

Simultaneous determination of four food allergens using compact disc immunoassaying technology

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Abstract A multiplex competitive microimmunoassay for the simultaneous determination of gliadin, casein, β -lactoglobulin, and ovalbumin is presented. The assay in microarray format is performed on a DVD where the allergens are physisorbed on the polycarbonate surface of the disc. The immunointeraction is detected using a mixture of specific gold-labeled antibodies and the signal amplified with the silver enhancement method. The optical density of the precipitate, read by a DVD drive, is related to the concentration of the four allergens in sample. An optimized protocol for the simultaneous extraction of the allergen proteins from food samples is also addressed. The suitability of the method is demonstrated for the simultaneous quantitative extraction and determination of the targeted allergens in spiked baby foods, juices, and beers. The sensitivity (EC₅₀) of the multiplexed assay was 0.04, 0.40, 0.08, and 0.16 mg L⁻¹ for gliadin, casein, β -lactoglobulin, and ovalbumin, respectively, and the recovery results from the analysis of food samples ranged from 72 to 117%. A portable, easy-to-use, array-based bioanalytical method is developed for quantification of food allergens with a limit of detection below the accepted levels of the international legislations, which allows promotion of food safety and quality.

Keywords Food allergy · Gliadin · β -lactoglobulin · Casein · Ovalbumin · Simultaneous extraction multiplex competitive microimmunoassay · Compact disc microarraying

Introduction

Food allergy is a major health problem affecting up to 5% of adults and 8% of children worldwide [1, 2]. It has been defined as an IgE-mediated abnormal response to very low quantities of certain food proteins [3]. Symptoms are diverse ranging from relatively mild to severe or sometimes even fatal consequences (anaphylaxis) [4]. The only possible way for sensitive persons to sidestep allergic reactions is to completely avoid the offending allergic foods.

According to the European Directive 1169/2011, a number of 14 allergenic food ingredients have to be declared on food labels [5]. Allergenic compounds can be found at any stage of food processing due to the presence of allergens in raw ingredients or caused by cross-contamination during manufacturing process [6]. Among them, wheat gluten, milk, and egg proteins account for the majority of the documented food hypersensitivity causing up to 90% of food allergies. The sources of these allergens, wheat, barley, rye, eggs, whey, and casein milk fractions, are widely used in food manufacturing. Consequently, they can be found in many types of nourishment such as baby foods, ice creams, sausages, pizzas, bread, cookies, and ready-to-serve prepared meals, which makes a declaration of the presence of these allergens in food a must.

The established limit of accepted concentration for gluten-free products is 20 mg kg⁻¹ as stated by FAO/WHO CODEX (STAN 118–1979, and its amendments 1983 and 2015, revised 2008) [7]. However, there is no accepted concentration limits for other food allergens. Most regulatory agencies have focused on regulating the labeling irrespective of the amounts

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of allergens in the food. In fact, today, there is not an international consensus to establish maximum allergen content for most food allergens except for gliadin. This is due in part because the allergic effect varies greatly between individuals and the consumption of low amount of allergens can upset some individuals but others are not affected at all. It is therefore necessary to develop highly sensitive methods to alert consumers about the presence or absence of certain allergens in food. The only way to confirm that foods are allergen-free is simple, but it depends on having reliable analytical methods which allow quantification of trace amounts of allergens.

A variety of methods is currently used for food allergen determination. The most popular are immunoassays in ELISA plate format, employed by food industries and food control agencies, for monitoring the presence of allergens. Immunoassays are based on specific antibodies released against whole protein extract or a unique allergen protein [8]. These methods can reach a limit of detection of $70 \mu\text{g kg}^{-1}$ for peanuts in food products [9]. Another type of immunoassay applied for allergen detection is represented by lateral flow assays (LFA) which are rapid, inexpensive, and portable and it does not require sophisticated instrumentation or trained personnel. However, it is commonly used for qualitative detection of food allergens in the food industry with relatively good sensitivity ($\sim 5 \text{ mg kg}^{-1}$ of allergen) [10]. In this line, a two-site monoclonal antibody LFA was described for bovine β -lactoglobulin detection with a sensitivity of 0.2 ng mL^{-1} [11]. A magnetic particles-based chemiluminescence enzyme immunoassay (MPs-CLEIA) for ovalbumin detection was introduced; the assay detection limit was $0.24 \mu\text{g L}^{-1}$ with average recoveries between 92 and 97% [12].

