

A flow-cytometry-based method for detecting simultaneously five allergens in a complex food matrix

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Abstract To avoid carry-over contamination with allergens, food manufacturers implement quality control strategies relying primarily on detection of allergenic proteins by ELISA. Although sensitive and specific, this method allowed detection of only one allergen per analysis and effective control policies were thus based on multiplying the number of tests done in order to cover the whole range of allergens. We present in this work an immunoassay for the simultaneous detection of milk, egg, peanut, mustard and crustaceans in cookies samples. The method was based on a combination of flow cytometry with competitive ELISA where microbeads were used as sorbent surface. The test was able to detect the presence of the five allergens with median inhibitory concentrations (IC₅₀) ranging from 2.5 to 15 mg/kg according to the allergen to be detected. The lowest concentrations of contaminants inducing a significant difference of signal between non-contaminated controls and test samples were 2 mg/kg of peanut, 5 mg/kg of crustaceans, 5 mg/kg of milk, 5 mg/kg of mustard and 10 mg/kg of egg. Assay sensitivity was influenced by the concentration of primary antibodies added to the sample extract for the competition and by the concentration of allergenic proteins bound to the surface of the microbeads.

Keywords ELISA · Flow cytometry · Food allergens · Multiplex

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Introduction

Food allergies increased steadily over the past 20 years, leading to a 500% surge in the number of severe reactions that required hospitalization (Gupta et al. 2007). Between 2 and 3% of adults and 4–6% of children (Worm et al. 2010) suffer from this pathology characterized by an excessive immune response upon ingestion of an ingredient that is usually harmless. Strict exclusion of the allergy-causing ingredient from the diet of the sensitized individual is the main way to prevent accidents ranging from benign cutaneous itching to potentially lethal anaphylactic shock. Consequently, having access to the complete list of ingredients present in food preparations is indispensable to minimize the risk of inadvertent exposure to the triggering ingredient. Canada, the United States, and the European Union have adopted legislations on the essential information to be communicated to consumers. Eight major allergens, i.e. peanut, tree nuts, milk, egg, fish, crustaceans, soy, and gluten, constitute the common focus of these different legislations (Gendel 2013) and represent over 90% of the agents triggering serious allergic reactions. In addition to these so-called «big 8», the following have been added to the list of priority allergens: mollusks, sesame, mustard, and sulfites (in Europe and Canada) and lastly, celery and lupin (in Europe only). Most product recalls ordered by food chain control bodies concern carry-over contaminations where the presence of an allergen is accidental. This is often due to the increasing variety of ingredients stored at production sites and to the use of a single production line to manufacture several food preparations (Alvarez and Boye 2012; Gendel 2013). To minimize this risk, checking for the absence of allergens in finished products has become a routine quality control procedure in the agro-food industry. Yet because of the ever-increasing volume

of foods produced, an effective control policy must be based on the use of quick, cheap tests in order to analyze a representative sampling of the preparations available on the market.

Immunological methods (the enzyme-linked immunosorbent assay or ELISA and the lateral flow device or LFD) are routinely used for the detection of allergen traces in finished products due to their ease of use and competitive prices. Polymerase chain reaction (PCR), which detects allergens indirectly by quantifying their DNA instead of their proteins, is less commonly used for routine. However, it possess the advantage of being able to detect the DNA of several allergens simultaneously in a single test, in contrast to immunological methods, which can only detect one allergen (Poms et al. 2004; Kirsch et al. 2009; Monaci and Visconti 2010). On the other hand, PCR may lack sensitivity for food allergens with low DNA to protein ratio such as milk and egg. An effective control strategy thus requires multiplying the number of immunological assays, which increases the cost of food product quality control.

