to detect the target allergens at low concentrations. However, in most cases, due to the presence of matrix effects in complex food samples, an adequate sample pretreatment is mandatory before the analysis. In this sense, microextraction techniques have emerged in the sample preparation field to overcome the limitations of traditional extraction methods, allowing miniaturization, reduction of sample and reagent consumption, and low-cost operation, as well as automation in off-line and on-line configurations [9]. Indeed, certain microextraction techniques, such as (micro)solid-phase extraction (μSPE) [10,11], magnetic solid-phase extraction (MSPE) [12,13], and liquid-phase microextraction [14], among others, have been described for protein purification. Nevertheless, most of these reported methodologies use phases of reduced selectivity or limited active surface area to properly retain proteins. Additionally, the miniaturized extraction flow-through devices used in many cases (i.e. microcartridges, pipette tips, etc.) are susceptible to be blocked or damaged when complex samples are processed.

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Stir bar sorptive extraction (SBSE) is a miniaturized sample preparation technique, where the solutes are extracted using a magnetic stir bar coated with an appropriate phase. This technique has been satisfactorily applied to the analysis of trace amounts of small organic molecules in complex samples due to its advantages of versatility, high recoveries, and user-friendly application [15]. Despite these benefits, the application of SBSE to protein isolation is rather unusual. This could be explained by the limited selectivity of the typically available polydimethylsiloxane (PDMS) coatings. Recently, attempts to integrate biorecognition elements, such as antibodies, molecularly imprinted polymers (MIPs), or aptamers in SBSE have been described [16]. However, antibodies show low thermal stability, costly production, and cross-reactivity issues, whereas significant challenges remain for the development of MIPs against biomacromolecules, such as proteins.

Aptamers have been described as a novel type of selective, effective, and stable affinity ligand. They have been involved in different analytical applications, including as sorbents for sample preparation [17–21]. They typically consist of single-stranded DNA or RNA oligonucleotides, which have been appropriately selected to interact with the target molecules. Among their benefits, it should be highlighted their cost-effective and reproducible synthesis, high thermal and chemical stability, reusability, and easy modification in the 3′ or 5′ ends for further covalent immobilization onto solid supports [17–19]. However, the functionalization of SBSE coatings with aptamers has been scarcely investigated and mainly applied to small molecules (such as organic pollutants) [22,23]. Recently, we have developed an aptamer-based SBSE coating, in which thiolated aptamers were attached to vinylized stir bars, for extraction of the lectin protein allergen concanavalin A (Con A) from grain legumes and flours [24]. Merits of cost-effective and reproducible preparation, high selectivity, good reusability, satisfactory recoveries, and low limits of detection (LODs) were obtained in this study. Consequently, these aptamer-functionalized stir bars open a new way to the selective isolation of other target protein allergens in food samples.

Inspired by these considerations, we have developed a novel aptamer-based SBSE coating aimed at the selective clean-up and preconcentration of the allergenic milk protein β-LG from chocolate, hypoallergenic infant formula, and white bread. Commercial polytetrafluoroethylene (PTFE) stir bars were vinylized and then employed as scaffolds for binding aptamers in order to produce extraction units with a great aptamer affinity surface area. The resulting aptamer-affinity coating was characterized and several parameters that affected SBSE efficiency were evaluated in terms of extraction parameters for β-LG and other performance features (i.e., selectivity, LOD, and reusability). The optimized aptamer-based SBSE method combined with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) analysis was successfully applied to the determination of low levels of β-LG in the different food samples.

## 2. Experimental

### 2.1. Reagents and materials

Acetic acid (HAc) (glacial), acetone, hydrochloric acid, ammonium hydroxide, ethylenediaminetetraacetic acid (EDTA), magnesium chloride, sodium chloride, trifluoroacetic acid (TFA), potassium chloride, and sinapinic acid (SA) were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN) and methanol (MeOH) were purchased from Panreac AppliChem (Barcelona, Spain). Azobisisobutyronitrile (AIBN), bovine serum albumin (BSA), glycidyl methacrylate (GMA), α-lactalbumin (α-LA), β-casein, β-lactoglobulin (β-LG, mixture of A and B variants), sodium bicarbonate, sodium hydroxide, tris(hydroxymethyl) aminomethane (Tris), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and urea were obtained from Sigma-Aldrich (Steinheim, Germany). N,N-dimethylformamide (DMF) was from VWR Chemicals (Fontenay Sous Bois, France). Triethylamine (TEA) was from Alfa-Aesar (Karlsrube, Germany). Sodium naphthalene solution (FluoroEtch®) for the treatment of PTFE stir bar surface was provided by Acton Technologies (County Limerick, Ireland). Deionized water was obtained using a Crystal B30 EDI Adrona deionizer (Riga, Latvia). Pierce bicinchoninic acid assay (BCA) protein assay kit was from Fisher Scientific (Kander, Germany).

The thiol modified single-stranded DNA-aptamer against β-LG with a C6 spacer arm (5′-SH-$(CH_2)_6$-AGCAGCACAGAGGTCA-GATGTTCGGCCTTTGCGTTAACGAAC TTCTAGCTATGCGG CGTACC-TATGCGTGCTACCGTGAA-3′, 80-mer, molecular mass ($M_r$) = 24,865 [25]) was synthesized and purified by HPLC by Integrated DNA Technologies (Leuven, Belgium). The aptamer was dissolved in nuclease-free water (VWR) to obtain a final concentration of 100 μmol/L.

A 1 $mg{\cdot}mL^{-1}$ stock solution of β-LG standard was prepared in water, then it was aliquoted and stored in the freezer. Aliquots were defrosted before use and working standard solutions were daily prepared by dilution in the proper buffer solution and stored in the fridge when not in use.

PTFE-coated magnetic stir bars (8 mm length × 2 mm diameter) were obtained from VWR.

