Anal Bioanal Chem
DOI 10.1007/s00216-015-8607-4

RESEARCH PAPER

Rapid and label-free detection of egg allergen traces in wines by surface plasmon resonance biosensor

Rosa Pilolli · Angelo Visconti · Linda Monaci

Received: 20 January 2015 / Revised: 25 February 2015 / Accepted: 27 February 2015

Abstract The development of a surface plasmon resonance (SPR)-based biosensor tailored to the fast detection of egg-related fining allergens in wines is herein described. Ovalbumin (OVA) was chosen as the target protein to be monitored due to its highest abundance in the egg white powder, a typical fining agent used by the winery industry to promote wine clarification. A direct assay was designed, basing on the use of polyclonal anti-OVA antibody as bio-specific receptor. With the aim of optimizing the assay conditions, different parameters able to influence the final biosensor response were carefully investigated (i.e., pH, ionic strength, and additional surfactant concentration). After the fine tuning of these parameters, the assay was tested in the direct analysis of OVA in commercial wines artificially contaminated with egg white powder at different concentration levels in order to assess the reliability of the biosensor in detecting traces of OVA in complex matrices. The devised assay allowed to trace, in a short analysis time and with a minimal sample pre-treatment required, the presence of egg allergens at the lowest concentration comprised between 0.03 and 0.2 μg/mL. Finally, the response provided by the developed biosensor was correlated with an established liquid chromatography mass spectrometry (LC-MS) method developed in our laboratories, and performances of both approaches were assessed for the fast monitoring of egg allergen contamination in fined wines.

Keywords Surface plasmon resonance (SPR) · Biosensor · Allergen · Ovalbumin · Egg white · Wine

R. Pilolli (✉) · A. Visconti · L. Monaci (✉)
Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR), Via Amendola 122/O, 70126 Bari, Italy
e-mail: rosa.pilolli@ispa.cnr.it
e-mail: linda.monaci@ispa.cnr.it

Introduction

Food allergy is regarded as a problem of public health relevance, the main concern being the undeclared presence of an allergenic ingredient in food commodities. Even a little intake of allergen can trigger unpredictable, highly variable reactions, depending on the dose and the sensitivity of affected individual, thus compelling the allergic consumer to avoid allergen-containing food totally.

Egg-derived products, containing allergic proteins, are widely employed as processing aids in winemaking, thanks to their ability to interact and promote precipitation of wine polyphenols and other compounds that might be responsible for undesirable phenomena (presence of haze, generation of off flavors or deposits, etc.) [1]. Fining proteins should be removed by decantation or filtration steps and eventual further residuals by a secondary fining procedure, using inorganic agents, such as bentonite [2]. Some investigations proved that the implementation of such removal steps for excess fining proteins can lead to non-allergenic end products [3]. However, the risk for wine consumers allergic to egg proteins cannot be completely excluded as already highlighted in the opinions issued by the European Food Safety Agency [4].

Directive 2007/68/EC [5] regulates, within European Union, the general labeling of 14 allergenic food ingredients, egg and products thereof included, and since July 1, 2012 [6, 7] also obliges European wine producers to indicate the use of allergenic aids of animal origin whenever added for fining purposes. Within this European legislative frame, the development of reliable and sensitive methods enabling the detection of egg proteins in foods and beverages can open new perspectives for the producers that might spot the real risk associated to certain categories of products where the allergens are likely to remain as residues. Although no specific reference analytical method for the determination of fining agent proteins has been prescribed, the international

Published online: 22 March 2015

Organization of Vine and Wine (OIV) resolution 427-2010 modified by the OIV/COMEX 502-2012 set up the analytical requirements to be fulfilled by methods under development [8]. In particular, enzyme-linked immunosorbent assay (ELISA) methods or commercial ELISA kits (direct, indirect, competitive, sandwich ELISA) must comply with the detection limits and the quantification limits of ≤0.25 and 0.5 mg/L, respectively. Mass spectrometry has recently entered the arena of residual fining agent analysis, as a confirmatory technique [9–12]. Typical bottom-up proteomic approach was successfully applied for the detection of residual egg and milk proteins in white and red wines, either as such or after being artificially fortified with typical milk- or egg-related fining agents, i.e., caseinate or egg white powders [13–19]. Complementary to confirmatory techniques, rapid diagnostic tools are increasingly being requested from producers, in order to evaluate the actual risk of the end products for allergic consumers [20]. The most commonly used rapid methods for routine monitoring are based on ELISAs in 96-well plate format [21]. Although some level of automation has been achieved, ELISAs remain laborious, time-consuming, and expensive, particularly when multiplex analyses are required. Biosensors represent a potential alternative to ELISAs and provide probably one of the most promising ways to achieve simple, fast, reproducible, and cheap multi-analyte detection. An overview on the most recent advances and future trends in biosensor systems applied to food allergen management has been recently published [22].