Recently, coupling flow cytometry to immunoassays made possible the development of multi-residue detection methods. This approach has been tested with success in detection of veterinary drug residues (Bienenmann-Ploum et al. 2012) and mycotoxins (Peters et al. 2011, 2013), but it is still little used in food quality control. (Haasnoot and du Pré 2007) were the first to publish results in this field, having developed a method for detecting fraudulent substances derived from plants (soy proteins, peas, and wheat) in powdered milk. The method described focused on the presence of fraudulent instead of allergens and was optimized to display 50% inhibition when the soy proteins or gluten concentrations reached 0.5% of the total protein content (5 g/kg). This 0.5% action level was chosen because a lower percentage of adulteration would not have been of commercial interest for the fraudster. More recently, (Gomaa et al. 2012; Gomaa and Boye 2015) compared the performances of three platforms (commercial ELISA kits, flow cytometry and mass spectrometry) to detect and quantify traces (10, 100 and 1000 ppm) of soy, milk and gluten in unbaked dough and in cookies. The limit of detection of the method was 0.4 ppm for each allergen (based on a serial dilution of allergen extracts used as standard curve). In these experiments, flow cytometry displayed similar performances to the ELISA tests.

The objective of the work was to develop a flow-cytometry-based test able to detect simultaneously five allergens (egg, milk, mustard, peanut, and crustacean) present at concentrations under 100 ppm in a cookie matrix. Secondary goals were to define the minimum allergen concentration able to induce a significant difference of signal between the negative controls and the

positive samples and to evaluate how the ratio of primary antibody in solution and allergenic proteins bound to the surface of the microbeads impacts assay performances.

Materials and methods

Instruments and reagents

Flow cytometry data were acquired and analyzed with a BD Accuri[®] C6 apparatus (Becton–Dickinson, Franklin Lakes, NJ, USA). ELISA plate absorbance measurements were done on a Multiskan-EX spectrometer (Labsystems, Helsinki, Finland) and washes were carried out with a PW 41 plate washer from Bio-Rad (Hercules, CA, USA).

The reagents required to prepare the buffers were supplied by Sigma-Aldrich (Saint-Louis, MI, USA). CytoPlex[™] carboxylated microbeads 4 μm in diameter were purchased from Thermo Scientific (Waltham, MA, USA). Anti-rabbit IgG antibodies coupled to a fluorophore (AlexaFluor 488) or to horseradish peroxidase (HRP) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Polyclonal antibodies raised against proteins of the allergens were purified on Protein G Sepharose 4 Fast Flow columns purchased from GE Healthcare (Little Chalfont, UK). Tetramethylbenzidine (TMB) used to develop the ELISA plates was from SurModics (Eden Prairie, MN, USA). The ingredients used to study cross-reactivity were purchased at a local store.

Buffers preparation

PBS buffer (pH 7.5) used to extract allergenic proteins contained NaCl (150 mM), Na₂HPO₄ (40 mM), KH₂PO₄ (5 mM), and NaN₃ (1 mM). The buffer (pH 6) used to activate the microbeads contained 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 100 mM *N*-hydroxy-succinimide (NHS), and 65 mM *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Antigen coupling to microbeads was done in borate buffer pH 8.5 (50 mM Na₂B₄O₇, 200 mM H₃BO₃). Microbead saturation was done in HPLC-grade water containing ethanolamine (0.1 M). The buffer used to elute antibodies from the affinity column was Na-carbonate buffer pH 9.6 (13 mM Na₂CO₃, 35 mM NaHCO₃). This buffer was also used to coat the ELISA plates. HRP activation was done in 1 mM Na-acetate buffer pH 4.5 (pH corrected with CH₃COONa and CH₃COOH) containing NaIO₄ (35 mM). Coupling to antibodies was done by adding NaBH₄ (4 mg/ml) and triethanolamine to the mix of activated HRP and antibody. The conjugated antibodies were then dialyzed overnight in 4 l PBS containing 10 mM glycine.

Preparation of the analysis matrix

A commercially available cookie-type food matrix was purchased and ground to powder with an industrial grinder (Blixer 4.V.V., Robot Coupe, France). According to the ingredient list, the product contained wheat flour, soy flour, sugar, salt, cinnamon, margarine containing plant oils (palm and turnip rape oils), water, caramel syrup, and sodium. The matrix was spiked with 100 ppm of purified proteins from each allergen (milk, egg, peanut, mustard, and crustacean) and then diluted in non-spiked cookie matrix to achieve the following concentrations: 100, 75, 50, 40, 20, 10, 2, 1, 0.5, and 0.25 ppm.

