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Food Allergens Profiling with an Imaging Surface Plasmon Resonance-Based Biosensor

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Food allergy is a growing health concern, which currently affects approximately 4% of adults and 8% of infants. For consumer protection purposes, food producers are required by law to disclose on the product label whether a major allergen is used during the production process. The commonly employed monitoring methods are highly laborious, time-consuming, and often expensive when screening for multiple allergens. Here, we utilize imaging surface plasmon resonance (iSPR) in combination with antibody array for rapid, quantitative, and multianalyte food allergens detection. We demonstrate how the use of this technology provides a complete allergen profile within short measurement time and with adequate sensitivity. The successful applicability of this approach is demonstrated by analyzing cookies and dark chocolate products from different manufacturers. Hazelnut content of the tested food products is also determined by enzyme linked immunosorbent assay and is found to correlate well with the hazelnut content determined by iSPR. This newly developed method opens the door to automated and high-throughput allergen analysis, ultimately aiming at providing the consumer with safer food.

Millions of people experience allergic reactions to food, presenting mild to life-threatening symptoms. Food allergy is also considered to be the leading cause for outside hospital anaphylaxis. So far, there is no cure for food allergy, and the only way to manage the health risk is by strict avoidance of the offending allergen. Even so, one of every four food allergic individuals suffer from allergic reaction due to accidental exposure.[1−3] For consumer protection, legislation requiring a mandatory declaration of allergenic foods has been put into place both in the EU and in the USA.[4,5] However, this legislation refers only to ingredients that are deliberately introduced to the product, leaving out food contamination with allergens during the production process. Since processed food contains multiple ingredients and shares storage and production facilities, an allergen-free end product is difficult to guarantee. A precautionary labeling is voluntary implemented by the manufacturers to indicate possible allergens presence at trace levels.

Food allergens are abundantly occurring proteins, and many foods contain multiple allergens at variable amounts. For instance, a peanut contains several allergenic proteins which belong to the seed storage globulin family.[6−8] Traditional allergen monitoring techniques include protein-based methods in various formats (immunoblotting, enzyme or radio-allergosorbent test, rocket-immuno electrophoresis, etc.) and DNA-based methods (PCR and real-time PCR).[9,10] Immunoassays which employ polyclonal antibodies usually target the total protein extract of the offending food, whereas immunoassays which employ monoclonal antibodies target one specific allergen or another protein as a marker.[10,11] Currently, the most commonly and routinely used method for food allergen detection is the 96 well plate-based enzyme linked immunosorbent assay (ELISA), which is usually used to test for each allergen separately. Even in its fastest format, ELISA requires at least 30 min for 14 samples to be analyzed for a single allergen at an approximate materials cost of 15 euros per sample. Considering the required sample dilutions, replicates, and manual labor, screening multiple samples for multiple allergens becomes fairly unrealistic and, therefore, only few selected samples are analyzed for specific allergens. Moreover, for some allergens, the methods are not readily available and/or are expensive. The lack of adequate analytical tools for multiplex allergen detection, among other factors, leads to difficulties in consumer protection driven legislation and probably also to falsely labeled products. The latter, might endanger sensitive consumers and unnecessarily narrow down their nutritional choices. There is an evident need for a rapid screening device with multianalyte diagnostic facilities, which can provide a detailed food profile, enabling improvement in food safety and quality monitoring.

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Several immunoassays for multiple allergen detection in different formats have been reported recently. A multiallergen ELISA in the competitive indirect format has been described by Rejeb et al. for the simultaneous determination of peanut and several tree nut allergens in chocolate with limits of detection below 1 mg kg^{-1} protein for each allergenic food.[12] They assembled multiallergen microtiter plates by combining 8-well strips coated with proteins from each of the five allergenic foods. Additionally, a multiplex reverse dot blot enzyme immunoassay system, using spots of egg yolk antibodies (IgY) specific for different allergens on a strip of polyester cloth in combination with allergen specific enzyme-labeled antibodies, has been developed for the multiple detection of allergens with a limit of detection (LOD) of 0.1 mg kg^{-1} for peanut allergens in various food and for hazelnut and Brazil nut allergens in chocolate ice cream.[13] Resonance-enhanced absorption (REA)-based near-field immunoassay was also proposed as a novel platform to detect ovalbumin and ovomucoid with a sensitivity of 1 μg L^{-1} by Maier et al.[14] Despite these advances in food allergen detection methods, the techniques mentioned above still suffer from time consumption and labor intensiveness. To fulfill the need in rapid and automated analysis, several biosensors were developed for food allergen screening. The multianalyte biosensors reported so far include the highly sensitive (ppb levels) naval research laboratory (NRL) array biosensor and a flow channel-based surface plasmon resonance (SPR)-based biosensor (Biacore Q).[15,16] Biacore Q was used to develop both direct and sandwich singleplex immunoassays for the detection of proteins from milk, egg, hazelnut, peanut, shellfish, and sesame in food samples with detection levels down to 1−12.5 mg kg^{-1}.[16] The application of an array biosensor for fluorescent sandwich immunoassays on the surface of a planar waveguide was demonstrated for the detection of ovalbumin as an indicator of egg contamination within 16 min and with limits of detection of 25 ng L^{-1} in buffer and 1.3 μg L^{-1} in 10 times diluted nonegg pasta extract.[15] A miniature biosensor technology SPREETA was also evaluated as a method for peanut allergen detection.[17] Several other multianalyte systems have been reported for various environmental and food contaminants, including electrical microarrays for the detection of multiple pathogens, microbead-based suspension arrays for the identification of genetically modified organisms, magnetic nanotag-based detection platform for mycotoxins, and automated parallel affinity sensor array (PASA) with a chemiluminescent read-out for the rapid analysis of antibiotics.[18−21] Despite the significant reduction in analysis time, automation, and good sensitivities of the described multianalyte systems, so far, none of them demonstrated simultaneous detection of the above four allergens.