Several commercial label declared dairy-free food samples were analyzed (i.e. a 100 % cocoa dark chocolate, a hydrolyzed hypoallergenic infant formula (substitute for milk), and a white bread. Two additional white bread samples were also analyzed (trace levels of milk were only declared in one of them). All samples were purchased from a local market in Valencia (Spain).

### 2.2. Instrumentation

Attenuated total reflection Fourier-transform infrared (FT-IR) spectra of the stir bar surfaces were monitored using a Tensor 27 FT-IR spectrometer (Bruker, Bremen, Germany) with a DuraSamplIR II accessory from Smiths Detection Inc. (Warrington, UK) equipped with nine reflection diamond/ZnSe DuraDisk plates. SEM images of both bare stir bars and modified with aptamer were taken with a Hitachi S-4800 scanning electron microscope (Ibaraki, Japan), provided with a field emission gun, and an EMIP 3.0 image data acquisition system (Rontec, Normanton, UK). Surface analysis of these substrates was also carried out by X-ray photoelectron spectroscopy (XPS). Samples were placed onto a molybdenum plate with scotch SI-5 tape film followed by air drying. The measurements were performed on a K-Alpha™ XPS system using a monochromatic Al K(alpha) source (1486.6 eV).

The phosphorous content in bare and aptamer-functionalized stir bars was determined, after removing the Teflon coating using a cutting blade, with a 7900 inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Waldbronn, Germany). The quantification of P was carried out by measuring the signal ratio of this element (specifically, $^{31}P$) to the isotope $^{72}Ge$, selected as internal standard. The calibration curve ranged from 0 to 2000 $\mu g\ L^{-1}$ of P.

All sample-stir bar incubations were carried out in a TS-100 thermoshaker (Biosan, Riga, Latvian Republic). The aptamer concentration in the supernatant solution before and after the immobilization reaction was estimated by measuring the absorbance at 260 nm with a DeNovix DS-11 NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, EUA). Along the SBSE optimization steps, the protein concentration was estimated by the BCA colorimetric assay [26] by measuring the absorbance at 562 nm with the same spectrophotometer. Centrifugal filtration was carried out in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Identification and quantification of β-LG in food samples was carried out by MALDI-TOF-MS in a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Waltham, MA, USA). Data acquisition and processing were performed using the 4000 Series Explorer™ and Data Explorer® software (Applied Biosystems), respectively.

### 2.3. Preparation of aptamer-functionalized PTFE stir bars

The PTFE stir bar surface vinylization was carried out as described in our previous works [27–29] and can be found in the Electronic Supplementary Material (ESM).

After that, and prior to the attachment of the aptamer onto the vinylized stir bar surface, 50 μL of the aptamer solution (100 μmol/L) was treated with TCEP according to the manufacturer's aptamer reduction protocol [30] to break the disulfide bond and generate thiol groups. Then, aptamer folding treatment was performed by heating the solution at 95 °C for 10 min and cooling at 4 °C for 10 min in a thermoshaker. Next, the preparation of the aptamer-functionalized stir bar was done via "thiol-ene" click chemistry according to the method described by Wang *et al.* [31], with slight modifications. Each vinylized stir bar was introduced into 1 mL solution containing 0.9 % (m/v) AIBN (prepared in ACN:$H_2O$ (30:70 (v/v))). Then, 50 μL of 100 μmol/L solution of the folded thiolated aptamer were added and the mixture was stirred gently at 400 rpm and 55 °C for 5 h. The aptamer coupling efficiency to the stir bar surface was estimated by measuring the absorbance of the supernatant at 260 nm before and after this reaction. Afterwards, the aptamer-functionalized stir bar was rinsed with water and air-dried. The SBSE device was used immediately or was stored in water at 4 °C until use.

### 2.4. Extraction of β-LG from food samples

Non-powdered food samples (i.e. chocolate and bread) were firstly ground into a fine powder using a mixer grinder. For the β-LG extraction, the method described by Shi *et al.* [32] was followed with certain modifications. Briefly, 2 g of food sample (ground chocolate or bread, or hypoallergenic formula) were mixed with 10 mL of Tris-HCl (pH 7.4) at 40 °C for 30 min in a shaking water bath. Extracts were clarified by centrifugation at 8,000 rpm and 4 °C for 15 min. The resulting supernatant was acidified to reach pH 4.6 with 1 M HCl. After that, the extract was left to stand for 20 min at room temperature, and then centrifuged (at 3,500 rpm and 4 °C for 10 min) to sediment caseins. The supernatant was collected, neutralized until pH 7.4 with 1 M NaOH and filtered through a 0.22 μm polyvinylidene difluoride syringe filter (Ultrafree-MC, Millipore, Bedford, MA, USA). For spiked samples, an appropriate volume of 100 μg $mL^{-1}$ β-LG standard solution was added to this filtered whey extract to reach a final concentration of 0.01 mg β-LG per g of food (2 μg·$mL^{-1}$). Then, an aliquot of 5 mL of this solution was subjected to SBSE. Before MALDI-TOF-MS analysis, low $M_r$ compounds were removed from the eluate frations by centrifugal filtration using 3,000 $M_r$ cut-off cellulose acetate centrifugal filters (Amicon® Ultra.0.5, Millipore) as reported in our previous work [20,21,24]. Sample solution (eluate fractions 0.25 mL) was transferred to this filter and centrifuged at 10,000 × g for 10 min at 25 °C. The filtrate was collected and adjusted to a final volume of 0.25 mL before MALDI-TOF-MS analysis.

