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Accurate determination of the milk protein allergen β-lactoglobulin by on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry

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ABSTRACT

An on-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry (AA-SPE-CE-MS) method was developed to purify, preconcentrate, separate, and characterize the milk allergenic protein β-lactoglobulin (β-LG) in food samples. The sorbent to pack into the SPE microcartidges was prepared by immobilizing an aptamer against β-LG onto magnetic bead particles. After optimizing the SPE-CE-MS method, the sample (ca. 75 μL) was loaded in separation background electrolyte (BGE, 2 M acetic acid pH 2.2), while a solution of 100 mM NH_4OH (pH 11.2) (ca. 100 nL) was used for the protein elution. The linearity of the method ranged between 0.1 and 20 μg mL^{-1} and the limit of detection (LOD) was 0.05 μg mL^{-1}, which was 200 times lower than by CE-MS. The method was repeatable in terms of relative standard deviation (RSD) for migration times and peak areas (<0.5% and 2.4%, respectively) and microcartridge lifetime was more than 25 analyses. The applicability of the method for the determination of low levels of β-LG was shown by analyzing milk-free foods (i.e. a 100% cocoa dark chocolate, a hypoallergenic formula for infants, and a dairy-free white bread) and milk-containing white breads. Results were satisfactory in all cases, thus demonstrating the great potential of the developed method for accurate food safety and quality control.

1. Introduction

Food allergy is an immune response due to a sensitization to a food or food component [1]. Nowadays, there is a considerable interest in the food allergy field due to the wide spread of this pathology, which is concretely affecting around 3% of adults and 7% of children (below 3 years) [2–5]. The Food and Agriculture Organization (FAO) divides most foods that can cause food allergies into eight main categories, being milk one of those categories [6]. In particular, bovine milk and dairy products are considered to be the most common foods causing allergies in infants, being β-lactoglobulin (β-LG) the main allergenic protein [3–5,7]. In fact, β-LG can produce allergenic reactions even at low concentrations, being the most common symptoms: atopic dermatitis, urticaria, allergic rhinitis, asthma, angioedema or chronic cough [8]. Thus, in order to prevent the incidence of allergic diseases and ensure the consumer safety, it is necessary to establish accurate and sensitive detection and quantification methods for β-LG determination.

Until now, several analytical strategies have been reported for qualitatively or quantitatively determine β-LG, including chromatographic methods coupled to UV [9,10] or mass spectrometry (MS) detection [11,12]. Also, DNA-based methods, such as polymerase chain reaction [13], immunoassays [4,14–17], and other biosensors [5,18–21] have been described. These last types of affinity-based methodologies are especially appropriate for a rapid allergen screening in raw materials and final products [5,18–21]. However, the typically applied electrochemical or spectroscopic detection, which do not allow an accurate identification and characterization of β-LG, may promote false-negative or false-positive results due to undesired sample matrix effects.

Recently, aptamers have been described as a new type of affinity ligands for the isolation of a wide range of compounds, including small molecules or biomacromolecules, such as proteins, or even biological entities (e.g. cells) [22–26]. The most common aptamers consist of

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single-stranded oligonucleotides that are selected by an iterative *in vitro* process named systematic evolution of ligands by exponential enrichment (SELEX) [24]. Aptamers offer some well-known features such as their high-affinity, cost-effective and reproducible post-SELEX synthesis, high thermal and chemical stability, poor immunogenicity, reusability, and easy modification in the 3′ or 5' ends for further covalent attachment to solid supports [22,23]. Several authors have integrated aptamers into biosensors for β-LG analysis with electrochemical and spectroscopic detection [4,5,19–21], but not yet with MS detection. In recent studies we have demonstrated that aptamer affinity solid-phase extraction can be coupled on-line to capillary electrophoresis-mass spectrometry (AA-SPE-CE-MS) for the accurate determination of protein biomarkers in biological fluids [25,26]. In AA-SPE-CE-MS, a microcartridge containing an AA sorbent against the target analyte is integrated into the inlet of the separation capillary allowing the introduction of a large volume of sample (i.e. from tens to hundreds of microliters) before the elution in a nanoliter volume of an appropriate eluent. This results in a selective sample clean-up and analyte preconcentration followed by the electrophoretic separation and the MS detection for the accurate identification and quantification [25,26].

In this study, an AA-SPE-CE-MS method is presented for the clean-up, preconcentration, separation, and characterization of the allergenic protein β-LG in several food samples. For this purpose, an aptamer against β-LG previously described by Qui et al. [5] was used to prepare the AA sorbent for AA-SPE-CE-MS. After method optimization and validation with standards, the practical applicability and versatility of the developed method was evaluated by analyzing the target protein in several food matrices at a threshold dose able to induce allergy symptoms [27,28].

2. Experimental

2.1. Reagents and materials

Acetic acid (HAc) (glacial), ammonium hydroxide (NH_4OH) (25%), formic acid (HFor) (99.0%), potassium chloride (99.0%), potassium dihydrogenphosphate (≥99.0%), sodium chloride (≥99.5%), and sodium hydrogenphosphate (≥99.0%) were provided by Merck (Darmstadt, Germany). Acetonitrile (LC-MS) and ethanol (96%) were supplied by Panreac AppliChem (Barcelona, Spain). Propan-2-ol (LC-MS grade) was purchased from Scharlau (Barcelona, Spain). Ammonium acetate (NH_4Ac) (≥99.9%), bovine serum albumin (BSA), β-lactoglobulin (β-LG, >90%), and Tween® 20 were obtained from Sigma-Aldrich (Steinheim, Germany). Water (LC-MS grade) was provided by Fisher Scientific (Loughborough, UK).