Here, we utilized imaging SPR technology for rapid and quantitive allergen detection as a novel approach to food profiling.[22,23] While SPR is widely applied for kinetic studies of biomolecular interactions, its application for routine concentration analysis has been limited due to high costs of the dedicated instrumentation and low throughput.[24−29] The imaging surface plasmon resonance (iSPR) platform, on the other hand, offers multiplex analysis in a single measurement by combining SPR-based detection with spatial modifications of a surface, such as microarrays.[22] The reduction in labor costs, through automation, together with high multiplexing capabilities and reusability of the sensor chips compensate for the cost of the iSPR instrument, which is approximately 7 times higher than that of the ELISA reader. In this study, we used an angle scanning iSPR system in combination with an antibody microarray directed against 13 major food allergens for direct food allergens profiling (Figure 1a).

EXPERIMENTAL SECTION

Antibodies Generation. Production of antipeanut (mAb 51-12D2), antihazelnut (mAb 48-10F3m), anti-κ-casein (mAb 33-4G10), and antisoy (mAb 3G12) was described previously.[26,29,28] Polyclonal antibodies against lupine (PAb MH22) and egg (PAb MH7) were raised in rabbit according to the same immunization protocol as previously described for the development of antiflumequine PAbs.[27] Polyclonal antibodies against pine nut, almond, macadamia nut, brazil nut, cashew, pistachio, and pecan were kindly provided by Dr. P. Delahaut fromCER Groupe−Laboratoire d'Hormonologie, Belgium.

Food Samples and Allergen Extraction. Three dark chocolates (Degustation intense 70% cacao, Cote d'or; Excellence 70% cacao, Lindt; Noir 76% de cacao, Poulain) and seven cookies (Top crunch cookie, Nestle; Hello cookies, LU; Brownies, Auchan; Brownie mini, Brossard; Cookie dough mix, Alsa; Biscuits, Auchan; Luxury cookies, Cadbury) were screened for allergens. To 1 g of food sample (melted chocolate or ground cookie) 20 mL of preheated (60 °C) RIDASCREEN allergen extraction buffer (R-Biopharm AG, Darmstadt, Germany) was added and mixed intensively. To reduce unspecific binding, either 1 g of skimmed milk powder (MARVEL, UK) or, when determining the milk content, 1 g of bovine serum albumin (BSA) was added to

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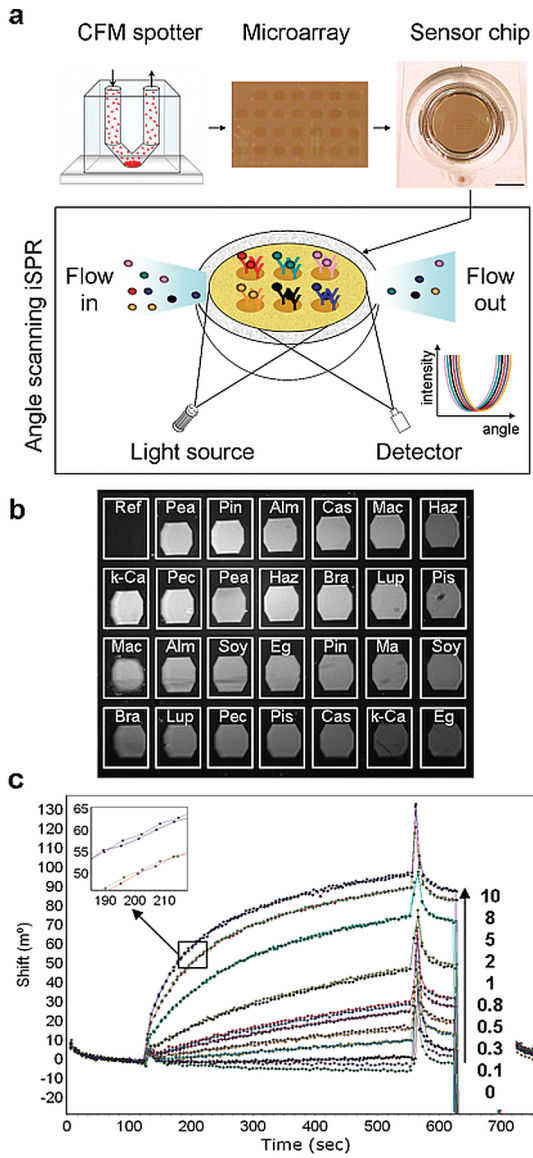