### 2.5. SBSE protocol

The SBSE stir bar was firstly conditioned with 1 mL of water for 5 min at 600 rpm. Then, water was removed, and 5 mL of β-LG standard solutions or food protein extracts were added, being the extraction carried out at 1,500 rpm and 55 °C for 3 h. Next, the stir bar was rinsed with 0.5 mL of water for 5 min to remove the non-retained compounds. Elution of the retained protein was achieved with 0.25 mL of 75 mM $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl and 2 M urea, keeping the same stirring rate and temperature for 1 h. After each extraction, a regeneration step was accomplished by washing the stir bar with the eluent and water (0.5 mL × at 600 rpm and room temperature for 5 min, each one). Next, the extraction device was stored in water at 4 °C until the next extraction cycle. Along the extraction optimization with standards, SBSE fractions were collected and subjected to BCA assay in order to evaluate β-LG retention and recovery. The retention was calculated as the ratio between the β-LG concentration in the remanent solution (after contact with the extraction device) and in the loaded solution. The recovery was established in a similar manner but considering the protein concentration in the eluate. Once the SBSE method was optimized, it was validated combined with MALDI-TOF-MS and later applied to food extracts, which were pretreated before SBSE as described in the previous section. All experiments were performed at least in triplicate-.

### 2.6. MALDI-TOF-MS

MALDI-TOF-MS analyses were performed in mid mass positive mode within a range of 5,000–30,000 *m/z*, with the laser intensity set to 80 % of the maximum (7900 V). Sample-MALDI matrix mixtures were freshly prepared according to a previous work [24] Briefly, the following layers of reagents were deposited onto a stainless steel MALDI plate: 1 μL of SA in 99:1 (v/v) acetone:water (final SA concentration 27 mg·mL-1), 1 μL of sample solution, again 1 μL of sample solution (to increase sample homogeneity), and finally, 1 μL of SA in 50:50 (v/v) ACN:water with 0.1 % (v/v) of TFA (final SA concentration 10 mg·$mL^{-1}$). Spots were allowed to dry at room temperature between the addition of each layer to ensure maximum homogeneity and, therefore, reproducibility in the MALDI-TOF-MS analyses. Three replicates (spots) of each sample were prepared and analyzed.

## 3. Results and discussion

### 3.1. Aptamer-functionalized stir bar preparation and characterization

As previously introduced, a few studies have been devoted to increasing extraction selectivity and efficiency in SBSE using aptamers [22–24]. In this context, a critical aspect in the preparation of these coatings involves the development of an efficient method for immobilizing aptamers onto the SBSE surface for the selective capture and enrichment of the target analyte from a complex sample matrix. Among the immobilization strategies of aptamers onto solid supports, the "thiol-ene" click chemistry has attracted significant attention due to its simplicity, mild reaction conditions, and high yield [17–19]. We have recently prepared an aptamer-based SBSE coating for Con A following this approach [24]. The preparation procedure started with the chemical etching of low-cost commercial PTFE stir bars, followed by a vinylization process to finally immobilize the aptamer through click chemistry (Fig. S1). The same preparation procedure was applied now to prepare the aptamer-functionalized SBSE coating for β-LG. Several studies have demonstrated that the aptamer coupling time has a great effect on the final performance of the aptamer affinity sorbent [17,24,33,34]. Thus, the influence of this variable on the aptamer immobilization onto the vinylized stir bar surface was considered (Fig. S2). Coupling efficiency was assessed by measuring the aptamer absorbance at 260 nm before and after the coupling reaction. The results revealed a progressive increase in the coupling efficiency with the immobilization time, from 1 to 5 h. Afterwards, it reached a plateau, with an average coupling efficiency of about 80 % because the stir bar surface became fully covered with aptamer. Hence, 5 h was selected as the optimized coupling time to immobilize the aptamer onto the stir bar surface.

To confirm the immobilization of the aptamer onto the stir bar surface, FT-IR measurements were carried out (Fig. 1). As observed, strong absorption bands (1,100 to 1,300 $cm^{-1}$) were observed for the bare PTFE stir bar attributed to C-F bonds (Fig. 1**A**). After etching and vinylization (Fig. 1**B**), a band around 3,300 $cm^{-1}$ due to the resulting hydroxyl groups was found, as well as peaks at 1,600–1,700 $cm^{-1}$ attributed to the C = C stretching mode, which confirmed the vinylization. After the coupling of the aptamer onto the vinylized stir bar surface (Fig. 1**C**), there was a slight decrease in the absorption peak of vinyl groups at 1,600–1,700 $cm^{-1}$ and appeared a small band at 950 $cm^{-1}$, which could be due to the C-S vibration.

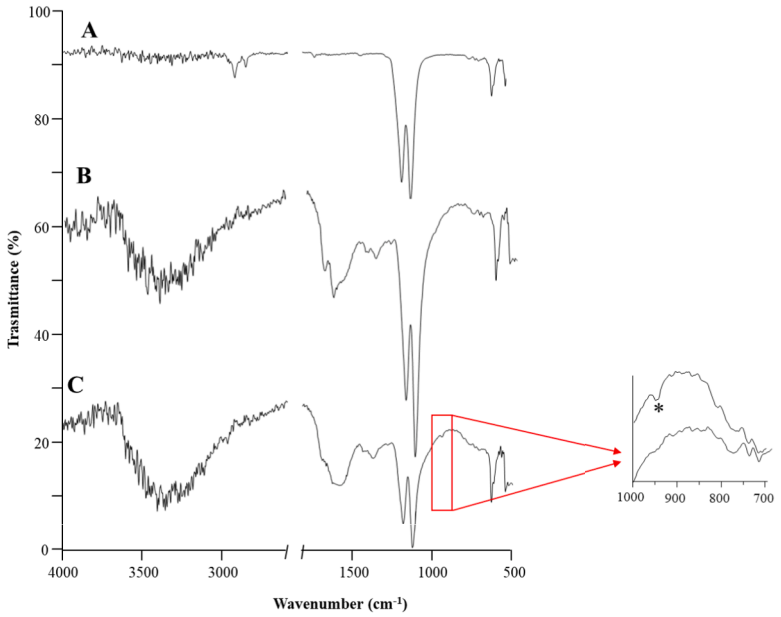


**Fig. 1.** FT-IR spectra of bare PTFE stir bar (A), PTFE stir bar after etching with Fluoroetch® and vinylization (B), and final aptamer-functionalized stir bar (C).