The amino modified single-stranded DNA-aptamer against β-LG (5′–NH_2–$(CH_2)_6$- AGCAGCACAGAGGTCAGATGTTCGGCCTTTGCGTTA ACGAACTTCTAGCTATGCGGCGTACCTATGCGTGCTACCGTGAA-3', 80 -mer, molecular mass (M_r) = 24,865 [5]) was synthesized and purified by HPLC by Integrated DNA Technologies (Coralville, IA, USA). Magnetic beads (MBs) LOABeads™ AffiAmino of 45–165 μm diameter were purchased from Lab on a Bead (Uppsala, Sweden). Fused-silica capillaries (75 and 250 μm inner diameter (i.d.) × 375 μm outer diameter (o.d.)) were supplied by Polymicro Technologies (Phoenix, AZ, USA).

2.2. Background electrolyte, sheath liquid, protein standards and food samples

For CE-MS and AA-SPE-CE-MS analyses, an acidic BGE composed of 2 M HAc (pH 2.2) was used. The sheath liquid consisted of a hydro-organic mixture of 60:40 (v/v) propan-2-ol:water with 0.25% (v/v) of HFor and it was delivered at a flow rate of 3.3 μL/min with a KD Scientific 100 series infusion pump (Holliston, MA, USA). All solutions were degassed by sonication for 10 min and filtered through 0.20 μm nylon filters (Micron Separations Inc, Westborough, MA, USA) before use.

A stock β-LG standard aqueous solution (1 mg mL^{-1}) was prepared, aliquoted and stored in the freezer when not in use. Working standard solutions were daily prepared by dilution in water.

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

2.3. β-LG extraction from food samples

β-LG was extracted from the food samples as part of the whey fraction, following the classical method for fractionation of milk proteins. Briefly, 1 g of sample was dissolved in 10 mL of phosphate buffered saline (PBS, 10 mM Na_2HPO_4/NaH_2PO_4, 137 mM NaCl, and 2.7 mM KCl at pH 7.4). After that, the suspension was skimmed by heating at 40 °C for 30 min in a TS-100 thermoshaker (Biosan, Riga, Latvian Republic) and then the suspension was centrifuged at 2000×*g* at 4 °C for 15 min in a Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The resulting supernatant was subsequently acidified with 1 M HCl to pH 4.6 and the mixture was allowed to precipitate during 20 min. After that, the acidified suspension was centrifuged at 2000×*g* at 4 °C for 10 min to sediment and discard the caseins, and the supernatant, which contained the whey proteins, including β-LG, was neutralized with 1 M NaOH (until pH 7.2). This whey fraction (i.e. extract) was filtered through a 0.22 μm polyvinylidene difluoride centrifugal filter (Ultrafree-MC, Millipore, Bedford, MA, USA). For the spiked food samples, 500 μL of the filtered whey fraction was spiked with a proper volume of 100 μg mL^{-1} β-LG standard solution to obtain a final concentration of 0.1 mg β-LG per g of food sample (10 μg mL^{-1}). Then, low M_r compounds were removed with 10,000 M_r cut-off cellulose acetate centrifugal filters (Amicon Ultra-0.5, Millipore) [25]. The unspiked or spiked filtered whey fractions were centrifuged at 25 °C for 10 min at 10,000×*g*, being the residue washed three times with 50 μL of water for 10 min in the same way. The final residue was recovered by inverting the upper reservoir in a vial and spinning once more at a reduced centrifugal force (2 min at 300×*g*). Finally, sufficient water was added to adjust the final volume to 500 μL, being the resulting solution subjected to AA-SPE-CE-MS.

2.5. AA sorbent preparation

The amino-modified aptamer immobilization onto the MBs was carried out according to our previous study [25]. Two hundred μL of MBs solution (equivalent to 20 μL of sedimented MBs) was vortexed and the supernatant was removed by magnetic separation using a neodymium cube magnet (12 mm, N48, Lab on a Bead). The MBs were washed three times with 200 μL of PBS-T (PBS with 0.1% Tween 20) and the supernatants removed. The resulting MBs were resuspended in the same volume of PBS-T, 10 μL of activation buffer was added, and the mixture was incubated at room temperature for 15 min. The supernatant was removed, the MBs were washed with 200 μL of PBS-T, and then resuspended with 150 μL of PBS-T. Then, 50 μL of a 100 μM β-LG aptamer aqueous solution was added to the MBs suspension and the mixture was incubated with moderate shaking at room temperature for 40 min. After discarding the supernatant, the AA-MBs were washed three times with 200 μL of PBS and resuspended in the same volume of PBS. In order to prevent the non-specific binding of other proteins to the AA-MBs, 200 μL of blocking buffer (5% (m/v) BSA in PBS-T) was added to the suspension and the mixture was incubated at 37 °C for 2 h under moderate shaking. Finally, the supernatant was removed and the AA-MBs were subsequently washed three times with 200 μL of PBS. The AA-MBs were used immediately or stored in PBS with 20% (v/v) ethanol at 4 °C when not in use.