Figure 1. On-chip direct allergen screening using imaging surface plasmon resonance (iSPR). (a) Principle of the allergen detection using the iSPR system. Hydrogel coated SPR chip is spotted with antibodies against allergens using a continuous flow microfluidic (CFM) spotter. The antibody-microarrayed chip is mounted on a glass prism, assembled with the flow cell, and placed in the iSPR instrument. The surface of the chip is illuminated at different light angles and images of the surface are taken by a CCD camera. For each spot, the SPR angle is determined from angle versus intensity plots. The sample is delivered to the chip using a flow cell; present allergens bind to spotted antibodies, and a shift in the SPR angle occurs. Scale bar, 1 cm. (b) SPR image of the microarrayed chip with antipeanut (Pea), antipine nut (Pin), antialmond (Alm), anti-κ-casein (κ-Ca), antimacadamia (Mac), antihazelnut (Haz), antipecan (Pec), antibrazil nut (Bra), antilupine (Lup), antipistachio nut (Pis), anticashew nut (Cas), antiegg (Egg), antisoy (Soy), and buffer (ref). Spot dimensions are 400 $\times$ 600 μm. (c) The shift in SPR angle is monitored in real time (sensorgram) on an anti-κ-casein spot during injection of the following: buffer (0−120 s), sample (120−540 s), buffer (540−660 s), and regeneration solution (660−720 s). Standard solutions of κ-casein at different concentrations (0−10 mg L^{-1}) were injected in duplicate over the antibody-microarrayed chip. Zeroed sensorgrams measured on the κ-casein spot are shown. The inset shows a close-up on duplicate injections of 10 and 8 mg L^{-1} κ-casein.

chocolate samples. Extraction was done in a 60 °C water bath with shaking for 10 min. The aqueous fraction was collected by centrifugation (3220*g* for 10 min) and, subsequently, centrifuged again at 20 000*g* for 10 min to remove residual fat and insoluble particles. The food extracts were divided into small aliquots (1 mL) and stored at −20 °C until used. For protein extracts of the allergenic food, 2 g of raw ground nut (pistachio nut, cashew nut, almond, macadamia, brazil nut, pine nut, and pecan) was diluted in 20 mL of PBS (20 mM phosphate buffer, pH 7.4, 150 mM NaCl) and agitated for 0.5 h at 60 °C. Crude extracts were centrifuged at 3220*g* for 20 min. The fat layer was discarded, and the supernatant was filtered through a 0.45 μm HT Tuffryn acrodisc syringe filter (Pall Life Sciences, UK). Lupine and soy extracts were prepared in the same way from raw beans purchased locally. For egg extraction, a whole egg powder (NIVE, The Netherlands) was used. Raw peanut and hazelnut extracts were kindly donated by R-Biopharm AG. The protein content of each protein extract was determined using a BCA protein assay (PIERCE, Rockford, USA).

Antibody Microarraying. Sensor chip coated with linear polycarboxylate hydrogel (approximately 200 nm thick), preactivated with *N*-hydroxysuccinimide (NHS) for amine coupling (XanTec bioanalytics, Duesseldorf, Germany), was spotted with antibodies using a continuous flow microfluidic spotter (Wasatch Microfluidics, Salt Lake City, USA). Optimal pH for each antibody immobilization was determined beforehand in a surface preconcentration experiment using Biacore 3000. The spotter was washed with 0.1% (w/v) sodium dodecyl sulfate (SDS) followed by 60 °C warm reverse osmosis (RO) water, conditioned with 0.01% (v/v) Tween 20, and primed with 5 mM acetic acid. The antibodies were prepared beforehand in 10 mM acetate buffer at pH and concentration as follows: antipeanut pH 4.5, 0.005 g L^{-1}; anti-κ-casein pH 4.5, 0.005 g L^{-1}; antilupine pH 5, 0.01 g L^{-1}; antisoy pH 4.5, 0.005 g L^{-1}; antihazelnut pH 4, 0.005 g L^{-1}; antialmond pH 4.5, 0.01 g L^{-1}; antibrazil nut pH 5, 0.005 g L^{-1}; antimacadamia nut pH 5, 0.01 g L^{-1}; anticashew nut pH 4.5, 0.01 g L^{-1}; antipine nut pH 5, 0.01 g L^{-1}; antipistachio nut pH 5, 0.01 g L^{-1}; antipecan pH 5, 0.01 g L^{-1}. Each antibody was spotted in duplicate in a randomized manner over the sensor chip surface. During the spotting, six immobilization cycles were performed, each including 5 min contact time with the surface. Unreacted ester groups in the hydrogel were blocked with 0.5 M ethanolamine, pH 8.5, for 10 min at RT. If not used immediately, the sensor chip was washed with RO water, dried under nitrogen stream, and stored at 4 °C.