Optical biosensors measure changes in the surface properties of a sensor chip, caused by the analyte capture to a specific biological receptor. Among various optical transduction mechanisms, surface plasmon resonance (SPR) plays an important role.

Few examples were reported about SPR-based systems applied for the detection of egg proteins. In 2006, a sandwich immunoassay for the detection of conalbumin was for the first time developed and applied for the detection of residual amounts of egg in pasta due to cross-contamination in shared production lines [23]. Rebe Raz et al. in 2010 developed an imaging SPR biosensor for the multiplex detection of food allergens, also including egg, in cookies and dark chocolate [24]. Further recent examples report the development of lysozyme biosensors using molecular imprinted nanoparticles [25] or specific aptamers [26, 27] as biorecognition element. However, it must be pointed out that these investigations are far from the food allergen management field; indeed, lysozyme was selected either merely as a model protein to prove the correct operation of the developed biosensor [25, 26] or as a case study of investigation of protein self-association by SPR aptasensor [27].

To the best of our knowledge, this is the first report of a SPR biosensor devised for screening the presence of egg allergen residues in wine matrix. In this paper, we describe the development of a label-free SPR-based immunoassay for tracing egg-derived fining agent residues by monitoring ovalbumin (OVA) as a protein marker. The assay reliability was assessed by analyzing the OVA in several white and roseè wines fortified with egg white at different concentration levels. Performances of the method were evaluated and discussed.

Finally, the proposed analytical approach was compared with a liquid chromatography mass spectrometry (LC-MS) method developed in our laboratories [17, 28]. The responses of two different mass analyzers were considered, equipped with a dual pressure linear ion trap and a stand-alone Orbitrap™ platform, respectively. Both *selective reaction monitoring* MS/MS (SRM) and *full-scan* high-resolution MS (HR-MS) approaches enabled to reach limit of detection comparable with SPR-based direct immunoassay, thus proving to be a semiquantitative tool for a fast monitoring of allergen contamination in spiked wine samples.

Experimental section

Chemicals

Albumin from chicken egg white, egg white, whole egg powder, skim milk powder, caseinate, lysozyme, ovotransferrin, albumin from bovine serum, acetic acid, sodium hydroxide, sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, and Tween 20 were purchased from Sigma-Aldrich. Rabbit anti-ovalbumin polyclonal antibody (pol-anti-OVA) raised against the native protein was purchased from Millipore. Cellulose acetate syringe filters, 0.20 μm, size 25 mm were purchased from LABOCHEM Science S.r.l., and polytetrafluoroethylene syringe filters, 0.20 μm, size 4 mm were purchased from Sartorius. Ultrafiltration tubes with 10-kDa cutoff membranes were purchased from Sartorius.

CM5 chips, amine coupling kit (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 1.0 M ethanolamine HCl (pH 8.5), HBS-EP buffer solution (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % *v*/*v* Surfactant P20), rabbit anti-β2 microglobulin polyclonal antibody (neg-Ab), and PD-10 desalting columns were purchased from GE Healthcare Life Sciences. All the wine samples were purchased from a local retailer and were preliminary checked for their absence of residual egg proteins.

Standard/stock solutions

Solutions of OVA and egg white (EW) were prepared by dissolving 1 and 5 mg of powder, respectively, in 1 mL of ammonium bicarbonate 50 mM. Serial dilutions in 10 mM

sodium acetate buffer (pH 4.8) were prepared in the range 0.5–50 μg/mL for OVA and 1–400 μg/mL for EW. All solutions were stored at +4 °C and warmed up at room temperature before their use. All diluted solutions were analyzed within 2 days from their preparation. A 5,000-μg/mL EW stock solution was used for wine fortification.