Preparation of immunogens and antibodies

Polyclonal antisera were obtained by immunizing rabbits (New Zealand White) with protein extracts from the five selected allergens (milk, egg, mustard, peanut, and crustacean). Purified casein was purchased from Calbiochem (Darmstadt, Germany). Egg powder was a reference standard from the National Institute of Standards and Technologies (NIST, Gaithersburg, MD, USA). Crustacean (*Panaeus vannamei*), peanut (*Arachis hypogaea*), and mustard (*Sinapis alba*) extracts were prepared in-house. Briefly, soluble proteins were extracted from 2 g grinded samples diluted in 20 ml PBS preheated to 60 °C. The solutions were shaken for 30 min in a water bath at 60 °C and centrifuged for 15 min at 2700 g. The supernatants were filtered on Acrodisc 0.8/0.2 µm (Millipore). The Bicinchoninic Acid Kit (Sigma-Aldrich) was used to quantify the extracted proteins. Immunological response was triggered by subcutaneous injection of 0.2 mg of purified allergen proteins emulsified with Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (all following injections). Injections were administered on a fortnightly basis and then, from the third injection onward, at the rhythm of one injection every 28 days (Huet et al. 2008). Test bleeds were collected 10 days after each immunization (from the third immunization onward).

ELISAs for serum evaluation

The presence of antibodies of interest in the sera of immunized animals was confirmed by incubating dilutions of the sera for 30 min at room temperature in ELISA plates coated with proteins extracted from the allergens (2 µg/well). After washing and incubation with HRP-conjugated anti-rabbit IgG antibody, detection was done by incubating with TMB/H₂O₂ for 30 min. After blocking the reaction by adding H₂SO₄, the absorbance was read at 450 nm (Huet et al. 2008). The antibodies from the sera that recognized

the proteins of the allergens were then purified on a protein G affinity column and eluted in carbonate buffer pH 9.6.

Coupling of HRP to purified antibodies

HRP was activated with sodium periodate and conjugated to antibodies by reductive amination (Hermanson 2008). Two milligrams HRP were dissolved in 600 µl HPLC-grade water containing NaIO₄ and shaken for 20 min at room temperature in the dark. The solution was then dialyzed for 16 h at 4 °C against 4 l acetate buffer. The solution containing activated HRP was adjusted to pH 9.4 with 0.2 M carbonate. Five hundred microliters of solution containing purified antibodies (8 mg/ml) was added to the solution of activated HRP and the mixture was shaken gently for 2 h at room temperature in the dark. 50 µl of NaBH₄ (4 mg/ml) and 50 µl triethanolamine were added to the HRP-coupled antibodies and shaken for 2 h at 4 °C in the dark. The solution was then dialyzed for 16 h at 4 °C against 4 l PBS containing glycine (10 mM). An equal volume of ethylene glycol was then added to allow storing the HRP-conjugated antibodies at –20 °C.

Specificity and cross-reactivity

The specificity of the allergen-targeting antibodies was evaluated in a sandwich ELISA with 93 ingredients commonly used in prepared foods (almond, apple, apricot, asparagus, avocado, bamboo, banana, basil, bean, beef, beet, blackcurrant, black olive, black radish, Brazil nut, broccoli, butter, carrot, cashew, cherry, cheese, chicken/turkey, chive, raw chocolate (86% cacao), white chocolate, cilantro, clementine, clove, coconut, cod, cream, cucumber, cumin, curry, dried dates, boiled egg, powdered egg, raw egg, eggplant, endive, fennel, garlic, gherkin, ginger, grapefruit, green cabbage, hazelnut, honey, kiwi, laurel, leek, lemon, lettuce, Macadamia nut, maize, melon, milk, powdered milk, mint, mushroom, mussels, mustard, mustard pickles, nutmeg, onion, orange, palm oil, paprika, passion fruit, pea, peach, pear, peanut, pecan, pepper, yellow pepper, red pepper, green pepper, pineapple, pine nut, pistachio, pork, potato, rabbit, rapeseed, red currant, red wine, rice, salmon, shrimp, soy (yellow soybean flour), sesame seed, spinach, sunflower seed, thyme, tomato, turmeric, vinegar, walnut, wheat, white celery and zucchini). Soluble proteins from two grams of each ingredient were extracted as described under "Preparation of immunogens" and incubated in 96-well plates coated with antibodies of interest for 30 min at room temperature. After washing, HRP-coupled antibodies were added and incubated for 30 min at room temperature. Detection was done with TMB/H₂O₂. For extracts generating a positive signal, proteins concentrations were quantified using the