2.6. CE-MS analysis

CE-MS experiments were carried out in a 7100 CE coupled with an orthogonal G1603A sheath-flow interface to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany). CE and TOF mass spectrometer control, data acquisition and processing were done with the ChemStation and MassHunter softwares (Agilent Technologies). The parameters of the mass spectrometer were: capillary voltage 4000 V, drying gas temperature 300 °C, drying gas flow rate 4 L min^{-1}, nebulizer gas 7 psi, fragmentor voltage 325 V, skimmer voltage 80 V, and OCT 1 RF Vpp voltage 300 V. Data were collected in profile at 1 spectrum/s between 100 and 3200 *m/z*, with the mass range set to high resolution mode (4 GHz).

Separations were performed at 25 °C in a 60 cm total length (L_T) × 75 μm i.d. × 365 μm o.d. capillary. All capillary rinsing steps were performed flushing at 930 mbar. Prior to first use, new capillaries were activated by sequentially flushing off-line, to prevent the contamination of the mass spectrometer, with 1 M NaOH (15 min), water (15 min), and BGE (30 min). The hydrodynamic injection of sample was accomplished at 50 mbar for 10 s (ca. 55 nL, estimated using the Hagen–Poiseuille equation [29]). A voltage of +20 kV (normal polarity, cathode in the outlet) and 50 mbar of pressure were applied for the electrophoretic separation. The capillary was conditioned between runs, rinsing with water (2 min) and BGE (2 min), while among working days, capillaries were washed off-line with 1 M NaOH, water, and BGE (5 min each).

Samples were kept at 12 °C in the CE instrument autosampler using an external water bath (Minichiller 300, Peter Huber Kältemaschinenbau AG, Offenburg, Germany).

2.7. AA-SPE-CE-MS

The AA-SPE-CE-MS analyses were performed using fritless particle-packed microcartridges, since the average size of the sorbent particles (AA-MBs) was larger than the inner diameter of the separation capillary. For microcartridge preparation, a piece of capillary (0.7 cm L_T × 250 μm i.d. × 365 μm o.d.) was vacuum-filled with the AA-MBs and then connected with plastic sleeves between two capillary fragments (7.5 cm L_T × 75 μm i.d. × 365 μm o.d. (inlet) and 52.5 cm L_T × 75 μm i.d. × 365 μm o.d. (outlet)), which were activated before cutting, as a conventional CE-MS capillary [25,26].

All experiments and capillary rinses were performed at 25 °C and 930 mbar. Under the optimized conditions, AA-SPE-CE-MS capillaries were first conditioned flushing with BGE for 5 min. Then, standard β-LG solutions, blank or spiked food extracts were loaded at 930 mbar for 10 min (ca. 75 μL [29]), followed by a flush with BGE for 1 min to eliminate non-retained molecules and fill the capillary before the elution and electrophoretic separation. Through these steps, the nebulizer gas and the ESI capillary voltage were switched off to prevent the mass spectrometer contamination. Then, both were switched on and the retained protein was eluted by injecting 100 mM NH_4OH (pH 11.2) at 100 mbar for 20 s (ca. 100 nL [29]). For a rapid and repeatable protein elution, the small plug of eluent was pushed with BGE at 50 mbar for 100 s, before applying the separation voltage (+20 kV), and a small pressure (50 mbar) to compensate the microcartridge counter-pressure. Between consecutive runs, to avoid carry-over, the capillary was flushed with water (1 min), followed by an injection of the eluent (50 mbar for 40s), and a final flush with water (1 min).

2.8. Quality parameters

Migration times and peak areas from the extracted ion electropherograms (EIEs) of the detected β-LG proteoforms (+17 to +12 molecular ions of the A and B variants were the most abundant) were used to determine the quality parameters. The linearity range and LOD (defined as S/N = 3) of the CE-MS and AA-SPE-CE-MS method were experimentally established analyzing in triplicate (n = 3) 5 concentration levels of β-LG standard solutions from 25 to 1000 μg mL^{-1} and 0.05–20 μg mL^{-1}, respectively. Repeatability was measured at 50 μg mL^{-1} (CE-MS) and 5 μg mL^{-1} (AA-SPE-CE-MS) as the relative standard deviation (RSD) of migration times and peak areas (n = 3), which was expressed as percent by multiplying the standard deviation by 100 and dividing by the mean. The microcartridge lifetime was investigated by repeatedly analyzing a 5 μg mL^{-1} β-LG standard solution and protein extracts from food samples. The microcartridge was discarded when a significant loss of extraction efficiency was noticed, concretely, when the β-LG peak area decreased more than 25% compared to the mean value of the first three analyses with the microcartridge under consideration [25].