iSPR Measurements. iSPR measurements were conducted using the IBIS iSPR instrument (IBIS Technologies B.V., Hengelo, The Netherlands) as described elsewhere.[30] Briefly, a flow cell (20 μL volume) was used to deliver the buffer, the sample, and the regeneration solution to the sensor chip surface. The SPR angle was scanned on each predefined region of interest in the range between −2.5 and +2.8 degrees in steps of 200 millidegrees. SPR curves were fitted automatically by IBIS software while curve parameters were limited to 20 points before and after the dip. All the measurements were performed in the "analysis mode", recording SPR angle shift (m^0) as a function of time (s). Subsequently, SPR data were analyzed using SPR inspection tool software ver. 1.6.0.0 (IBIS Technologies B.V. Hengelo, The

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Netherlands). Post measurement data sampling for each angle shift was done by averaging at least five data points collected around the desired time. Raw sensorgrams were first zeroed to the angle before the injection and then referenced to the angle of the blank spot. The maximum responses were calculated from the angle shift during the dissociation phase (around 600 s).

Allergens Screening with iSPR. Thirteen direct immunoassays for allergens were multiplexed as follows. A freshly prepared sensor chip, microarrayed with antibodies directed against major food allergens, as described in the sensor chip preparation section, was conditioned with serial injections of 10 mM hydrochloric acid, pH 2, in order to remove any antibodies which are noncovalently bound to the sensor chip surface. To monitor baseline stability, after each HCl injection, the sensor chip surface was equilibrated with the 10 mM Hepes pH 7.4, 150 mM sodium chloride, 3 mM EDTA, and 0.005% (v/v) surfactant polysorbate (P20) (HBS-EP) buffer and the difference between the SPR angle before HCl injection and after HCl injections was evaluated, measuring both with HBS-EP buffer on the surface. To test spot to spot cross-contamination and antibodies cross-reactivity, each protein extract of allergenic food at a 5 mg L^{-1} concentration was injected separately (7 min contact time) in duplicate over the surface of the preconditioned sensor chip. Next, multistandard solutions containing all the protein extracts were prepared in HBS-EP buffer at concentrations ranging from 0.1 to 10 mg L^{-1}. These mixtures were injected over the sensor chip arrayed with the antibodies in duplicate, starting with blank solution containing only running buffer. For measurements in food matrixes, blank cookie and blank chocolate were spiked with all 13 allergen protein extracts simultaneously at concentrations ranging from 0.1 to 10 mg L^{-1}. For background signal determination, unspiked blank cookie and chocolate were injected prior to the multistandard solutions. Each measurement cycle included sample injection (7 min contact time) and one injection of regeneration solution (1 min contact time). All measurements were performed in duplicate and repeated at least on two different days on every chip. The maximal binding responses were also averaged between the spots containing the same antibody. To generate calibration curves, maximal responses were plotted against protein concentrations for each antibody. The calibration curves were fitted with a nonlinear one-phase model using GraphPad Prism software ver. 5.02 (GraphPad Software, Inc.). The immunoassays were characterized by the limit of detection (LOD) and limit of quantitation (LOQ) which were calculated by adding three or ten, for LOD and for LOQ, respectively, standard deviations to the average background responses of blank cookie samples and blank dark chocolate samples. For allergenic profiling of cookies and dark chocolates, the extracts of these food samples were measured following the multiallergen standards, in duplicates and in two different dilutions, all on the same sensor chip.