The morphology of the stir bar was also investigated by SEM. Fig. S3 depicts the SEM micrographs of the bare PTFE stir bar, after etching and vinylization, and functionalized with aptamer. As it can be seen, both the chemically etched PTFE and aptamer grafted stir bars exhibited similar morphological features, revealing deep cracks on the PTFE surface compared to the untreated stir bar. These results were consistent with previous studies involving PTFE materials treated with Fluoroetch® as chemical etchant [27,28,35]. Further characterization was conducted by XPS to examine the surface elemental composition and prove aptamer immobilization. Fig. S4 illustrates the XPS survey spectra of the stir bars before and after aptamer functionalization. As observed, a P2p peak located at 133 eV was identified in the aptamer-functionalized stir bar, which corresponds to the phosphate groups present in the aptamer's backbone. Additionally, an N1s peak at 400 eV

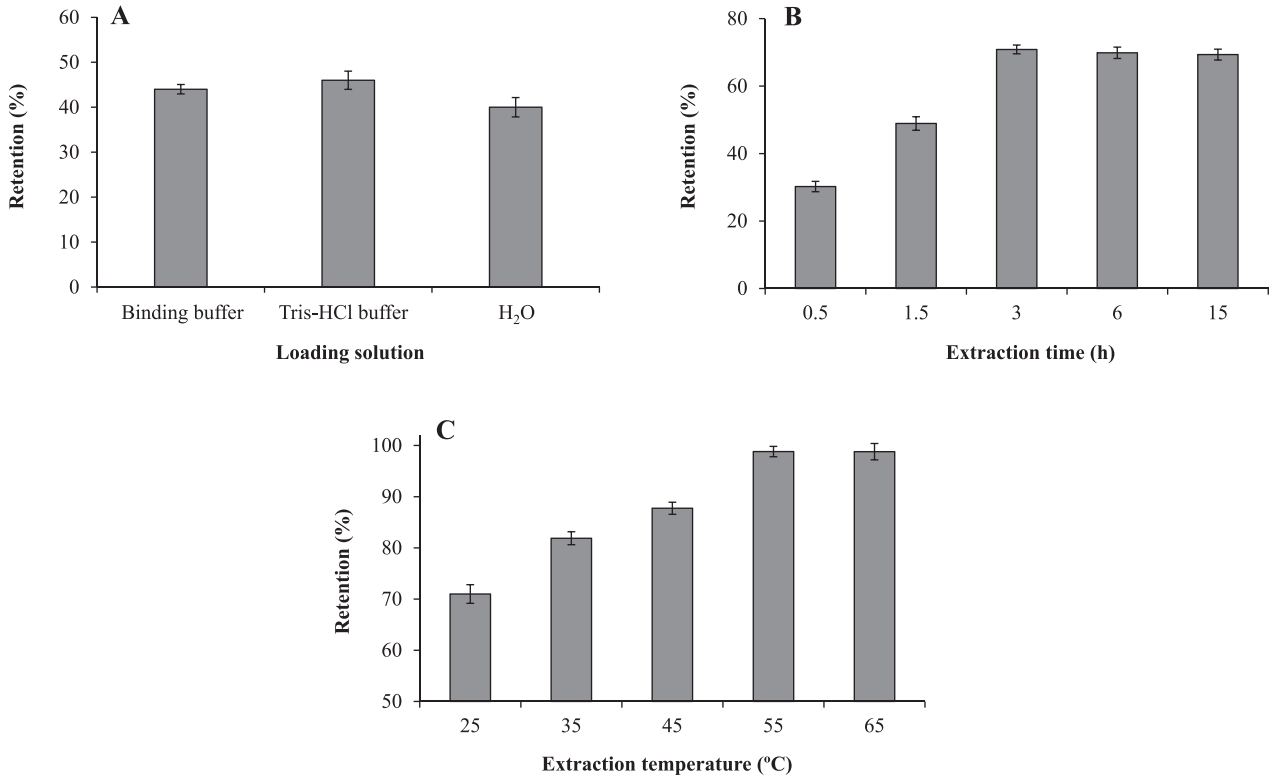


**Fig. 2.** Influence of several extraction parameters on the β-LG retention using the aptamer-functionalized stir bars: loading solution (A), extraction time (B), and extraction temperature (C). Error bars show the standard deviation of the results (n = 3). Experimental conditions (except the parameter under investigation): sample volume, 1 mL aqueous standard solution of β-LG (10 μg·mL$^{-1}$); extraction time, 1.5 h; stirring at 1,500 rpm and 25 °C.

confirmed the presence of the aptamer's nitrogenous bases on the stir bar surface. To complement the XPS studies, the phosphorous content was determined by ICP-MS before and after the aptamer functionalization process, revealing contents of this element in the aptamer-functionalized coating (0.0497 ± 0.0005 %) higher than in the control vinylized coating (0.0056 ± 0.0002 %). The residual phosphorous content found in the control would be associated to the reagents and water used in the aptamer functionalization.