DNA-based assays detect the specific oligonucleotide sequences which encode for the allergenic protein. They are preferred when used as confirmatory techniques. A tetraplex real-time PCR method was established to quantify simultaneously trace amounts of the four allergens soybean, celery, and white and brown mustard, achieving a limit of detection of 8.5, 3.7, 36.8, and 2.6 mg kg^{-1} , respectively [13]. Immunoassays and PCR-based techniques complement well each other, depending of the processed level of the food product.

Bioanalytical techniques represent a potential alternative method, and provide probably one of the most promising ways to solve some problems concerning simple, fast, reproducible, and cheap multi-analyte detection [14]. For example, a silicon-based optical thin-film was developed, that simultaneously permits the visible detection of eight food allergens on the basis of two tetraplex PCR systems [15]. Recently, an electrochemical assay was developed in combination with the disposal pencil graphite electrode (PGE) for detection of gluten, reaching a detection limit of 7.11 mg L^{-1} [16]. Another sensitive approach based on magneto immunosensor was

established for the determination of β -lactoglobulin, reaching a limit of detection of $0.8 \mu\text{g L}^{-1}$ [17].

Nevertheless, the performances of the technologies used for the determination of allergen compounds are very dependent on the food extraction protocol, which is a critical step when the simultaneous detection of food allergens is the goal. An optimization of a simultaneous extraction protocol for food allergen proteins is a challenge. The extraction buffer used could affect the resulted values depending on the total amount of extracted protein. Due to the different nature of proteins and food matrices effect, it is not easy to develop a unique extraction protocol, able to extract simultaneous and quantitatively all allergenic proteins in tested sample. Optimization of an extraction protocol which can give acceptable recovery values for several allergen proteins would save time of the assay particularly in a multiplexed detection format [18].

The purpose of this work was twofold. First, to develop a sensitive and selective multiplexed biosensing methodology; second, to select a reliable and cost-effective extraction protocol for gliadin, casein, β -lactoglobulin, and ovalbumin quantitative and simultaneously from food samples. The analytical biosensing approach is based on a competitive immunoassay adopted on DVD as an analytical platform using specific gold-labeled antibodies as detector species and a disc drive as detector.

Experimental

Chemical reagents

Casein, β -lactoglobulin, ovalbumin, 5 nm diameter gold spherical nanoparticles (AuNPs), bovine serum albumin (BSA), trizma base, Tween 20, and silver enhancer solutions were from Sigma-Aldrich (Madrid, Spain). Monoclonal antibodies for gliadin (R5), casein, and ovalbumin, polyclonal antibodies for β -lactoglobulin, HRP-conjugated antibodies, and gliadin standard solution were kindly provided by Ingenasa, S.A. (Madrid, Spain). Tetramethylbenzidine (TMB) substrate was from SDT GmbH (Baesweiler, Germany). Printing buffer was 50 mM sodium carbonate/bicarbonate buffer, pH 9.6, assay (and washing) buffer (PBST $1\times$), 10 mM sodium phosphate buffer, 150 mM NaCl, 0.05%, Tween 20, pH 7.5, Tris base 20 mM, pH 8.5, 1 and 10% BSA-Tris base 20 mM, pH 8.5; conditioning buffer for liquid samples (1 M Tris base buffer, 5% Tween, pH 9.0). The tested extraction buffers were (I) 2-ME (250 mmol L^{-1}) in guanidine-HCl (2 mol L^{-1}) in PBS, pH 7.4; (II) high salt buffer, 20 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 1 M NaCl, pH 7.4; (III) carbonate buffer, 0.1 M, pH 9.6; and (IV) 40% ethanol in PBST, pH 7.4.

Antibody labeling

The conjugation of specific antibodies to spherical gold particles was performed as previously described [19]. Briefly, the pH of 1.0 mL of colloidal gold solution was adjusted to 8.5 adding two drops of 1 M Tris buffer, pH 8.5. Next, 30 μg of antibody dissolved in 20 mM Tris base, pH 8.5 was added to the colloidal solution and left it for 30 min at room temperature on stirring. Then, 100 μL of 10% BSA-Tris base 20 mM, pH 8.5 were added and incubated as before. The suspension was collected in Eppendorf vials and centrifuged for 30 min at 15,000 rpm, controlling the temperature, 4 °C. Supernatant was discarded and pellet washed with 1.0 mL Tris base 20 mM, pH 8.5 then centrifuged again as before. Supernatant was discarded and the pellet was resuspended in 100 μL 1% BSA-Tris 20 mM, pH 8.5. The gold conjugates were kept at 4 °C until use.