Hazelnut Protein Determination with ELISA. Hazelnut protein was quantified in the food samples, using sandwich ELISA (The RIDASCREEN FAST kit, R-biopharm, Darmstadt, Germany). Food extracts were diluted using extraction buffer supplied with each kit. Each standard solution (150 μL) and series of food sample extracts were first prepared in a noncoated 96-well microtiter plate, and then, 100 μL was transferred to an antibody coated plate. The rest of the assay was performed according to the guidelines from the manufacturer. The absorbance was measured at 450 nm using an ELx808 ultra Microplate Reader (BIO-TEK instruments, USA). Qualitative ELISA for hazelnut protein traces was performed as described previously.[31]

RESULTS AND DISCUSSION

Biosensor Analytical Performance Evaluation. For antibody microarray fabrication, hydrogel-coated SPR chips and a continuous flow microfluidic (CFM) spotter were used. The CFM spotter applies a microfluidic interface to enable antibody immobilization on each spot individually, offering many advantages over the conventional spotting techniques, including high-quality spot formation on hydrophilic surfaces and a substantial increase in the spot load.[32−34] Even if only a fraction of immobilized antibodies is active toward the analyte, the response will be sufficient. This enables direct spotting of polyclonal antibodies without prior affinity purification. In this study, the panel of antibodies was chosen according to the designated food product group, cookies and chocolates, and included both monoclonal and polyclonal antibodies. Seven major allergens (peanut, milk, lupine, soy, egg, hazelnut, and almond) and six additional tree nut allergens (cashew nut, brazil nut, pine nut, pecan, macadamia nut, and pistachio nut) were targeted (Figure 1b). Every spot on the chip surface essentially is a specific sensing region for a particular allergen. For each antibody spot, a dose response curve with the specific allergen was constructed (Figure 1c). Each measurement cycle (including chip stabilization, interaction with the sample, and chip regeneration) produced quantitative data on the concentration of 12 allergens within 12 min (Figure 1c).

Total protein extracts of the selected allergenic food were used as standards to study the sensitivity and the selectivity of the chip. Multianalyte standard solutions (containing total protein extracts of all targeted allergens) in a concentration range from 0.1 to 10 mg L^{-1} were injected over the antibody-microarrayed chip. For each protein extract of the allergenic food, a calibration curve was constructed by plotting the maximum binding response as a function of the concentration. Calibration curves obtained for all allergenic food protein extracts showed dose dependency on the concentration, even though they had different sensitivities. Most of the curves showed that the optimal working range of the chip is below 2 mg L^{-1}, similarly to the allergen assay described by Yman et al., suggesting a relevant analytical capability for food allergen detection (Figure 2a,b).[16] Optimal curve fitting was obtained using a nonlinear one-phase association model, making use of the entire concentration range (Figure 2a,b and Table 1 in Supporting Information).

Next, the selectivity of the chip was assessed. Detection of a single allergenic protein in food products, which contain a

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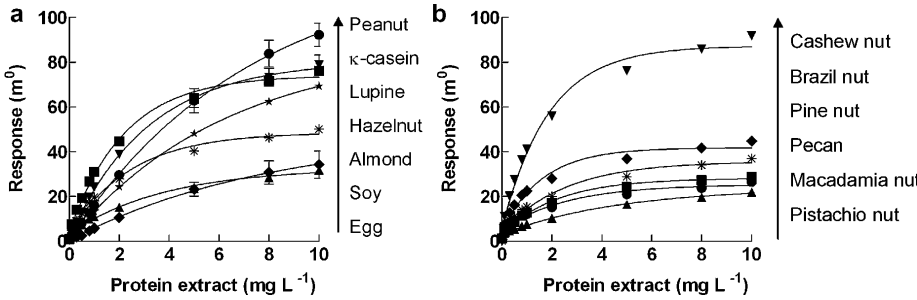

Figure 2. Multianalyte calibration curves in buffer. (a) and (b) Dose response curves of 13 protein extracts: peanut (circle), κ-casein (reverse triangle), lupine (square), hazelnut (star), almond (asterisk), soy (diamond), egg (triangle), cashew nut (reverse triangle), brazil nut (diamond), pine nut (asterisk), pecan (square), macadamia nut (circle), and pistachio nut (triangle) measured on a single antibody-microarrayed chip using iSPR. Multistandard solutions containing all the proteins were prepared in HBS-EP buffer at concentrations ranging from 0 to 10 mg L^{-1}. These mixtures were injected over the antibody-microarrayed chip, and maximal binding responses were measured at 480 s after sample injection. To generate calibration curves, maximal binding responses were plotted against protein concentration for each antibody. Solid lines show curves fitted with a nonlinear one-phase model. Error bars represent standard deviations ($n = 4$).