Bicinchoninic Acid test. The ingredient extracts were then diluted to known concentrations (500, 50 and 5 ppm) and quantified against a standard curve generated with known proteins concentrations of the allergen of interest. Cross-reactivity was expressed as the ratio of protein concentrations calculated with the allergen standard curve and protein concentration calculated with the Bicinchoninic Acid test.

Immunogen-microbead coupling

The microbeads used are supplied as a stock suspension containing 10^8 microbeads/ml. For coupling, 10^7 microbeads were collected in a 1.5 ml Eppendorf tube and centrifuged for 3 min at 5500 g. The coupling of the proteins to the microbeads was conducted as described earlier (Haasnoot and du Pré 2007) with the following modifications: 500 μ l MES containing NHS and EDC was used as activation buffer; coupling solution was borate buffer pH 8.5 containing the different concentrations of allergenic proteins (1, 10, 100, and 1000 μ g per 10^7 beads) and HPLC-grade water containing 0.1 M ethanolamine was used as blocking buffer. After removing the ethanolamine solution, the beads were rinsed three times with 500 μ l PBS and then resuspended in 500 μ l PBS buffer.

Flow cytometry protocol

Uncontaminated/contaminated cookie samples (0.5 g) were incubated for 30 min with shaking (140 rpm) in 20 ml PBS buffer preheated to 60 °C. After centrifugation at 2700 g, 200 μ l supernatant was incubated with purified anti-allergen rabbit antibodies at room temperature with shaking (250 rpm) for 15 min. Protein-coated microbeads were added to the solution (7500 microbeads for each allergen to be detected) and incubated with shaking (250 rpm) for 15 min at room temperature in the dark. The suspension was filtered on a 96-well plate equipped with an MSBVN1250 filter (Millipore) to remove reagents that had not reacted. The beads were then incubated with fluorophore-coupled (Alexafluor 488) anti-rabbit IgG antibody for 15 min under shaking (250 rpm) at room temperature in the dark. The fluorescence of each bead at 488 and 700 nm was measured by the cytometer.

Variation of the response with concentrations of allergenic proteins coated on the beads and concentration of anti-allergen antibodies in solution

Several concentrations of purified anti-allergen antibodies in solution (10, 2, 0.5, and 0.17 μ g/ml) and of allergenic proteins coated on the beads (1, 10, 100, and 1000 μ g per

10^7 microbeads) were tested in order to evaluate the impact of these factors on the fluorescence emitted at 488 nm. For each combination of concentrations (antibodies in solution-antigens per bead), two parameters were measured at 488 nm: the signal of the negative controls (matrix without allergens) and the signal decrease between the positive controls (matrix spiked with 1 ppm egg, mustard, or milk antigens or 5 ppm peanut or crustacean antigens) and the negative controls. Results are reported as follows:

$$(MFI/MFI_0) \times 100\%$$

where MFI is the median fluorescence intensity at 488 nm of the tested samples and MFI_0 is that of the negative controls. MFI and MFI_0 are arithmetic medians of the fluorescence intensities calculated for the whole set of microbeads having a similar fluorescence intensity at 700 nm (beads of the same allergen). The above formula represents the signal intensity decrease of the positive samples, normalized with respect to the intensity recorded for the negative controls.

IC50 calculation

The median inhibitory concentration (IC50) is the concentration of contaminating allergen resulting in a 50% decrease of the signal at 488 nm as compared to the negative control. Standard curves consisting in cookie matrices spiked with known concentrations of allergenic proteins (100, 75, 50, 40, 20, 10, 5, 2, 1, 0.5, 0.25 and 0 ppm) were used to calculate, by the means of a logistic regression, the IC50 values for each allergen. The median fluorescence intensity at 488 nm of negative controls (MFI_0) represents 100% of the activity and the remaining standards were normalized relative to the median fluorescence intensity of MFI_0 . Three independent replicates were used to evaluate the reproducibility of the method. For each allergen, the ratio of MFI (positive control) to MFI_0 (negative control) provides a measure of the sensitivity of the flow-cytometry-based method. The lowest contaminant concentration able to induce a statistically significant difference of fluorescent signal between contaminated and uncontaminated cookie samples was calculated for each allergen.