Assay protocol

Two types of immunoassays, sandwich and competitive, were tested for food allergen determination in terms of analytical performances. For the sandwich immunoassay, specific antibodies against gliadin, casein, β -lactoglobulin, and ovalbumin were prepared in printing buffer at 40 mg L^{-1} . The solutions were dispensed (50 nL) on the polycarbonate surface of a regular DVD in microarray format, using a noncontact printer (AD 1500 BioDot, Inc., Irvine, CA, USA). The disc arrays were incubated overnight at room temperature. The disc layout consisted of 48 microarrays, each one of 3×3 spots. Then, the allergen proteins in PBST (0–20 mg L^{-1}) were dispensed over the microarrays identified by four corner marks (30 μL /array). After 30 min incubation at room temperature, each disc was washed with deionized water and dried by centrifugation, using a laboratory centrifuge adapted for planar surfaces. Next, 30 μL of a mixture solution prepared from HRP-labeled specific antibodies (1/1000 dilution anti-gliadin and anti- β -lactoglobulin, 1/20,000 anti-casein, and 1/50,000 anti-ovalbumin) were dispensed over the arrays. The immunoreaction was incubated for 30 min and the disc washed and dried as before. The immunoreaction was developed by homogeneously distributing 1.0 mL TMB solution along with the whole disc surface using a 12 cm diameter 0.6-mm-thick dummy disc. After 10-min incubation, the reaction was stopped by washing with deionized water and drying the disc as before.

For the multiplex competitive immunoassay, discs were printed in microarray format with the allergen proteins at a concentration of 5.0 and 10 mg L^{-1} , for gliadin and casein, respectively, and 20 mg L^{-1} for β -lactoglobulin and ovalbumin. The microarrays consisted in 4×4 spots in which each row corresponded to one coating allergen protein. The discs were coated as described before.

Standard proteins, diluted in PBST at a concentration range of 0–10 mg L^{-1} , were incubated for 15 min with the mixture of specific gold-labeled antibodies at room temperature, before dispensing the solution onto the disc (1/500 dilution for all gold-labeled antibodies except for anti-casein that 1/1000 dilution was used). Then, 30 μL of the mixture were dispensed on the array and incubated for 30 min, followed by washing and drying as described above. The immunoreaction was detected using silver enhancer solution for 10 min. Finally, the disc was washed and dried. A scheme of simultaneous determination of four food allergens using compact disc immunoassaying technology is depicted in Fig. 1.

Signals were quantified by a modified DVD drive and custom software was used to perform the microarrays acquisition and data analysis as previously described [20]. Briefly, during the DVD scanning, the laser hits the immunoreaction products that modify the reflection properties of the DVD surface, attenuating the laser beam intensity according to the optical density of the immunoreaction product. The displayed signal is direct and inversely proportional to the analyte concentration for the sandwich and the competitive assay, respectively. The standard data points (measurements vs. semi-log concentration) were plotted and a four-parameter logistic (4PL) curve was fitted through the points, using SigmaPlot 11 software. The concentrations of the samples were determined from the fit with specified dilution factors applied, if needed. Standard deviation was calculated for each concentration and sample. The limit of detection and the signal-to-noise ratio were calculated as the concentration of the allergen that inhibits 10% the maximum signal, and as the ratio of the maximum signal and the standard deviation of the background noise, respectively.

Food sample preparation

Solid and liquid food samples were bought from local stores and kept at 4 °C until use. Infant jar food with meat from an international food brand was used as a solid sample. Both the solid and liquid samples (apple juice) were declared by the manufacturer as “allergen-free” products.

Solid samples undergo an extraction protocol starting with adding 9 mL of extraction buffer to 1 g of representative sample ($n = 5$). Each sample was homogenized with ART-MICCRA D-1 at speed scale of 10,000 min^{-1} for 5 min. Then the sample was incubated for 30 min at room temperature over stirrer. Next, samples were sonicated and centrifuged for 10 min at 13,000 rpm. The supernatant was collected and filtrate through 0.45- μm nylon membrane filters. Extracts were diluted 1/10 (v/v) with PBST before the analysis. The extraction buffers were tested with infant spiked food with known concentrations of allergen proteins. Samples were analyzed using the optimized multiplexed competitive immunoassay.