3. Results and discussion

3.1. CE-MS

In CE-MS, it is highly recommended the use of volatile BGEs with low conductivity to avoid salt precipitation, electrical arcing, and contamination of the mass spectrometer. In addition, acidic conditions are usually preferred for intact protein analysis to ensure a proper ionization efficiency. In this sense, the combination of BGEs and sheath liquids composed of aqueous HAc and propan-2-ol/water with HFor have been often reported in sheath-flow CE-MS [25,26,30,31]. Consequently, in this study, BGEs of HAc (0.1 M (pH 2.8), 1 M (pH 2.3), and 2 M (pH 2.2)) combined with sheath liquids of 60:40 (*v/v*) propan-2-ol/water with 0.05% or 0.25% (*v/v*) of HFor were evaluated for the determination of β-LG. The best results in terms of sensitivity were obtained with 2 M HAc (BGE) and 60:40 (*v/v*) propan-2-ol/water with 0.25% (*v/v*) of HFor (sheath liquid), applying +20 kV and 50 mbar during the electrophoretic separation. Under these CE-MS conditions, β-LG was detected as a mixture of two proteoforms (i.e. β-LG A and β-LG B variants), which were found at similar relative abundances (60% and 40%, respectively) as shown in the EIEs of Fig. 1A for a 50 μg mL^{-1} standard solution. The mass spectrum in the protein peak detection window is illustrated in Fig. 1C (molecular ions with charges from +17 to +12). As can be observed, each ion charge state consisted of a "doublet", which was due to the comigration of the two proteoforms. Mass spectrum deconvolution resulted in M_r experimental values of 18,364.20 and 18,277.50 for β-LG A and B (E_r < 194 ppm), respectively (Fig. 1D). These results agree with other studies related to the analysis of β-LG [32,33]. It is well-known that the two most abundant β-LG proteoforms in bovine milk are the A and B variants, which are found at a 60:40 ratio and differ by two amino acid substitutions at sequence positions 64 and 118, resulting in a nominal mass difference of 87 units [33]. The CE-MS method was linear in the β-LG concentration range comprised between 25 and 1000 μg mL^{-1} (R^2 < 0.996), while the LOD was 10 μg mL^{-1}, hence 6 and 4 μg mL^{-1} for the A and B variants, considering their respective relative abundances. The repeatability of the CE-MS method was evaluated by three consecutive analyses of a 50 μg mL^{-1} β-LG standard solution, reaching satisfactory RSD values for migration times and peak areas (0.6% and 2.4%, respectively).

3.2. AA-SPE-CE-MS

For the AA-SPE-CE-MS analyses, an AA-based sorbent involving an aptamer with high selectivity and affinity against β-LG was prepared [25,26]. For method optimization, the CE-MS conditions for β-LG analysis were considered (2 M HAc BGE with a sheath liquid of 60:40 (*v/v*) propan-2-ol/water with 0.25% (*v/v*) of HFor), as well as the AA-SPE-CE-MS method developed in a previous study, which required a BGE composed of 0.1 M HAc (pH 2.8) and a basic eluent of 0.1 M NH_4OH (pH 11.2) [25,26]. Accordingly, BGEs containing HAc at different concentrations (0.1, 1, and 2 M) were explored, and the best performance in terms of sensitivity for β-LG was obtained with the BGE of 2 M HAc (pH 2.2). Using this BGE, the injection time of the eluent plug at 50 mbar was established at 20 s because longer injection times (i.e. greater eluent

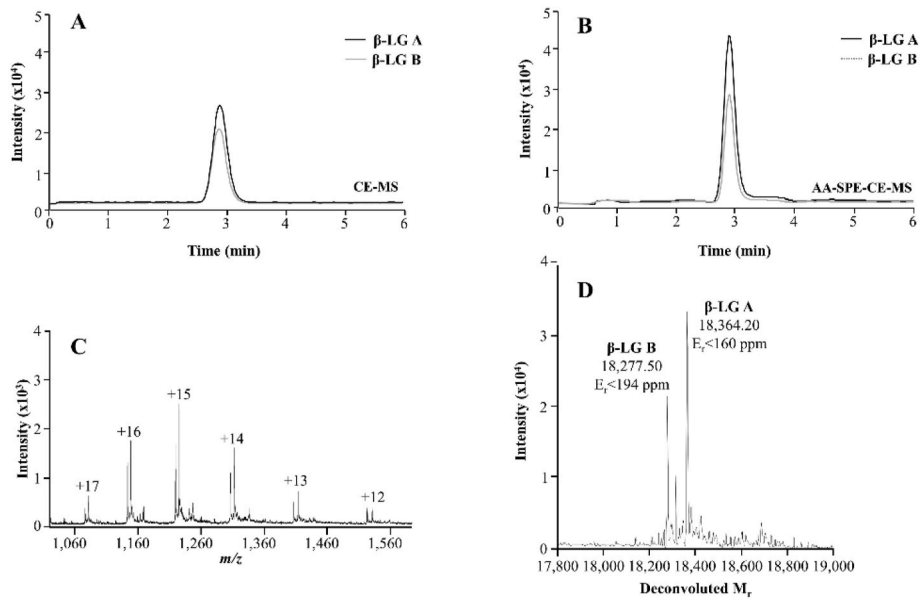

Fig. 1. (**A**) Extracted ion electropherograms (EIEs) for the CE-MS analysis of a 50 μg mL^{-1} β-LG standard solution; (**B**) EIEs for the AA-SPE-CE-MS analysis of a 1 μg mL^{-1} β-LG standard solution; **(C)** mass spectrum (by CE-MS) in the time window of the β-LG peak (*ca.* 3 min); and **(D)** deconvoluted mass spectrum (by CE-MS). (E_r (ppm) = $M_{r\ experimental} - M_{r\ theoretical})/M_{r\ theoretical} \times 10^6$).

volumes) resulted in broader and less intense β-LG peaks. Carry-over between consecutive analyses was prevented washing with water, a small plug of eluent, and again water. Under these conditions, the sample loading time was evaluated with 5 μg mL^{-1} β-LG standard aqueous solutions by flushing at 930 mbar from 5 to 30 min (Fig. 2). As observed, the maximum β-LG peak area was achieved between loading times of 10 and 20 min, since a loading time of 30 min resulted in smaller β-LG peak area due to exceeding the sample breakthrough volume [25]. Therefore, a sample loading time of 10 min was selected as the best compromise between total analysis time and protein extraction efficiency.