Statistical analysis

Variation of the response with concentrations of allergenic proteins coated on the beads and concentration of anti-allergen antibodies in solution as well as the ratio MFI/MFI_0 for cookies contaminated with increasing concentrations of allergens are reported as mean \pm standard deviation. Mean comparisons were performed (Student *t* test with unequal variance) to determine at which concentration of allergens in cookies the difference in fluorescence intensities between

the negative controls (MFI_0) and the positive samples (MFI) becomes statistically significant ($p \leq 0.05$). Mean comparisons were done with the statistical package of Microsoft Excel.

Results and discussion

Antibody specificity

The following cross-reactions were observed with tested ingredients: anti-casein antibodies with apple (0.7%); anti-peanut antibodies with turmeric (1%); anti-mustard antibodies with rapeseed (100%), anti-egg antibodies with salmon (0.2%). No cross-reactivity was observed with the anti-crustacean antibodies. Sandwich ELISA tests were used to confirm the capacity of antibodies raised against white mustard (*Sinapis alba*) to detect other mustard species commonly used in cooking (yellow mustard *Brassica Juncea* and black mustard *Brassica nigra*) and the capacity of antibodies raised against one crustacean (*Panaceus vannamei*) to detect proteins from other species of this branch (shrimp, crab, lobster, crayfish) (data not shown).

Despite confirmation of cross-reaction with some tested ingredients, none of the developed antibodies reacted with any component of the cookie matrix, making them suitable for use in the described application. Interestingly, mustard antibodies did not react with turnip oil (*Brassica rapa*) present in the matrix while strongly cross-reacting with rapeseed (*Brassica napus*), which was another closely related *Brassicaceae*. Cross-reactivity between members of the gender *Brassica* (*B. napus*, *B. rapa*, *Brassica oleracea*, *B. nigra*, *B. juncea* and *S. alba*) due to important genetic homology between species has been previously described in the literature (Figueroa et al. 2005). Several scientific publications investigating the effect of refining process on oil allergenicity concluded that highly refined vegetable oils contained very low amounts of proteins (Crevel et al. 2000; Zitouni et al. 2000; Koppelman et al. 2007). This absence of significant amount of allergenic proteins can explain the lack of positive signal for mustard in the experimental cookie matrix.

Variation of the response with concentrations of allergenic proteins coated on the beads and concentration of anti-allergen antibodies in solution

The fluorescence of the negative controls was found to increase proportionally to the concentration of antibodies in solution and to the concentration of microbead-bound antigens (Fig. 1). Coating the microbeads with 1 or 10 μg protein proved insufficient as the fluorescent signal

obtained did not exceed the background level (data not shown). The fluorescence intensities observed with egg proteins coated beads were 100 times lower than those observed with the other allergens. The standardized egg sample used for antigen extraction (NIST RM 8415) was irradiated to extend its storage life. In an ELISA test where this reference material was compared to a more recent one specifically developed for allergen detection (NIST RM 8445), the latter displayed a 10-fold superior affinity for the anti-egg antibody used in the study (data not shown). We hypothesize that irradiation or another manufacturing process may have altered the conformation of the allergenic proteins bound to the beads, thus decreasing the sensitivity of the test.