large variety of other proteins at much higher amounts, requires an extreme specificity of the antibody used. However, antibodies directed against a specific protein might also bind, mostly to a minor extent, to other proteins, presenting so-called cross-reactivity. This cross-reactivity might cause a false positive test result in a screening assay for a single allergen. In multiplex assays, however, the combination of antibodies with overlapping specificities and variable degree of selectivity is often beneficial due to the fact that it provides a greater confidence level in positive results and reduces the number of false negatives during the screening process. In this study, the cross-reactivity (CR) of each antibody was assessed by injecting each protein extract separately to the antibody-microarrayed chip and measuring binding responses on all the antibody-containing spots. CR was expressed as the ratio (in %) of nonspecific binding (with any protein extract) to specific binding (with the antigen) at the same concentration (Figure 3). The results showed that peanut, κ-casein, egg, and hazelnut were detected with highest specificity, displaying less than 1% CR with other allergens. Lupine, soy, and almond as well as pine, brazil, and macadamia nuts were detected with a moderate degree of CR to other protein extracts. The cashew nut and pistachio nut antibodies were found to be highly cross reactive with each other's antigens, and the pecan antibody exhibited an extensive degree of binding to all the protein extracts tested, except for κ-casein and peanut, and thus was used for a positive control as a generic binder (Figure 1 in Supporting Information). Of course, the antibody panel may be altered in accordance to a specific analytical need. When looking at signal stability and reproducibility, the chip was found to be highly robust. Figure 4 shows the responses of lupine and hazelnut antibodies during seven calibration curve repetitions, each including 20 measurement cycles. In total, including the food sample measurements, over 200 measurements were conducted before reduction in responses occurred (Figure 2 in Supporting Information).

Food Allergens Profiling with iSPR Biosensor. Food matrixes are often complex mixtures, containing a large variety of molecules which could mask the presence of the allergen either by lowering allergen extraction efficiency or by interfering with the analytical assay. The ability to measure in food extracts is of utmost importance to the method's applicability and was studied as follows. The mixtures of the protein extracts of the allergenic

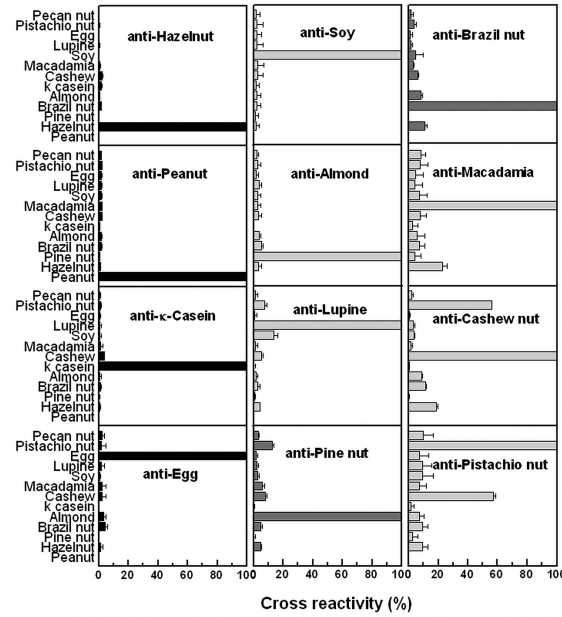

Figure 3. Cross-reactivity of the antibodies toward different proteins. Standard solutions containing each protein extract were prepared in HBS-EP buffer at concentrations of 5 mg L^{-1} and injected separately over the antibody-microarrayed chip. Maximal binding responses were measured at 480 s after sample injection and compared to the maximal binding response of the specific antigen at the same concentration (100%). Shades of black indicate the specificity of the antibody, from most selective (black) to the least selective (light gray). Error bars represent standard deviations ($n = 3$).

foods were spiked into cookie and dark chocolate extracts and injected over the antibody-microarrayed chip. The calibration curves were constructed in the same manner as in buffer measurements. Expectedly, the introduction of the food matrix caused reduction in binding of some antibodies but did not significantly affect the sensitivity. For example, egg measurements remained unchanged, but the peanut antibody produced somewhat lower responses in dark chocolate in comparison to buffer (Figure 4). Overall, the effect of the food matrix on the analytical performance was minor, most likely due to a proper extraction method used for sample preparation (Figure 3 in Supporting Information). This observation is especially significant considering that the measurements are based on monitoring direct binding of the allergens in the food extract to the chip without any

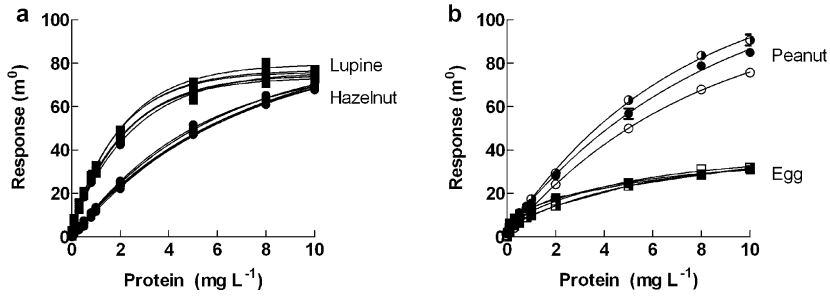

Figure 4. Sensor chip stability and food matrix effect. (a) Seven calibration curves, each including 20 injections, measured on antilupine (square) and on antihazelnut (circle) spots are shown. Solid lines show curves fitted with a nonlinear one-phase association model. (b) Peanut and egg calibration curves in buffer (black and white), in cookie extract (black), and in dark chocolate extract (white). Solid lines show curves fitted with a nonlinear one-phase association model. Error bars represent standard deviations ($n = 4$).