The EIEs obtained under the optimized conditions for the analysis of a 1 μg mL^{-1} β-LG standard solution by AA-SPE-CE-MS is shown in Fig. 1B. As observed, β-LG was again detected as a mixture of β-LG A and β-LG B with similar relative abundances as by CE-MS (Fig. 1A). Migration times were similar compared to CE-MS and the AA sorbent had no preference for any of the β-LG proteoforms, as their relative abundance was very similar to CE-MS. Therefore, it was possible to accurately identify and quantify both β-LG proteoforms, which could be relevant to design novel studies to evaluate allergenicity at the proteoform level.

The optimized AA-SPE-CE-MS method was validated in terms of linearity, LOD, and repeatability using β-LG standard solutions. Linearity was satisfactory from 0.1 to 20 μg mL^{-1} of β-LG ($R^2 < 0.998$, Fig. S1). The LOD was 0.05 μg mL^{-1} for β-LG (which corresponds to 0.03 and 0.02 μg mL^{-1} for the A and B variants, respectively). These LOD values were 200 times lower than by CE-MS, as well as below the values

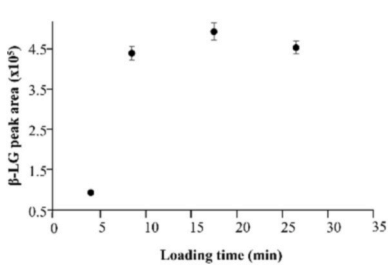

Fig. 2. Influence of the loading time at 930 mbar on the peak area of the detected β-LG (5 μg mL^{-1} β-LG standard solution in water) under the optimized elution and separation conditions.

reported for other bioassays, including ELISA with spectroscopic detection [13], aptamer-based paper sensor with smartphone detection [20], or liquid chromatography with UV [9] or MS detection [11,12]. Repeatability was evaluated by analyzing in triplicate a 5 μg mL^{-1} β-LG standard solution, and the RSD values for migration times and peak areas (0.5% and 2.4%, respectively) were similar to CE-MS. The reusability of the microcartridges was also investigated at this concentration and there was not a significant reduction in extraction efficiency after 25 analyses. All these results indicated the excellent performance of the developed AA-SPE-CE-MS method.

3.3. *Analysis of β-LG in food samples*

Different food matrices were analyzed by AA-SPE-CE-MS to demonstrate the applicability of the developed method [5,14,20], as well as the selectivity of the AA sorbent against β-LG in presence of other milk protein and matrix components, which could mask recognition of β-LG at low concentration. For this purpose, not only a representative group of milk-free samples (a 100% cocoa dark chocolate, a hydrolyzed hypoallergenic formula for infants, and a dairy-free white bread) were included in the study, but also two randomly selected samples of white bread (milk trace levels were declared in only one of them).

As expected, and can be observed in Fig. 3, no β-LG proteoforms were detected in the EIEs corresponding to the extracts from the three milk-free food samples. Therefore, these food extracts were spiked at 0.1 mg β-LG per g of food sample (10 μg mL^{-1}), which was the threshold concentration able to induce allergy symptoms [27,28]. The resulting EIEs for the analysis of the three spiked extracts are also shown in Fig. 3. As observed, and also confirmed with the mass spectra (data not shown), the β-LG A and B peaks were clearly detected at the expected migration time with no interfering signals. These results supported that the AA sorbent was able to efficiently and selectively isolate the β-LG proteoforms at low concentration, while allowing an accurate quantification, even from complex matrices, with no possibility of false positives due to the accurate MS identification. Table 1 shows the recovery percentages of β-LG calculated for the spiked milk-free food matrices, with adequate values comprised between 83% and 94%. As expected with food samples, RSD values for migration times and peak areas were slightly higher than for standards, but below 5.3% and 5.2%, respectively (Table 1),