For microbeads coated with 100 and 1000 μg of proteins, a continuous decrease of the result (MFI/MFI_0) was observed in presence of all allergens studied (except crustaceans) when anti-allergen antibody concentrations ranged from 2 to 0.17 $\mu\text{g}/\text{ml}$ (Table 1). It indicates that the difference of response intensity between negative controls and the contaminated sample is more significant for lower concentrations of primary antibody. Above 2 $\mu\text{g}/\text{ml}$ of primary antibody in the solution, the response difference between the sample and control is low (0–6%), indicating a saturation of the microbeads by the antibody and a loss of assay sensitivity. In the case of crustaceans, the result (MFI/MFI_0) is greater than 90% for the three lowest concentrations of antibodies (Table 1). In this case, almost all the antibodies are bound to the free allergens and very little to the microbeads, resulting in a significant difference of response between the negative controls and positive samples. At a concentration of 10 $\mu\text{g}/\text{ml}$ primary antibody, a larger number of antibodies binds to the microbeads, resulting in a decrease of the signal difference at 488 nm.

In conclusion, the difference of signal intensity at 488 nm between positive samples and negative controls increases when the concentration of primary antibodies in solution decreases. Paradoxically and with the exception of milk, the amount of antigens per microbead had little effect. Although the trend was similar for all five allergens, the amplitude of the signal decrease differed according to the antigen. These differences in amplitude might reflect differences in soluble primary antibody-bound antigen binding affinity. In the case of egg, the signal of the negative controls was too low to allow detection of trends related to changes in test conditions (Table 1).

Assay IC50 values

For each allergen, the lowest antibody concentration that resulted in a signal for the negative control above 100,000 fluorescence units (representing two orders of magnitude above the background signal) was selected as