Table 1. Limits of Detection and Quantitation Determined for the iSPR-Based Multiallergen Screening[a]

	cookies		dark chocolates	
protein extract	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
peanut	3.2	9.2	3.9	12.6
κ-casein	0.2	5.4	na[b]	na
lupine	0.8	3.4	0.6	2.3
egg	0.5	2.2	0.8	6
hazelnut	1.5	4.3	4.6	14.7
almond	1.9	9.3	1.5	7.5
macadamia nut	1.6	15.4	2.9	22.8
brazil nut	0.6	2.5	0.4	1.7
pine nut	0.5	4.5	0.6	6.7
pistachio nut	1	6.1	0.8	4.3
cashew nut	0.4	2.3	0.9	3.1
pecan	1.5	8.2	5	36.6
soy	5[c]	14.8[c]	na	na

[a] Limit of detection (LOD) and limit of quantitation (LOQ) were calculated by adding three or ten, for LOD and for LOQ, respectively, standard deviations to the average maximum response of blank cookie samples and blank dark chocolate samples and by interpolating from the calibration curves in blank cookie samples and blank dark chocolate, respectively. The calculated LODs and LOQs were then multiplied by 20, to compensate for the food sample dilution during the extraction procedure. LODs and LOQs are expressed in units of mg protein extract in kg tested food product. The blank dark chocolates used in this study tested positive in the κ-casein assay. Thus, the LODs and LOQs for κ-casein were determined in cookies only. For LOD and LOQ of the κ-casein assay, the chocolate samples were extracted in the presence of bovine serum albumin. [b] na: not available. [c] For soy protein extract, the LOD and the LOQ were calculated only from the measurements in buffer due to the fact that the blank cookies and chocolate used in this study were found to be positive in soy assay.

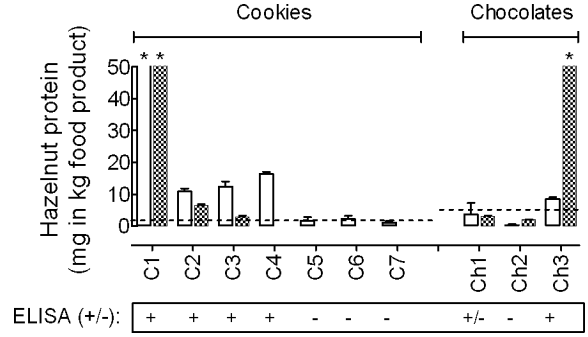

Figure 5. Comparison of the hazelnut content determined by iSPR-based screening assay to the hazelnut content determined by qualitative and quantitative ELISA. Seven cookies and three chocolate samples were screened for hazelnut protein content using on-chip direct measurements with iSPR (white bars), and the results were compared to results obtained previously with qualitative (±) and quantitative ELISA (plaid bars). Dotted lines represent LOD values for iSPR. Asterisks indicate hazelnut concentration more than 50 mg per kg food product. Error bars represent standard deviations ($n = 4$).

additional steps, in contrast to ELISA which includes multiple washing, labeling, and color development steps. The sensitivity of on-chip allergen detection (Table 1), expressed in limit of detection (LOD) and limit of the quantitation (LOQ) of allergenic food protein extracts, was found to be in the low mg kg^{-1} range both in cookies and dark chocolates, adequately compatible with food allergen analysis and comparable to most commercially available ELISAs and Biacore-based assays.[16]

The applicability of the iSPR-based allergen screening was validated by analyzing commercially available food samples, seven cookies and three dark chocolates, which were previously used in an EU survey for hazelnut and peanut presence assessment.[31] For comparison, all samples were also analyzed for hazelnut content with an in-house qualitative ELISA and a commercially available, AOAC certified ELISA kit (Figure 5). The results obtained with direct on-chip iSPR measurements correlated well with the results obtained with both ELISA assays. All samples that were positive for hazelnut in ELISA showed positive responses when analyzed with our chip, and no false negatives were observed. However, hazelnut concentration in food samples determined by quantitative ELISA were somewhat lower in cookies and higher in chocolates than the values that were obtained with our chip (Figure 5). The variation between the two might be attributed to the different antibodies used, as well as to the immunoassay format. In ELISA, a sandwich immunoassay format is implemented, where as in this study direct measurements were performed. Moreover, since there is neither certified reference hazelnut material available nor a method for absolute allergen concentration determination, it is difficult to judge which value is the correct one. As long as the concentrations obtained with both methods are in the same order of magnitude, the values are generally considered to be comparable.