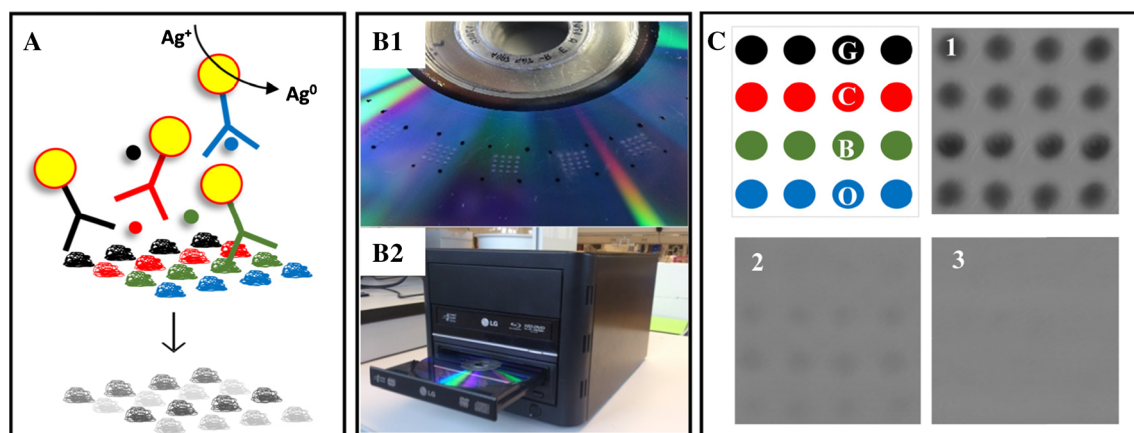


Fig. 1 Scheme of a competitive microimmunoassay on DVD. **A** The allergen proteins are immobilized on the DVD surface. Gold-labeled antibodies bind either to the specific immobilized allergens or to the analytes in solution (allergens). The immunointeraction is displayed as a black precipitate (gray scale) after silver enhancement step. Each array

(**B1**) is localized by four marks made by a waterproof pen and the disc is scanned by the DVD drive (**B2**). Home-made software was used to perform the microarrays acquisition and data analysis. In frame **C**, panels 1–3 correspond to the images of the arrays for allergen concentrations of 0, 1.25, and 10 mg L⁻¹, respectively

For the analysis of juice and beer samples, one volume of PBST 10 × as conditioning buffer was added to nine volumes of sample to adjust pH and ionic strength. If needed, additional dilutions (1/10–1/2000) were done in PBST to samples outside the range of the standards or the fit.

Results and discussion

Assay development

Immunoassays in microarray format are a good choice to develop multiplexed analytical approaches for the simultaneous determination of food allergens because of the high sensitivity and selectivity of antibodies. Two immunoassay formats (sandwich and competitive) were compared in terms of analytical performance (sensitivity, selectivity, and reproducibility). In a first study, the performance of the sandwich single immunoassay was evaluated in terms of sensitivity. For that, the optimal capture antibody concentration, ranging from 5 to 50 mg L⁻¹, and HRP-labeled detector antibody dilution (1/100 to 1/50,000) were selected in a checkboard titration assay. Each single assay was optimized by setting a calibration test using protein standards at a concentration range of 0–20 mg L⁻¹. The best results were obtained, using 25 mg L⁻¹ for anti-gliadin, and 40 mg L⁻¹ for both anti-casein and anti-β-lactoglobulin as the capture antibody concentration while 1/1000, 1/20,000, and 1/1000 dilution for anti-gliadin, anti-casein, and anti-β-lactoglobulin detector antibody, respectively. The single assays showed very good sensitivity and well-fitted calibration curves (R^2 was found 0.97, 0.99, and 0.98 for gliadin, casein, and β-lactoglobulin) with limits of detection of 1.2, 31, and 120 μg L⁻¹. According to the accepted criterion that gliadin fraction represents 50% of the whole gluten

molecule [7], the detection limit for gluten was 2.4 μg L⁻¹. Calibration curves are showed in the Electronic Supplementary Material (ESM, Fig. S1). As far as the ovalbumin single immunoassay is concerned, the sensitivity, referred to the concentration of allergen that inhibits the response halfway between the background and maximum signal (EC_{50}), was higher than 20 mg L⁻¹. This result limited the integration of ovalbumin system in a multiplex assay for the simultaneous determination of the four allergen proteins. It is worth mentioning that the sensitivity of the triplex assay (gliadin, casein, and β-lactoglobulin) was significantly similar to the sensitivity of each assay in the single mode.