### 3.2. Optimization of the SBSE method

In order to achieve a proper extraction performance of β-LG using the aptamer-functionalized SBSE coating, several parameters affecting retention and elution were optimized with β-LG standards. In these optimization studies, a test solution (1 mL) consisting of a 10 μg·mL$^{-1}$ aqueous solution of β-LG was used. Also, an extraction time of 1.5 h under stirring at 1,500 rpm and 25 °C was firstly selected. The retention and recovery of β-LG were used to evaluate the performance under the different tested conditions by using the BCA assay to estimate the protein concentration. First, the influence of the loading solution on β-LG retention was investigated using different buffer solutions, including the binding buffer used in the SELEX process to select the aptamer [25], a Tris-HCl buffer (50 mM Tris-HCl at pH 7.4), and water. As shown in Fig. 2**A**, the binding buffer and the Tris-HCl buffer provided slightly higher β-LG retentions (44 and 49 %) compared to water (40 %). This could be explained because these buffer media preserved the activity and affinity of the aptamers, which are typically maximized at neutral pH values [25,36]. Based on these findings, the Tris-HCl buffer was chosen for further studies due to the simplicity and convenience, as it was the extraction buffer solution for food sample preparation.

Next, the extraction time was evaluated (from 0.5 to 15 h) by keeping constant the rest of extraction parameters. As shown in Fig. 2**B**, β-LG retention was maximized at 3 h (70 %), while longer extraction times did not yield higher retention. Therefore, 3 h was selected as extraction time for further assays.

The influence of the extraction temperature on the β-LG retention was also investigated (Fig. 2**C**). The bar graph shows that the greatest retention was achieved at 55 °C. This could be explained considering that high temperatures would favour the mass transfer kinetics, without significative changes on the aptamer conformational structure.

Also, an efficient protein elution from the aptamer-functionalized stir bar is essential to achieve an effective SBSE protocol. Several authors have reported that protein elution from aptamer affinity sorbents can be addressed by varying pH, temperature, ionic strength, and by adding chelating or chaotropic agents, among others [17,18,33]. All these elution strategies can disrupt the aptamer-protein interactions, thus releasing the retained target protein. Based on these considerations, our previous studies [20,21,24], and those from other authors [37], several eluents were selected (with $NH_4OH$ (eluent 1), EDTA (eluent 2), and urea with NaCl (eluent 3); see caption of Fig. 3 for more details). The elution optimization was performed using 5 mL of eluent for 0.5 h under stirring at 1,500 rpm and 25 °C. The bar graph shows that the eluent containing urea and NaCl (eluent 3, Fig. 3**A**) allowed the greatest β-LG recoveries (66 %). Consequently, this eluent was selected to evaluate different elution times ranging from 0.5 to 3 h (Fig. 3**B**). As observed, when the elution time increased from 0.5 to 1 h, the extraction recovery of β-LG increased significantly, and then it remained nearly stable. Then, 1 h was selected to investigate the elution temperature (Fig. 3**C**) and the best recovery values (close to 100 %) were obtained at 55 °C, which was chosen for subsequent studies. Additionally, different elution volumes (from 0.25 to 5 mL) were tested. Eluent volumes below 0.25 mL were not assayed since they did not totally cover the stir bar. As shown in Fig. 3**D**, when the elution volume was reduced up to 0.25 mL recoveries were also nearly quantitative (98 %). This elution volume was selected as

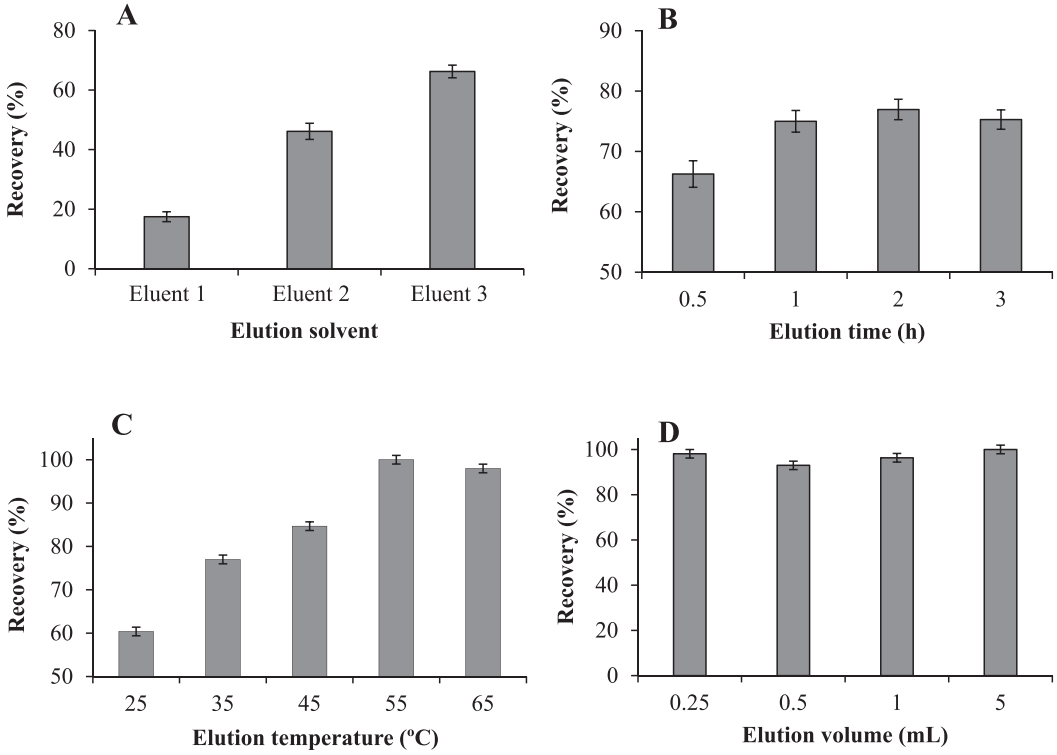


**Fig. 3.** Influence of several elution parameters on the β-LG recovery using the aptamer-functionalized stir bars: elution solvent (A), elution time (B), elution temperature (C), and elution volume (D). Error bars show the standard deviation of the results (n = 3). Experimental conditions (except the parameter under investigation): sample volume, 1 mL standard solution of β-LG (10 μg·mL$^{-1}$) in Tris-HCl buffer (pH 7.4); extraction time, 3 h; stirring at 1,500 rpm and 55 °C. The investigated elution solvents were: eluent 1, 100 mM $NH_4OH$ (pH 11.2); eluent 2, 20 mM Tris-HCl buffer (pH 7.4) with10 mM EDTA, and eluent 3, 75 mM $NaHCO_3$ (pH 8.3) with 2 M urea and 0.5 M NaCl.

optimized because it ensured the largest preconcentration factors. These high recovery values were maintained when the loaded sample volume was investigated from 0.5 to 5 mL, but significantly decreased with 10 mL. Therefore, 5 mL of loaded sample volume was adopted for the optimized SBSE method.