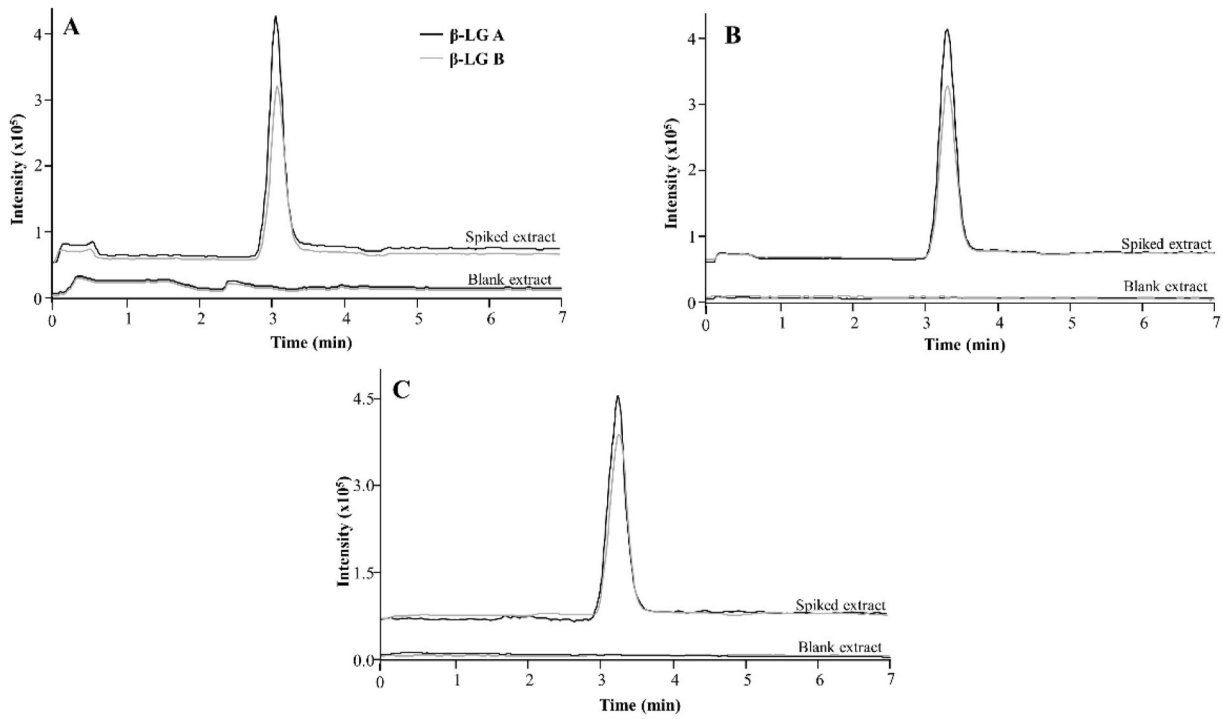

Fig. 3. EIEs for the AA-SPE-CE-MS analyses of different food samples (blank extracts and spiked extracts with 0.1 mg β-LG per g of food sample). **(A)** 100% cocoa dark chocolate, **(B)** hypoallergenic formula for infants, and **(C)** dairy-free bread.

Table 1
Recovery values (standard deviation, SD, n = 3), migration time and peak area RSD values (n = 3) of β-LG proteoforms for the analysis of spiked food samples (0.1 mg per g of food sample) by AA-SPE-CE-MS.

Sample	β-LG proteoform	Recovery (%) ± SD	Migration time RSD (%)	Peak area RSD (%)
100% cocoa dark chocolate	A	86 ± 5	5.3	5.2
	B	83 ± 6	5.8	5.1
Hypoallergenic formula	A	83 ± 6	1.8	2.3
	B	81 ± 4	1.5	2.4
Dairy-free white bread	A	94 ± 5	3.7	4.4
	B	91 ± 4	2.5	4.9

due to the complexity of the sample matrix. Sample matrix complexity also affected microcartridge lifetime that with food samples could be only reused until 10 analyses without reduction in the extraction efficiency.

Finally, as example of positive and suspicious samples, two white breads were analyzed (milk trace levels were declared in only one of them). The obtained EIEs by AA-SPE-CE-MS are shown in Fig. 4, and the β-LG proteoforms were detected in both samples. The concentration of β-LG was determined to be 0.200 (±0.004) mg per g sample in the white bread sample with declared milk trace levels and 0.0101(±0.0004) mg per g sample in the white bread with undeclared milk. These results corroborated the potential of the developed method for purification, preconcentration, separation, quantification, and characterization of β-LG in food samples below the typical threshold concentration that can elicit allergic symptoms.

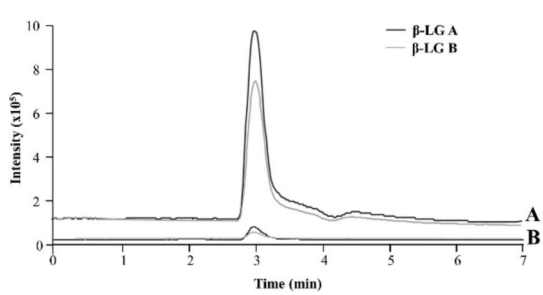

Fig. 4. EIEs for the AA-SPE-CE-MS analyses of (A) a white bread with declared milk trace levels and (B) a white bread with undeclared milk.

4. Conclusions

A novel AA-SPE-CE-MS method was presented to purify, preconcentrate, separate, and characterize the proteoforms of the milk allergenic protein β-LG in food samples. Under the optimized conditions, the analytical performance of the AA-SPE-CE-MS method was satisfactory, including low LODs for β-LG (0.05 μg mL^{-1}, which was 200 times lower than by CE-MS, 10 μg mL^{-1}) and satisfactory repeatability (below 0.5% and 2.4% RSD for migration times and peak areas, respectively). The method was successfully applied to the analysis of β-LG in different white bread samples at the typical threshold concentration that can cause food allergy symptoms (0.1 mg per g of food sample) and below in a suspicious sample with undeclared milk. Besides, the reusability of the sorbent was successful at least for 25 and 10 times with standard solutions and food matrices, respectively, without noticeable losses in the extraction efficiency. This study demonstrated that AA-SPE-CE-MS can be regarded as a promising approach for the accurate identification and quantification of β-LG at trace levels in complex matrices, thus avoiding the main limitations of typical non-MS based techniques. Furthermore, AA-SPE-CE-MS allows obtaining detailed information at the proteoform level that can be relevant to design novel studies to understand the mechanisms of β-LG allergenicity. More widely, the described approach can be adapted for the analysis of diverse allergens in complex samples, provided that appropriate selective aptamers are available.