In a way to implement the determination of the four allergens in a multiplex assay, the competitive format was evaluated. For that, the selection of the optimal concentration of each coated allergen protein and gold-labeled antibody dilution (1/25–1/2500) was carried out by checkboard titration assays, employing allergen standards ranging from 0 to 10 mg L⁻¹. The criteria used for the selection of the appropriate concentration of the immunoreagents were sensitivity, reproducibility, and signal-to-noise ratio ($S/N > 60$). The optimal coating allergen concentration was 5.0 and 10 mg L⁻¹ for gliadin and casein, respectively, and 20 mg L⁻¹ for β-lactoglobulin and ovalbumin. On the other hand, the optimal dilutions of the labeled antibodies were 1/500 for anti-gliadin, β-lactoglobulin, and ovalbumin and 1/1000 for anti-casein gold-labeled antibodies.

To demonstrate the reliability of the assays, the intradisc and disc-to-disc relative standard deviations (RSDs) were calculated. For that, a set of nine precoated discs with 20 arrays each were tested. The intradisc RSD varied from 2.1 to 8.8%, whereas the disc-to-disc RSD ranged from 5.5 to 14.7%. These results demonstrate the good reproducibility of the assays and the consistency of disc modification.

The multiplexed assay calibration curves are shown in Fig. 2. The data obtained from curves showed EC_{50} values (sensitivity) of 0.04, 0.40, 0.08, and 0.16 $mg\ L^{-1}$ for gliadin, casein, β -lactoglobulin, and ovalbumin, respectively. The relative standard deviation ranged from 3 to 15%, and the linear dynamic range of the multiplex assay for gliadin, casein, β -lactoglobulin, and ovalbumin was 0.01–0.3, 0.08–2.0, 0.02–0.5, and 0.04–0.5 $mg\ L^{-1}$, respectively.

Cross-reactivity studies were also performed, using the four allergens as possible interfering compounds. For that, each individual assay was run in the presence of the other three protein allergens at concentrations of 0.1, 1.0, and 10 $mg\ L^{-1}$. The cross-reactivity values were calculated as a percentage of an equivalent concentration of the analyte found from the calibration curve, divided by the equivalent concentration result from the tested protein curve. As is shown in Table S1 (see ESM), the maximum cross-reactivity was 2.04% for the gliadin assay when testing casein protein, while the minimal cross-reactivity found was <0.36% for gliadin assay when testing ovalbumin. These results indicate that the competitive multiplex assay is selective and the four allergen proteins can be simultaneously determined.

The activity of the precoated protein-modified DVDs was tracked during 3 weeks in order to evaluate the storage stability of the immobilized proteins. For these experiments, three discs were printed with 20 arrays. One array of each disc was used every day using freshly prepared gold-labeled antibody dilutions. The average signals obtained for each of the 20 arrays (days 1–20) were significantly similar (Student's *t* test, 0.05 threshold level) to those of the reference arrays (day 0). These results indicate that the four immobilized proteins (gliadin, casein, β -lactoglobulin, and ovalbumin) were stable over the analyzed period without losing analytical performances. The results are in good agreement with those already

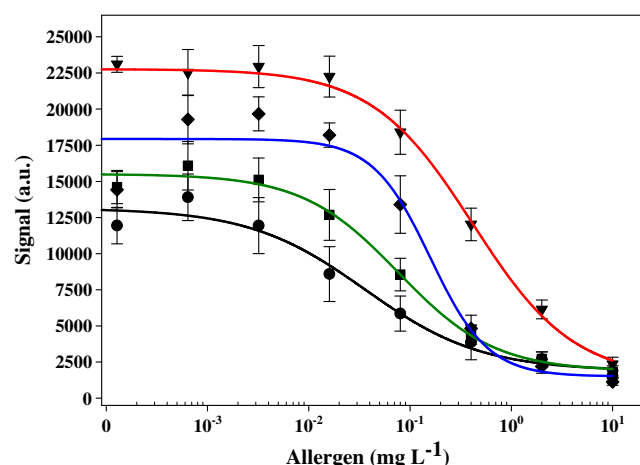


Fig. 2 Multiplexed immunoassay calibration curves. Black (bullet), red (down-pointing triangle), green (square), and blue (diamond) solid lines are for gliadin, casein, β -lactoglobulin, and ovalbumin curves, respectively

reported for immobilized proteins (coating conjugates) on the polycarbonate surface of standard DVDs [21]. During the experimental work, the discs were stored inside a slim box at 4 °C.