### 3.3. Selectivity of the aptamer-functionalized stir bars

The selectivity of the aptamer-functionalized stir bar coating was investigated by separately analyzing β-LG and different standard solutions of milk-related proteins at 2 μg $mL^{-1}$ (i.e. BSA, α-LA, and β-casein). Recoveries under the optimized SBSE conditions were evaluated again with the BCA assay. As shown in Fig. 4, recoveries below 15 % were found for BSA and β-casein, while for α-LA it did not exceed 3 %. These results demonstrated the outstanding selectivity of the aptamer-functionalized stir bar against β-LG. However, they also underlined the importance of considering an MS-based analytical technique as an alternative to the BCA colorimetric assay for accurate determination of β-LG.

### 3.4. Method validation of aptamer-based SBSE with MALDI-TOF-MS

Once optimized the SBSE method, it was combined with MALDI-TOF-MS analysis for the accurate identification and quantification of β-LG in complex samples, as well as to improve the overall sensitivity. Before method validation, β-LG standard solutions were analyzed by MALDI-TOF-MS. Fig. S5 shows a representative example of a MALDI-TOF-MS spectra of a 100 μg·$mL^{-1}$ β-LG standard solution. As it can be observed, two different β-LG proteoforms were identified, which corresponded to the A and B variants with $M_r$ experimental values of 18,364.6 and 18,282.7, respectively. This finding agreed with other studies that identified these two proteoforms, which have a nominal mass difference of 87 units due to the substitution of two amino acids at sequence positions 64 and 118 [38,39]. Also, the linearity of the MALDI-TOF-MS method was established, which ranged from 5 to 500 μg $mL^{-1}$ of β-LG ($R^2 > 0.993$), and the protein could be detected until 1.5 μg $mL^{-1}$ (signal-to-noise ratio, S/N > 3).

Then, the analytical performance of the SBSE MALDI-TOF-MS method was considered. A satisfactory linearity (with $R^2 > 0.991$) was obtained in the concentration range between 0.25 and 50 μg·$mL^{-1}$, with a LOD (established at signal-to-noise ratio of 3) of 0.08 μg $mL^{-1}$ of β-LG. The preparation reproducibility of the aptamer-functionalized stir bars was evaluated from the relative standard deviation (RSD) of the peak heights of the β-LG proteoforms analyzing a 2 μg $mL^{-1}$ β-LG standard solution. The RSD values were comprised between 1.8 % (n = 3) in one batch (or intra-day values (n = 3) using a single stir bar) and 5.2 % (n = 3) among different batches (or inter-batch values (n = 3) using different stir bars).

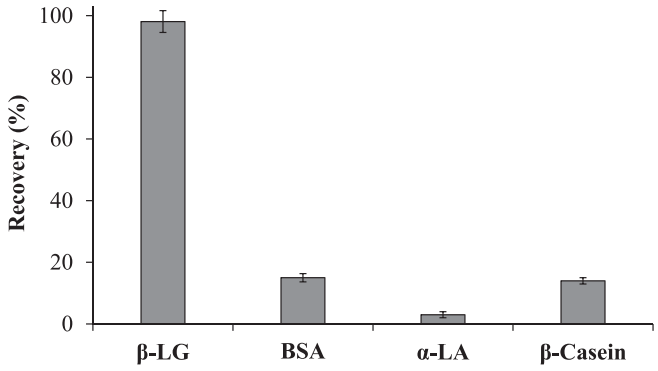


**Fig. 4.** Selectivity study of the aptamer-functionalized stir bar against β-LG and different milk-related proteins. Error bars show the standard deviation of the results (n = 3). Experimental conditions: sample volume, 5 mL standard solution of protein (2 μg·$mL^{-1}$) in Tris-HCl buffer (pH 7.4); extraction time, 3 h; stirring at 1,500 rpm and 55 °C; elution conditions, 0.25 mL of eluent 3 (75 mM $NaHCO_3$ (pH 8.3) with 2 M urea and 0.5 M NaCl); elution time, 1 h at 55 °C.

### 3.5. Analysis of β-LG in food samples

The developed SBSE MALDI-TOF-MS method was applied to the determination of β-LG in food samples. For this purpose, a representative series of label declared dairy-free food samples (i.e. a 100 % cocoa dark chocolate, a hydrolyzed hypoallergenic infant formula, and a white bread) jointly with two randomly selected white breads with declared and undeclared contents of milk were extracted and analyzed by SBSE MALDI-TOF-MS. In the case of the three dairy-free food samples, no β-LG proteoforms were detected after analyzing the sample extracts by SBSE MALDI-TOF-MS (see blank lines in Fig. 5). Consequently, the sample extracts were spiked at 2 μg·$mL^{-1}$ of β-LG (equivalent to 0.01 mg β-LG per g of sample), which was a concentration lower than the threshold value able to cause allergy symptoms [40,41]. As shown in Fig. 5, after spiking, β-LG proteoforms were unequivocally identified with no interfering signals, which corroborates the high affinity and selectivity of the aptamer against β-LG and the reliability of the MS identification. Regarding protein quantification, Table 1 shows the recovery values found in the spiked sample extracts, which were calculated for the total