The allergenic profile obtained with direct measurements on the chip revealed different fingerprints for each food sample (Figure 6a,b). For example, in "brownie mini", higher peanut, milk, lupine, egg, and hazelnut contents were found in comparison to "cookie dough mix". Even though multiple nuts and egg were found in the "cookie dough mix", no mentioning

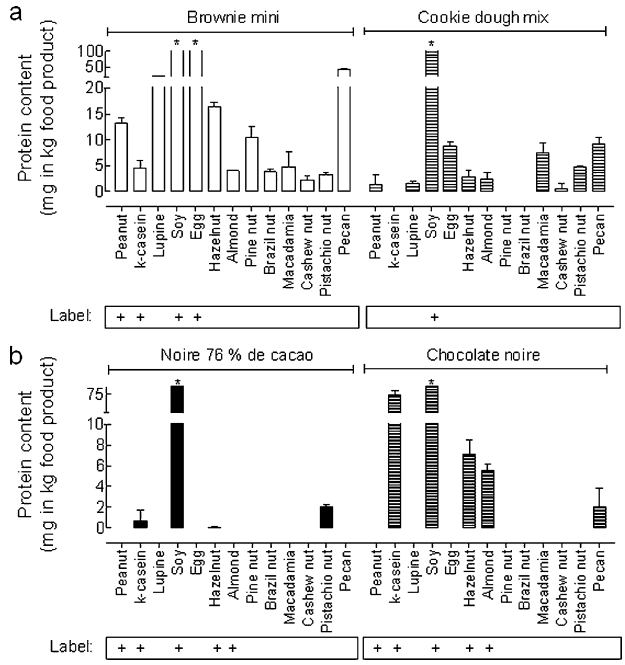

Figure 6. Allergen profiles of food samples. (a) Extracts of cookie dough mix (white) and brownie mini (white with stripes) were injected over the antibody-microarrayed chip, and maximal binding responses were measured on each spot at 480 s after sample injection. Asterisks indicate allergen concentration more than 100 mg per kg food product. Error bars represent the standard deviations between duplicate spots and duplicate sample injections. (b) Allergenic profile of dark chocolates from two different manufacturers (black and black with stripes). Chocolate extracts were injected over an antibody-microarrayed chip, and maximal binding responses were measured on each spot at 480 s after sample injection. Asterisks indicate allergen concentration more than 100 mg per kg food product. Error bars represent the standard deviations ($n = 3$).

of these allergens was stated on the product label (Figure 6a and Table 2 in Supporting Information). The analysis of two dark chocolates from different manufacturers showed differences in the milk, hazelnut, and almond content. In this case, the contents of peanuts and milk were misrepresented on the label (Figure 6b and Table 2 in Supporting Information). Usually, dark chocolate sample extraction buffer includes milk powder which blocks binding of tannins to the allergenic proteins, but it also causes saturation on anti-κ-casein spots (Figure 6b and Table 2 in Supporting Information). Thus, bovine serum albumin was used instead of milk powder, during dark chocolate extraction when screening for milk traces (Figure 4 in Supporting Information). High responses were also observed on antisoy spots both in dark chocolates and in cookies. Even though the presence of soy lecithin as an emulsifier is declared on the label, it is unlikely to cause these high responses unless it is heavily contaminated with soy protein. Since the antisoy antibody used in an iSPR assay is a monoclonal antibody with quite good specificity for the soy protein, the signals suggest product contamination with soy derived ingredients.

These examples demonstrate the power of direct allergen profiling on chip using iSPR. The obtained food profiles provide an extensive overview on the potential allergenicity of the food products, offering valuable information for both manufacturers and monitoring authorities. It is clear that this information is not readily accessible using currently employed single-analyte techniques, leading to incorrect labeling, as presented above.

CONCLUSIONS

This study showed how direct on-chip allergen screening using iSPR can be applied to food profiling, offering a powerful analytical alternative to existing methods. Multiple allergen detection was achieved using on-chip direct iSPR-based analysis without labeling, signal amplification, and washing steps, in a single reagent format. The obtained sensitivity was in the analytically relevant range and comparable to commercially available ELISA. Excellent applicability to allergens screening in food samples was demonstrated together with a broad detection spectrum and high robustness. The analytical performance of the iSPR-based biosensor is still heavily dependent on the employed antibodies. However, given the availability of adequate immuno-reagents, with iSPR, each food sample can be analyzed within several minutes, faster than any other method currently available, providing a detailed and quantitative allergenic profile. Additionally, high multiplexing capabilities and multiple measurements using a single chip contribute to reduction in the analysis costs. The automation of the iSPR system combined with the sensor chip's stability also presents a promising potential of implementation to in-line measurements, integrated into the manufacturing line. We believe that the method described here presents a cornerstone in food allergen analysis. It allows multianalyte and high-throughput monitoring of food production equipment and food products, and its routine application will contribute to correct product labeling, adequate legislation, and foremost, safeguarding the health of allergic consumers.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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