Credit author statement

María Vergara-Barberán: Methodology, Validation, Investigation,

Writing original draft, Writing – review & editing. **Ernesto Francisco Simó-Alfonso:** Supervision, funding acquisition, Writing – review & editing. **José Manuel Herrero-Martínez**: Conceptualization, Supervision, Investigation, Writing – review & editing, Funding acquisition. **Fernando Benavente**: 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.

Data availability

Data will be made available on reasonable request

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Appendix A. Supplementary data

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References

[1] H.T.T. Tan, K.J. Allen, in: J. Thyssen, H. Maibach (Eds.), Filaggrin and Non-cutaneous Disease: Food Allergy, Filaggrin Springer, Berlin, Heidelberg, 2014, pp. 195–207, https://doi.org/10.1007/978-3-642-54379-1_19.

[2] EFSA, Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes, EFSA J. 12 (2014) 3894–3907, https://doi.org/10.2903/j.efsa.2014.3894.

[3] P. Satitsuksanoa, K. Jansen, A. Głobińska, W. Van de Veen, M. Akdis, Regulatory immune mechanisms in tolerance to food allergy, Front. Immunol. 9 (2018) 2939, https://doi.org/10.3389/fimmu.2018.02939.

[4] P. Galan-Malo, S. Pellicer, M.D. Pérez, L. Sánchez, P. Razquin, L. Mata, Development of a novel duplex lateral flow test for simultaneous detection of casein and β-lactoglobulin in food, Food Chem. 293 (2019) 41–48, https://doi.org/10.1016/j.foodchem.2019.04.039.

[5] S. Qi, N. Duan, Y. Sun, Y. Zhou, P. Ma, S. Wu, Z. Wang, High-affinity aptamer of allergen β-lactoglobulin: selection, recognition mechanism and application, Sensor. Actuator. B Chem. 340 (2021), 129956, https://doi.org/10.1016/j.snb.2021.129956.

[6] FAO/WHO, Report of an Ad Hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens, 2021 (available at, https://www.fao.org/3/cb4653en/cb4653en.pdf). (Accessed 24 January 2023). accessed.

[7] M. Nehra, M. Lettieri, N. Dilbaghi, S. Kumar, G. Marrazza, Nano-biosensing platforms for detection of cow's milk allergens: an overview, Sensors 20 (2019) 32, https://doi.org/10.3390/s20010032.

[8] A. Fiocchi, H.J. Schünemann, J. Brozek, P. Restani, K. Beyer, R. Troncone, et al., Diagnosis and rationale for action against cow's milk allergy (DRACMA): a summary report, J. Allergy Clin. Immunol. 126 (2010) 1119–1128, https://doi.org/10.1016/j.jaci.2010.10.011.

[9] L.I. Boitz, G. Fiechter, R.K. Seifried, H.K. Mayer, A novel ultra-high performance liquid chromatography method for the rapid determination of β-lactoglobulin as heat load indicator in commercial milk samples, J. Chromatogr. A 1386 (2015) 98–102, https://doi.org/10.1016/j.chroma.2015.01.081.

[10] O. Rotkāja, J. Goluško, P. Mekšs, Reversed-phase high-performance liquid chromatography analysis of β-lactoglobulin and α-lactalbumin in different types of milk, Mat. Sci. Appl. Chem. 33 (2016) 36–39, https://doi.org/10.1515/msac-2016-0007.

[11] J. Ji, P. Zhu, F. Pi, C. Sun, J. Sun, M. Jia, X. Sun, Development of a liquid chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens, Food Control 74 (2017) 79–88, https://doi.org/10.1016/j.foodcont.2016.11.030.

[12] P. Lutter, V. Parisod, H. Weymuth, Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection, J. AOAC Int. 94 (2011) 1043–1059, https://doi.org/10.1093/jaoac/94.4.1043.

[13] C. Villa, J. Costa, M.B.P. Oliveira, I. Mafra, Cow's milk allergens: screening gene markers for the detection of milk ingredients in complex meat products, Food Control 108 (2020), 106823, https://doi.org/10.1016/j.foodcont.2019.106823.

[14] S. He, X. Li, J. Gao, P. Tong, H. Chen, Development of sandwich ELISA for testing bovine β-lactoglobulin allergenic residues by specific polyclonal antibody against human IgE binding epitopes, Food Chem. 227 (2017) 33–40, https://doi.org/10.1016/j.foodchem.2017.01.060.

[15] S. He, X. Li, Y. Wu, S. Wu, Z. Wu, A. Yang, H. Chen, Highly sensitive detection of bovine β-lactoglobulin with wide linear dynamic range based on platinum nanoparticles probe, J. Agric. Food Chem. 66 (2018) 11830–11838, https://doi.org/10.1021/acs.jafc.8b04086.

[16] Y. Su, D. Wu, J. Chen, G. Chen, N. Hu, H. Wang, Y. Wu, Ratiometric surface enhanced Raman scattering immunosorbent assay of allergenic proteins via covalent organic framework composite material based nanozyme tag triggered Raman signal "turn-on" and amplification, Anal. Chem. 91 (2019) 11687–11695, https://doi.org/10.1021/acs.analchem.9b02233.