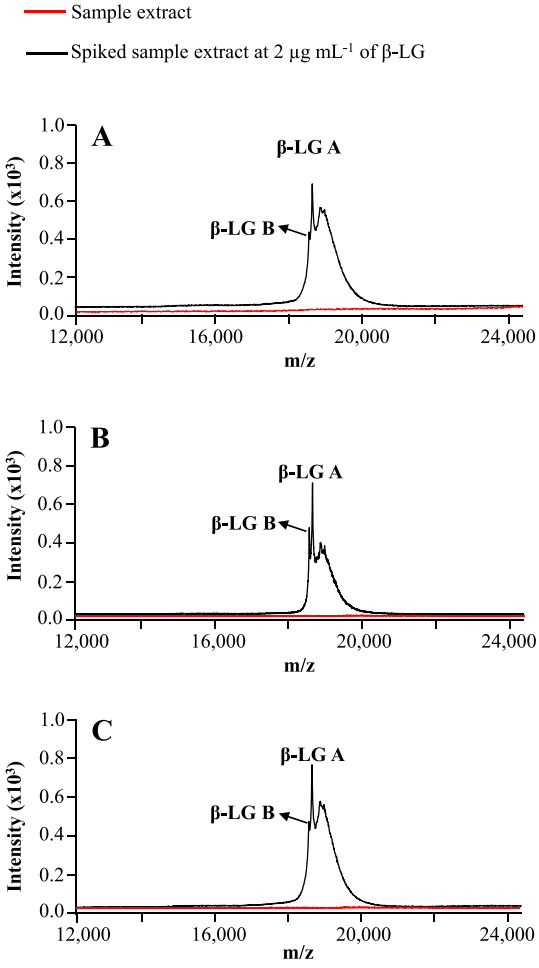


**Fig. 5.** MALDI-TOF mass spectra after aptamer-functionalized SBSE MALDI-TOF-MS analysis of a label declared dairy-free 100 % cocoa dark chocolate (A), a hydrolyzed hypoallergenic infant formula (B), and a white bread (C). The red and black lines correspond to the blank and spiked sample extracts at 2 μg $mL^{-1}$ of β-LG (0.01 mg β-LG per g of food sample), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
Total β-LG concentrations and recovery values obtained for food samples using the developed SBSE MALDI-TOF-MS method (standard deviation, SD, n = 3).

| Sample | Spiked concentration (mg/g sample) | Total β-LG[a] ± SD (mg /g sample) | Recovery ± SD (%) |
|---|---|---|---|
| 100 % cocoa dark chocolate (dairy-free) | 0.01 | 0.0086 ± 0.0002 | 86 ± 2 |
| Hypoallergenic formula (dairy-free) | 0.01 | 0.0084 ± 0.0002 | 84 ± 3 |
| White bread (dairy-free) | 0.01 | 0.0093 ± 0.0001 | 93 ± 1 |
| White bread (declared milk trace levels)[2] | | 0.226 ± 0.002 | |
| White bread (undeclared milk trace levels)[3] | | 0.0122 ± 0.0009 | |

[a] Total β-LG concentration was calculated as the sum of the A and B variants.

β-LG concentrations from the sum of A and B peak heights. As it can be seen, satisfactory recoveries were achieved for the three spiked sample extracts (above 84 %). The lifetime of the aptamer-functionalized stir bars with food samples was slightly shorter than with the standards (10 analyses), and the stir bars could be reused 5 analyses maintaining these recoveries (Fig. S6). In order to understand these findings, ICP-MS measurements of P content were conducted before and after the extraction device was reused 6 times in food sample extracts (Table S1). The results indicated negligible variation in the P content following these reuse experiments, which confirmed the stability of the covalent immobilization of aptamers onto the stir bar surface. Consequently, the observed complete loss of extraction capacity after the fifth analysis of food sample extracts was likely attributed to the irreversible alteration of aptamer conformation induced by the matrix components. This inevitably impacted analyte–aptamer interactions, hence influencing extraction recoveries.

Concerning the analysis of the two white breads with declared and undeclared contents of milk, Fig. 6 shows the obtained mass spectra. As it can be seen, β-LG proteoforms were detected in both sample extracts, and the total β-LG concentrations were 0.226 (±0.002) mg per g sample and 0.0122 (±0.0009) mg per g sample in the white breads with declared and undeclared milk trace levels, respectively.

All these results demonstrated the great performance of the combination of aptamer functionalized SBSE with MALDI-TOF-MS analysis, which allows a fast, simple, and accurate identification and quantification of the targeted protein, avoiding false positives or erroneous quantifications of non-MS based approaches.

### 3.6. Comparison with other methods

The developed procedure was compared with other recently reported aptamer-based methods for the determination of β-LG in food samples (Table 2). In general, our method showed similar sample pretreatment times compared to those reported in the literature [32,37,42,44–47], except for [43], which presented values exceeding 2.5 h. In terms of recovery values, our values were consistent with those obtained in all the considered studies. A similar observation applied to precision, although the current method demonstrated superior precision (RSD values below 6 %) compared to other dispersive SPE methods [32,42]. This is probably due to the need for centrifugation steps in this extraction mode, which promotes material or analyte losses during the operation. Regarding the LODs, our method provided comparable values to those found in most studies, except for certain methods with fluorescence [32] and electrochemical detection [37,47]. However, it is important to note that these methods lack reliable analyte identification by MS. Regarding the reusability of aptamer-based device, in most of these studies, this parameter was not investigated. Our aptamer-modified coating stir bar gave similar values to those obtained in a previous work [44]. However, while on-line SPE-CE-MS minimizes sample handling, it requires more sophisticated hyphenated equipment and skilled operators to conduct the experiments. Another advantage of our approach is the extraction device preparation process, enabling the simultaneous modification of multiple PTFE stir bars (approximately 10 in 12 h) at an estimated cost of 0.6 €/stir bar, which may increase up to 18 €/stir bar after aptamer functionalization. This cost-effectiveness renders the device potentially feasible for customization and commercialization.