Sample extraction study

Although there is a big progress in developing alternative analytical methods for food allergen detection, some of them deals only with single allergen detection, and others lack the unique extraction protocol, which is somewhat difficult to be optimized due to the different nature of proteins to be analyzed.

Allergen-free baby foods were used as a model of solid samples, with low-fat content (<10%). The extraction buffer selection was performed by recovery studies, using spiked samples, containing 20 $mg\ kg^{-1}$ of each tested allergen protein (a mixture of allergen proteins). Samples were extracted with four different extraction buffers: (I) 2-ME (250 $mmol\ L^{-1}$) in guanidine-HCl (2 $mol\ L^{-1}$) in PBS, pH 7.4; (II) high salt buffer, 20 $mM\ NaH_2PO_4 \cdot H_2O$, 1 $M\ NaCl$, pH 7.4; (III) carbonate buffer, 0.1 M , pH 9.6; and (IV) 40% ethanol in PBST, pH 7.4. These buffers are the most commonly used in commercial extraction kits; they were selected depending on their capacity to extract different types of allergen simultaneously. Also, both chemical and mechanical extraction conditions were combined to ensure maximum and regular recoveries.

The recovery of the mixed allergens with the extracting solution I resulted in a loss of the analytical performances of the multiplex assay; concretely, low S/N ratios (<12) and low reproducible results ($RSD > 28\%$) were obtained. Similarly, the use of high salt and carbonate buffers (II and III) affected particularly the determination of gliadin, achieving a recovery lower than 17% for this protein. On the other hand, the extraction of casein, β -lactoglobulin, and ovalbumin with buffers II and III worked successfully, achieving recoveries ranged from 85 to 98%.

Gliadin and its fractions are well-dissolved in aqueous ethanol solutions (40–70%), but ethanol at high concentration affects the analytical performance of the assay on disc. Since the other three allergen proteins are quite soluble in aqueous buffers, the study was also carried out using solution IV as extracting buffer. The minimum recommended concentration of ethanol (40%) was used to extract gliadin. First, allergen-free baby food samples were spiked with gliadin within the dynamic working range (0.1, 0.2, and 0.4 $mg\ kg^{-1}$). Also, non-spiked infant food samples were used as blank. Both spiked and non-spiked samples were extracted as described in the experimental section. Before analysis, the extracts were diluted in PBST (1/2, 1/4, and 1/10) to minimize the effect of ethanol on the analytical performances of the immunoassay.

The recovery results obtained with the extracts diluted 1/2 and 1/4 in PBST were below 35% for all the spiked

concentrations. It can be concluded that high ethanol content in the extracts (10 and 20% for 1/4 and 1/2 dilution, respectively) affected considerably the performances of the multiplex assay. On the contrary, 1/10 dilution of the extracts before analysis reduced significantly the ethanol effect, allowing to achieve good recoveries; 94, 88, and 86% for the samples spiked with 0.1, 0.2, and 0.4 mg kg⁻¹, respectively.

It is worth mentioning that the signal given by the 1/10 dilution of the blank was significantly similar (p value <0.05) to that obtained in PBST, what suggests that negligible sample interferences were found. According to these results, the buffered ethanol solution (40% ethanol in PBST, pH 7.4) as extracting medium allowed the quantitative extraction of gliadin from food samples. Further analysis was performed following the described extraction protocol and diluting the extracts 1/10 in PBST, before analysis.

The key point in the analysis of liquid samples consisted in adjusting the pH of the sample without practically diluting them to avoid loss of sensitivity. In particular, sample conditioning consisted in adding one volume of PBST 10 × to nine volumes of sample; this being the only required sample treatment. To test the suitability of this protocol, apple juice samples, which are completely free of allergen proteins, were spiked with gliadin, casein, β-lactoglobulin, and ovalbumin at 0.31, 1.25, 5.0, and 20 mg L⁻¹. Non-spiked samples were also used as blank. As it can be seen in Fig. S2 (see ESM), the

signals that correspond to the different allergen concentrations in conditioned juice samples are within the confidence interval of the curve in PBST. Only, in the case of β-lactoglobulin and ovalbumin, there was a significant difference for the quantification of these allergens above 1.0 mg/L. For the rest of the allergen proteins, there were negligible interferences.