[17] J. Zhou, Q. Qi, C. Wang, Y. Qian, G. Liu, Y. Wang, L. Fu, Surface plasmon resonance (SPR) biosensors for food allergen detection in food matrices, Biosens. Bioelectron. 142 (2019), 111449, https://doi.org/10.1016/j.bios.2019.111449.

[18] R. D'Aurelio, J. Ashley, T.L. Rodgers, L. Trinh, J. Temblay, M. Pleasants, I. E. Tothill, Development of a NanoMIPs-SPR-based sensor for β-lactoglobulin detection, Chemosensors 8 (2020) 94, https://doi.org/10.3390/chemosensors8040094.

[19] M. Shi, Y. Cen, M. Sohail, G. Xu, F. Wei, Y. Ma, Q. Hu, Aptamer based fluorometric β-lactoglobulin assay based on the use of magnetic nanoparticles and carbon dots, Microchim. Acta 185 (2018) 1–8, https://doi.org/10.1007/s00604-017-2569-5.

[20] J. Yang, Y. Zhang, L. Wu, Y. Lu, A coffee-ring effect-based paper sensor chip for the determination of beta-lactoglobulin in foods via a smartphone, Sensor. Actuator. B Chem. 374 (2023), 132807, https://doi.org/10.1016/j.snb.2022.132807.

[21] S. Eissa, M. Zourob, In vitro selection of DNA aptamers targeting β-lactoglobulin and their integration in graphene-based biosensor for the detection of milk allergen, Biosens. Bioelectron. 91 (2017) 169–174, https://doi.org/10.1016/j.bios.2016.12.020.

[22] V. Pichon, F. Brothier, A. Combès, Aptamer-based-sorbents for sample treatment - a review, Anal. Bioanal. Chem. 407 (2015) 681–698, https://doi.org/10.1007/s00216-014-8129-5.

[23] P. Röthlisberger, C. Gasse, M. Hollenstein, Nucleic acid aptamers: emerging applications in medical imaging, nanotechnology, neurosciences, and drug delivery, Int. J. Mol. Sci. 18 (2017) 2430, https://doi.org/10.3390/ijms18112430.

[24] Z. Zhuo, Y. Yu, M. Wang, J. Li, Z. Zhang, J. Liu, X. Wu, A. Lu, G. Zhang, B. Zhang, Recent advances in SELEX technology and aptamer applications in biomedicine, Int. J. Mol. Sci. 18 (2017) 2142, https://doi.org/10.3390/ijms18102142.

[25] R. Pero-Gascon, F. Benavente, Z. Minic, M.V. Berezovski, V. Sanz-Nebot, On-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry for the analysis of blood α-synuclein, Anal. Chem. 92 (2020) 1525–1533, https://doi.org/10.1021/acs.analchem.9b04802.

[26] H. Salim, R. Pero-Gascon, E. Giménez, F. Benavente, On-line coupling of aptamer affinity solid-phase extraction and immobilized enzyme microreactor capillary electrophoresis-mass spectrometry for the sensitive targeted bottom-up analysis of protein biomarkers, Anal. Chem. 94 (2022) 6948–6956, https://doi.org/10.1021/acs.analchem.1c03800.

[27] R. Sorva, S. Mäkinen-Kiljunen, K. Juntunen-Backman, Beta-lactoglobulin secretion in human milk varies widely after cow's milk ingestion in mothers of infants with cow's milk allergy, J. Allergy Clin. Immunol. 93 (1994) 787–792, https://doi.org/10.1016/0091-6749(94)90259-3.

[28] D.A. Moneret-Vautrin, G. Kanny, Update on threshold doses of food allergens: implications for patients and the food industry, Curr. Opin. Allergy Clin. Immunol. 4 (2004) 215–219, https://doi.org/10.1097/01.all.0000129454.42340.23.

[29] H.H. Lauer, G.P. Rozing, High Performance Capillary Electrophoresis, second ed., Agilent Technologies, Waldbronn, Germany, 2014, pp. 60–61, https://doi.org/10.1371/journal.pone.0016148.

[30] E. Giménez, F. Benavente, J. Barbosa, V. Sanz-Nebot, Analysis of intact erythropoietin and novel erythropoiesis-stimulating protein by capillary electrophoresis-electrospray-ion trap mass spectrometry, Electrophoresis 29 (2008) 2161–2170, https://doi.org/10.1002/elps.200700788.

[31] L. Pont, I. Compte, V. Sanz-Nebot, J. Barbosa, F. Benavente, Analysis of hordeins in barley grain and malt by capillary electrophoresis-mass spectrometry, Food Anal. Methods 13 (2020) 325–336, https://doi.org/10.1007/s12161-019-01648-8.

[32] U. M Elofsson, M.A. Paulsson, T. Arnebrant, Adsorption of β-lactoglobulin A and B in relation to self-association: effect of concentration and pH, Langmuir 13 (1997) 1695–1700, https://doi.org/10.1021/la9601061.

[33] C. Czerwenka, I. Maier, N. Potocnik, F. Pittner, W. Lindner, Absolute quantitation of β-lactoglobulin by protein liquid chromatography−mass spectrometry and its application to different milk products, Anal. Chem. 79 (2007) 5165–5172, https://doi.org/10.1021/ac062367d.