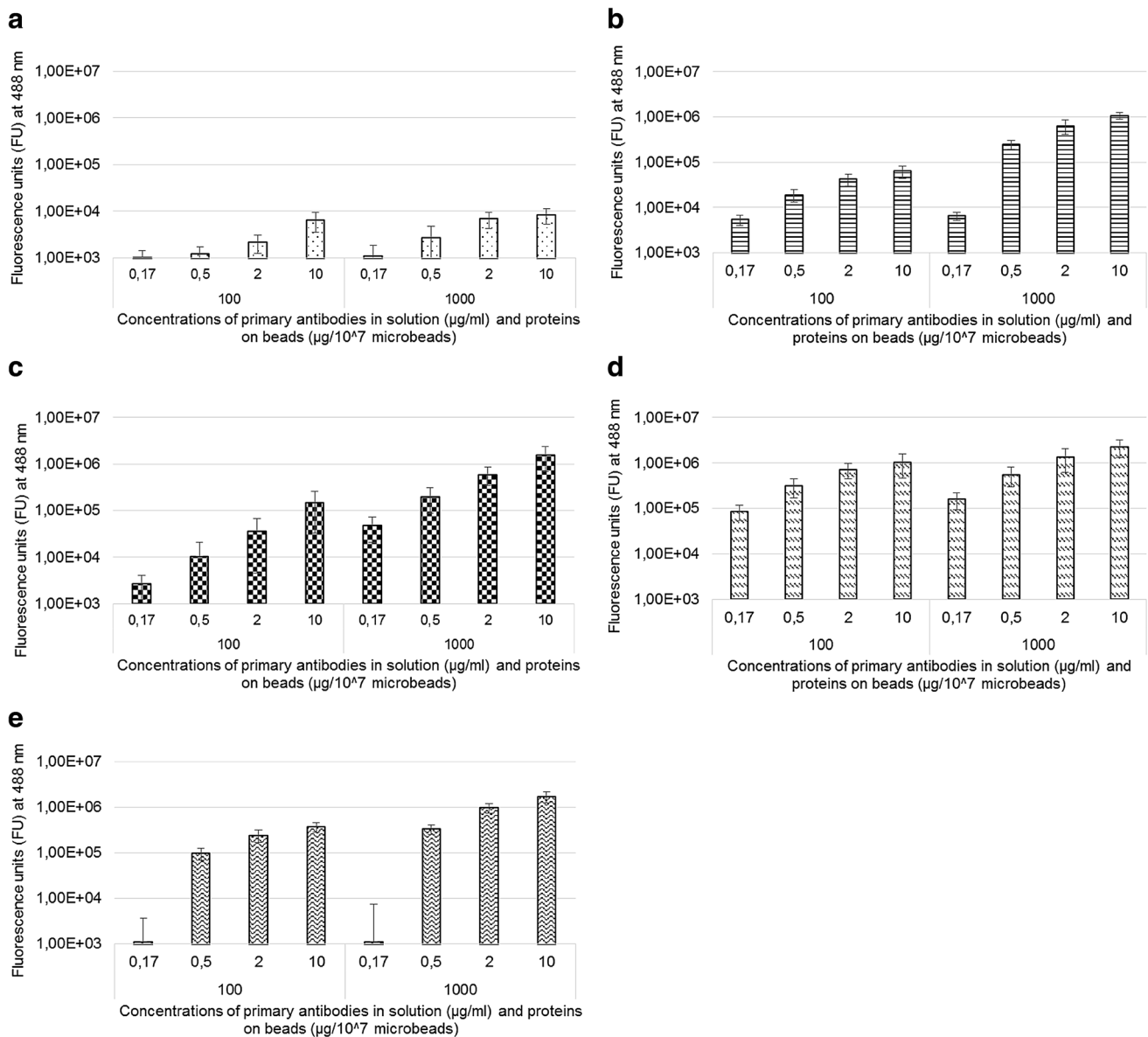


Fig. 1 Fluorescence intensities measured for negative controls with increasing concentrations of primary antibodies in solution (0.17, 0.5, 2, and 10 µg/ml) and at two concentrations of microbead-bound

antigens (100 and 1000 µg/10⁷ microbeads); **a** egg, **b** peanut, **c** mustard, **d** crustacean, **e** milk

antibodies/microbeads concentration ratio. Based on the results obtained for the variation of the response (Table 1), the microbeads coated with 1000 µg of allergenic proteins were selected to determine the IC₅₀ values of the method. Corresponding antibody concentrations used were 0.5 µg/ml for milk, 0.17 µg/ml for crustaceans, 0.5 µg/ml for peanut, 0.5 µg/ml for mustard and 2 µg/ml for egg.

Whatever the allergen studied, the signal at 488 nm was found to decrease with the concentration of allergen in the sample (Fig. 2). The lowest concentration of allergens able to induce a significant decrease in signal were 2 ppm for peanut, 5 ppm for crustaceans, 5 ppm for mustard, 5 ppm for milk and 10 ppm for egg. In the developed system, the

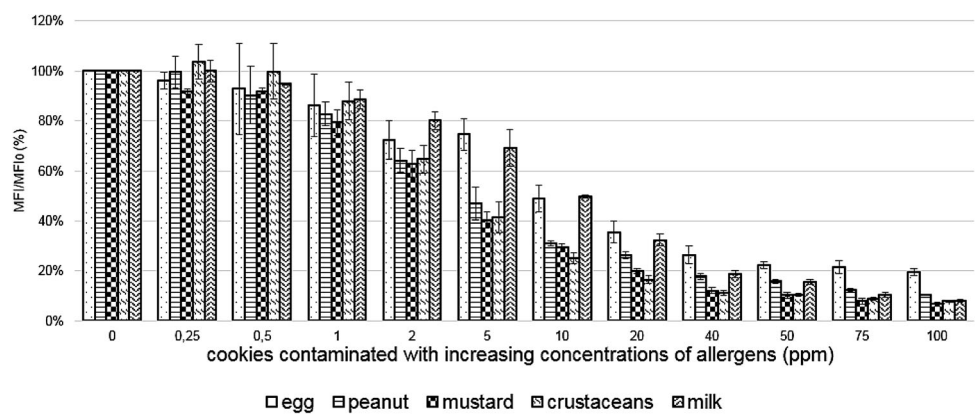
calculated IC₅₀ values were 3.5 ppm for peanut, 2.5 ppm for crustaceans 4.5 ppm for mustard, 8 ppm for milk and 15 ppm for egg.