Analysis of food samples

Recovery tests and validation studies were performed using five solid (S) and five liquid (L) spiked samples with different concentrations of allergen proteins, taking in consideration the presence of single, double, triple, and quadruple allergen mixtures. Meat infant food and apple juice samples were allergen-free as declared by the manufacturer. As is shown in Table 1, the recovery reached for solid infant food ranged between 77 and 108%, whereas for apple juice samples, the recovery percentage was between 82 and 117%. In most of the cases, the recovery values are within the analytical error range (±20%) and the minor discrepancy in recoveries between replicate samples are not statistically significant (p value <0.05). The results are in good agreement with those declared by the international food company as “allergen-free” food products.

As an interesting application for the developed methodology, 20 commercial beer samples, which represent major brands available in the Spanish market, were analyzed for

Table 1 Recovery results obtained for the competitive multiplex assay of spiked solid (S) and liquid (L) apple juice samples

Sample	At.	A. (mg kg ⁻¹)	F. (mg kg ⁻¹)	R. (%)	Sample	At.	A. (mg L ⁻¹)	F. (mg L ⁻¹)	R. (%)
S1	G	5	4.7 ± 0.2	93	L1	G	0.12	0.14 ± 0.01	117
	C	20	22 ± 1.8	108		C	0.50	0.49 ± 0.06	98
	B	0	< LD	–		B	0.12	0.13 ± 0.02	102
	O	10	9.9 ± 0.6	99		O	0	< LD	–
S2	G	5	4.5 ± 0.3	90	L2	G	0	< LD	–
	C	0	< LD	–		C	1	1.06 ± 0.07	106
	B	10	7.7 ± 0.4	77		B	0	< LD	–
	O	0	< LD	–		O	0.30	0.28 ± 0.04	95
S3	G	5	4.8 ± 0.2	95	L3	G	0.25	0.20 ± 0.02	82
	C	0	< LD	–		C	0	< LD	–
	B	0	< LD	–		B	0.30	0.29 ± 0.03	97
	O	0	< LD	–		O	0.15	0.13 ± 0.02	88
S4	G	0	< LD	–	L4	G	0	< LD	–
	C	5	5.1 ± 0.3	102		C	0	< LD	–
	B	0	< LD	–		B	2.5	4.8 ± 0.01	96
	O	0	< LD	–		O	0	< LD	–
S5	G	2	1.8 ± 0.1	92	L5	G	0	< LD	–
	C	2	2.1 ± 0.1	107		C	0	< LD	–
	B	2	1.6 ± 0.1	79		B	0	< LD	–
	O	2	1.9 ± 0.1	98		O	2.5	2.4 ± 0.21	94

At. analyte; A. added conc.; F. found; R.% recovery; LD limit of detection; G, C, B, and O are gliadin, casein, β-lactoglobulin, and ovalbumin, respectively; S1–S5 solid infant food samples; and L1–L5 liquid samples

gluten content by the developed disc competitive microimmunoassay.

Upon using the developed assay, samples were conditioned (see “Experimental” section) and analyzed by triplicate and diluted in PBST (1/10–1/100) accordingly, depending on the gliadin content. Gluten concentrations were found to be $<3.0 \text{ mg L}^{-1}$ in four samples of gluten-free beer. Eleven beer samples, mainly produced from barley or wheat, showed gluten concentrations above the threshold ($20 \text{ mg gluten kg}^{-1}$) of the EU 1169/2011 for gluten-free foods. The rest five samples contain acceptable gluten levels even; they were not labeled gluten-free (Table 2). These results show that there is no free beer produced from barley or wheat regarding the maximum tolerable daily intake (10 mg); however, some beers practically do not contain gluten while others have higher levels than 10 mg L^{-1} . The results were compared with those evaluated by ELISA, determining hydrolyzed prolamins. As it can be seen in Fig. 3, there is a good correlation between the results obtained by ELISA and DVD-array ($r^2 = 0.98$), corroborating the analytical performances of the proposed methodology.