## 4. Conclusions

An aptamer-functionalized SBSE coating for selective isolation and preconcentration of the allergenic milk protein β-LG has been developed. A simple and efficient strategy based on "thiol-ene" chemistry was adopted to prepare aptamer-functionalized stir bars from low-cost commercial PTFE magnets. The resulting SBSE coating demonstrated an outstanding selectivity against β-LG compared to other milk-related proteins, as well as good cost-effectiveness, reproducibility, and durability. Furthermore, the combination of SBSE with MALDI-TOF-MS resulted in an enhancement of the sensitivity (LOD as low as 0.08 μg $mL^{-1}$) and selectivity for the entire procedure. This fruitful synergy enabled the accurate identification and quantification of β-LG at low levels in complex food samples that may contain hidden dairy ingredients, with recovery values ranging from 84 to 93 %. This makes this method a promising tool in the research related to the worldwide issue of bovine milk allergy. The study also presents a very convenient approach for the successful preparation of other aptamer-based devices for the highly selective microextraction of other protein allergens. Consequently, it can be very useful for the development of accurate confirmatory analysis in the quality control of food, enhancing the food

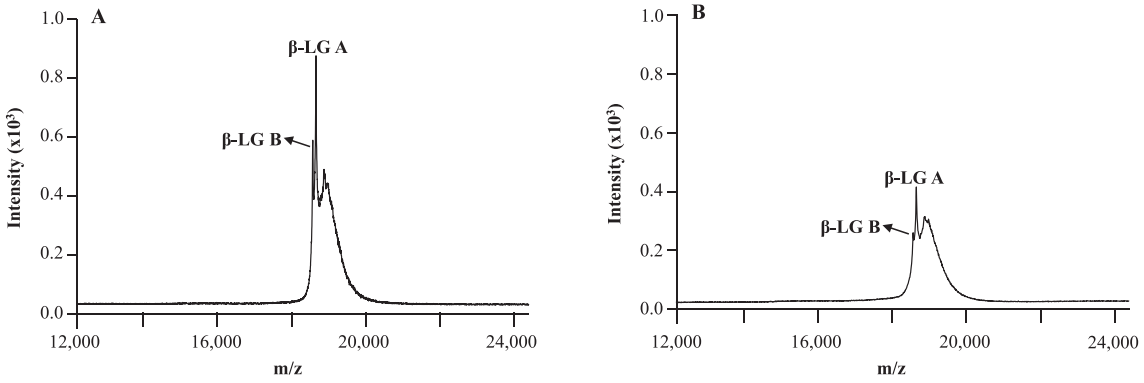


**Fig. 6.** MALDI-TOF mass spectra after aptamer-functionalized SBSE MALDI-TOF-MS analysis of two white breads with declared (A) and undeclared (B) milk trace levels.

**Table 2**
Comparison between the developed aptamer-based SBSE MALDI-TOF-MS method and other recent aptamer-based methods reported for β-LG determination in food samples.

| Method | Sorbent/sensor | Sample | Sample pretreatment time (h) | Recovery (%) | RSD (%) | LOD ($\mu g \cdot mL^{-1}$) | Reusability (analyses) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Dispersive SPE Fluorescence | Apt-MNPs-QD | Hypoallergenic formulations | 1.2 | 88–94 | ≤ 10 | $3.7 \cdot 10^{-5}$ | – | 32 |
| Dispersive SPE Fluorescence | QD-Apt-GO | Infant food | 1.0 | 83–115 | ≤ 13.3 | 0.10 | – | 42 |
| Dispersive SPE Fluorescence | Apt-$WS_2$ nanosheets | Pure milk and infant formula | 2.6 | 98–104 | ≤ 5.0 | 0.020 | – | 43 |
| On-line SPE-CE-MS | Apt-MBs | Food samples | 1.6 | 83–94 | ≤ 2.4 | 0.050 | 10–25 | 44 |
| Paper-based sensor chip | QD-Apt-GO | Food samples | 1.0 | 91–115 | ≤ 13.6 | 0.05 | – | 45 |
| Electrochemical biosensor | PANI/PAA-Apt electrode | Milk samples | 1.1 | 80–95 | ≤ 8 | 0.053 | – | 46 |
| Electrochemical biosensor | Apt-graphene electrode | Food samples | 1.3 | 90–95 | ≤ 5.5 | $2 \cdot 10^{-5}$ | – | 37 |
| Electrochemical biosensor | Apt-DNA-AuNPs-ITO electrode | Infant food formula | – | 92–103 | ≤ 5.4 | $7 \cdot 10^{-6}$ | – | 47 |
| SBSE MALDI-TOF-MS | Apt-PTFE stir bars | Dairy-free food samples | 1.3 | 84–93 | ≤ 5.2 | 0.08 | 5–10 | This work |

Abbreviations: CE, capillary electrophoresis; GO, graphene oxide; ITO, indium tin oxide; MBs, magnetic beads; MNPs, magnetic nanoparticles; QD, quantum dot.

security programs, and supporting the expansion of safer foods.

## CRediT authorship contribution statement

**Natalia Piqueras-García:** Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **María Vergara-Barberán:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Mónica Catalá-Icardo:** Methodology, Writing – review & editing. **Ernesto F. Simó-Alfonso:** Methodology, Resources, Writing – review & editing. **Fernando Benavente:** Conceptualization, Resources, Supervision, Writing – review & editing. **María Jesús Lerma-García:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **José Manuel Herrero-Martínez:** Conceptualization, Writing – review & editing, Supervision, Resources.

## 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.

## 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.microc.2023.109814.

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