Recent work by Taylor et al. (2014) conscientiously reviewed the scientific data on the allergens concentration thresholds able to trigger an allergic reaction in sensitized patients. Based on the meta-analysis of the lowest-observed adverse effect levels (LOAELs) recorded during oral food challenges, reference doses for the allergens tested in this study were established as follows: 0.2 mg of peanut proteins, 0.1 mg of milk proteins, 0.03 mg of egg proteins, 0.05 mg of mustard proteins and 10 mg of shrimp (crustaceans) proteins. These thresholds have subsequently

Table 1 Amplitude of the fluorescence signal decrease at 488 nm in spiked samples (milk, mustard, egg: 1 ppm; crustaceans, peanut: 5 ppm) as compared to negative controls

Proteins on beads ($\mu\text{g}/10^7$ microbeads)	Antibody concentration ($\mu\text{g}/\text{ml}$)	MFI/MFI ₀ (%)				
		Milk	Peanut	Mustard	Crustacean	Egg
100	0.17	33	28	81	9	56
	0.5	68	35	82	6	90
	2	96	73	100	5	100
	10	100	100	96	46	94
1000	0.17	3	29	83	11	51
	0.5	69	37	85	8	30
	2	92	62	98	9	40
	10	95	87	100	66	100

Fig. 2 Flow-cytometry-based detection of allergens in cookie samples spiked with different concentrations of allergenic proteins (0, 0.25, 0.5, 1, 2.5, 5, 10, 20, 40, 50, 75, 100 ppm). Concentration of microbead-bound antigens: 1000 $\mu\text{g}/10^7$ microbeads; primary antibodies concentrations: 0.17 $\mu\text{g}/\text{ml}$ for crustaceans, 0.5 $\mu\text{g}/\text{ml}$ for milk, peanut, mustard and 2 $\mu\text{g}/\text{ml}$ for egg



been recommended by the Australian system VITAL 2.0 (Voluntary Incidental Trace Allergen Labeling) as action levels for the food industry. The corresponding action levels for a serving of cookies of 50 g are: 4 ppm of peanut proteins, 2 ppm of milk proteins, 0.6 ppm of egg proteins, 0.1 ppm of mustard proteins and 200 ppm of crustacean’s proteins. Although sensitivity improvements are required for detection of milk, mustard and egg, the method is able to detect the presence of peanut and crustaceans at the levels used by the VITAL 2.0 system. Following successful optimization, flow-cytometry immunoassay may integrate the panel of screening tools available for monitoring the safety of the food chain in the near future.

Conclusion

The work described a flow-cytometry based approach for the simultaneous detection of five allergens (milk, egg, peanut, mustard, and crustacean) in a complex food matrix, extending previous achievement of a method able to detect three allergens (Gomaa et al. 2012, 2015). The lowest concentration of allergens proteins able to induce a significant signal difference between blank and contaminated

samples were: 2 ppm for peanut, 5 ppm for crustaceans, 5 ppm for mustard, 5 ppm for milk and 10 ppm for egg. IC50 values were 3.5, 2.5, 4.5, 8 and 15 ppm respectively. These calculated sensitivities are in the same range as those obtained with Gomaa’s method where all tested samples with contamination levels of 10 ppm were correctly flagged as positives. The test is sufficiently sensitive to detect peanut and crustaceans at the reference doses established by the VITAL expert panel (Taylor et al. 2014). Further improvement is needed for mustard, egg and milk for which the calculated thresholds for a serving of 50 g of cookies are respectively 0.1, 0.6 and 2 ppm.

Factors influencing the performances of the test were highlighted: below a minimal concentration of allergenic proteins bound to the microbeads, the signal does not differ significantly from the background. Over the antibody concentration range tested (0.17 to 10 $\mu\text{g}/\text{ml}$), the fluorescence signal of the negative controls increases proportionally to the concentration of antibodies in solution and is more easily distinguished from the background but sensitivity of the test decreases accordingly; competition for the antibodies between the microbead-bound and free antigens present in the extract is greater if there are fewer antibodies.

Manufacturing processes have a deleterious impact on the allergenic proteins (Alvarez and Boye 2012; Verhoeckx et al. 2015). The egg powder used had been irradiated to increase its storage life; this sterilization procedure may have resulted in the modification of allergenic epitopes and subsequent loss of recognition by the antibodies. It is therefore important to identify proteins and epitopes that resist manufacturing processes in order to develop robust immunoassays.

Flow-cytometry-based immunodetection may, in the near future, improve upon the performances of classic ELISAs by adding a new feature: simultaneous detection/quantification of multiple allergens. The only limitation of this technology is the possibility of purifying or synthesizing the immunogens required to produce the antibodies of interest.

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