Taking into consideration that food production increases day by day, from a practical point of view, sensitive, selective, rapid, and reproducible methodologies are demanded. The

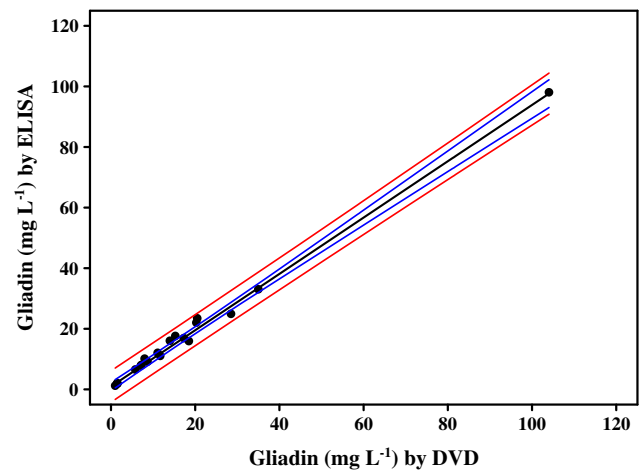


Fig. 3 Correlation of the results of gliadin concentration in beers determined by the DVD and direct ELISA approaches

commercially available kits for food allergens are time consuming and few dozen of samples can be measured in one standard ELISA plate format. Also, in general, the kits determine only one allergen per assay; this being a major disadvantage in comparison to the proposed methodology which, in the current version, four allergens can be simultaneously determined.

Table 2 Gluten content of commercial beers analyzed by the developed gliadin disc immunoassay

Sample	ABV (%) ^a	Gliadin (mg L^{-1})	Gluten (mg L^{-1}) ^b
1 ^c	5.4	1.0 ± 0.1	2.0 ± 0.2
2	4.8	20.3 ± 1.5	40.6 ± 3.0
3	5.0	5.8 ± 0.5	11.6 ± 1.0
4	<0.5	11.7 ± 1.2	23.4 ± 2.4
5	4.4	8.7 ± 0.9	17.4 ± 1.8
6	1.0	7.1 ± 0.7	14.2 ± 1.4
7	<0.5	11.0 ± 1.3	22.2 ± 2.6
8	2.6	14.1 ± 1.2	28.2 ± 2.4
9	5.4	7.3 ± 0.7	14.5 ± 1.4
10	5.1	85 ± 6.4	170 ± 12.8
11	6.5	104 ± 8.7	208 ± 17.4
12	6.3	18.5 ± 1.5	37.0 ± 3.0
13 ^c	4.2	1.2 ± 0.1	2.4 ± 0.2
14 ^c	4.8	1.4 ± 0.1	2.8 ± 0.2
15 ^c	5.7	1.3 ± 0.1	2.6 ± 0.2
16	5.1	20.5 ± 1.8	41.0 ± 2.8
17	2.6	8.0 ± 0.5	16.0 ± 1.0
18	4.5	15.3 ± 1.1	30.6 ± 2.2
19	5.0	28.5 ± 1.9	57.0 ± 3.8
20	4.0	17.3 ± 1.3	34.6 ± 2.6

^a Alcohol by volume

^b Gluten concentration was calculated by two times the concentration obtained for gliadin

^c Samples labeled gluten-free

Conclusions

A simultaneous screening method based on compact disc technology for the determination of multiple protein allergens in a competitive microimmunoassay format, using specific antibody-functionalized gold nanoparticles is a promising approach for food allergen testing. One DVD can hold huge number of micro spots (replicates) and dozens of samples can be analyzed simultaneously what become compact disc immunosensing technology an alternative analytical methodology for the determination of multiple allergens in a short time with high sensitivity and selectivity.

The multiplex assay was evaluated by the determination of allergen traces in both infant food (meat with vegetables) and apple juices and beers as liquid samples. The good limit of detection and low cross-reactivity demonstrate the good performances of the developed assay, also showing potential for application in food control analysis. Our methodology is capable of identifying allergen-free food products, considering jar infant food and apple juice and beers as model commodities.

The extraction protocol for solid samples includes using 40% ethanol in PBST, pH 7.4 as extracting buffer rendered good recovery figures for the four allergens. As high ethanol content affects the sensitivity of the assay, the aqueous dilution of the extracts is needed. For the analysis of apple juices and beers, adjusting the pH to 7.4 and ionic strength to that of the

working buffer (PBST) were the only sample treatment needed.

It is worth mentioning that the assay format is critical to set-up a practical multiplex assay. In this sense, according to our results, the sandwich format is in general more sensitive than the competitive. However, the competitive format is more practical to build a multiplex assay with the aim to determine simultaneously the four allergens. Also, having specific antibodies with suitable sensitivity and selectivity is key to scale-up the multiplex assay for other food allergens.

DVDs as a platform and a customized disc drive as the detector is in fact a powerful and cost-effective analytical tool with high potential for screening applications with further prospective to analyze a large number of food allergens and samples. These features prove the assay applicability for multi-allergen determination in food monitoring processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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