SPR system

For SPR detection, a Biacore® X instrument (GE Healthcare Life Sciences) equipped with Biacore® CM5 sensor chips (carboxymethylated dextran matrix) was used. HBS-EP buffer solution was used as running buffer for pH scouting experiments and ligand immobilization on the chip. Phosphate buffer 10 mM at pH 7.4 added with NaCl 0.1 M and 0.01 % Tween 20 was used as running buffer for all other experiments. The apparatus was equipped with a two-channel integrated microfluidic cartridge (IFC) which allowed the subtraction of non-specific interactions: flow cell 1 (FC1) used as sensing channel functionalized with rabbit poly-anti-OVA antibody and flow cell 2 (FC2) used as reference channel functionalized with neg-Ab, i.e., a generic rabbit polyclonal antibody. A real-time baseline correction of the unspecific binding was automatically performed via software during each measurement; all sensorgrams reported in the following were differential traces labeled as $RU_{1-2}=RU_{FC1}-RU_{FC2}$ [29].

The analytical signal of interest was given by the shift in resonance unit (ΔRU_{1-2}) measured at a fixed time (30 s) after the end of sample injection, averaged within a time window of 5 s [30]. All SPR measurements were carried out at 25 °C and flow rate of 5 and 10 μL/min in single and multichannel mode, respectively. All samples and buffer solutions were preliminary filtered through 0.20 μm cellulose acetate filters before analysis. The injection time was 3 min and a mean of three measurements was calculated unless otherwise reported. Regeneration of the sensor surface was performed by short injections (from 20 to 60 s) of a mixture standard solution of NaOH 50 mM and NaCl 1 M until complete recovery of the baseline RU value on both flow cells.

Immunosensor preparation

For the preparation of the direct assay, the ligand (poly-anti-OVA antibody) was covalently immobilized onto a CM5 chip by an NHS/EDC-based linking chemistry, and the analyte (OVA) was detected after direct interaction with the immobilized specific antibody [31]. The covalent binding of poly-anti-OVA was achieved on the sensing channel FC1 by commercially available amine coupling kit. Typically, a fresh sensor chip was conditioned with three consecutive 1-min injections of a solution containing 1 M NaCl and 50 mM NaOH, and afterwards, the surface was activated by a 7-min pulse of 0.05 M NHS/0.2 M EDC mixture, followed by a 7-min pulse of ligand solution at concentration of 100 μg/mL in 10 mM sodium acetate buffer, pH 4.8; the excess of reactive groups on the surface was deactivated with a 7-min pulse of 1 M ethanolamine hydrochloride pH 8.5 [31]. Under the standard conditions, an immobilized amount of ligand producing a $\Delta RU_{FC1}=19\pm3$ kRU was achieved; the mean and standard deviation values were estimated on the immobilization levels achieved for different sensor chips independently activated with the same procedure, thus being an estimate of the functionalization step reproducibility.

For the real-time baseline correction, the reference channel FC_2 was functionalized with a rabbit polyclonal antibody, referred to as negative antibody, achieving a total amount of immobilized antibody comparable to the sensing channel, $\Delta RU_{FC2}=17\pm2$ kRU.

Calibration curves of standard/stock solutions

Calibration curves were built up by monitoring the variation of differential response (RU_{1-2}) after injection of diluted solutions of OVA and EW. The averaged amount of analyte bound to the receptor (measured at 30 s after the end of sample injection and averaged within a 5-s-long window) was plotted against the analyte concentration. The results were fitted by a sigmoidal equation in semi-logarithmic scale: $y = A_2 - \frac{A_2 - A_1}{1+\left(\frac{x}{x_0}\right)^p}$. Moreover, ten replicates of buffer solutions were injected and averaged for each calibration curve ($y_0 \pm s_{y0}$). The limit of detection (LOD) and lower limit of quantification (LLOQ) were extrapolated from the sigmoidal equation and defined as the concentration at which the signal was y_0+3s_{y0} and y_0+10s_{y0}, respectively [24, 30].

Wine sample purification by size exclusion chromatography (SEC)

Two different wines, white produced from Falanghina grape and roseé produced from a mixture of Negramaro and Malvasia Nera grape varieties (referred to as Salice Salentino), were purchased from a local retailer and analyzed to check the applicability of the developed method. For calibration purposes, typically, 3-mL wine aliquots were fortified with EW stock solution to reach the following concentrations: 1, 3, 5, 8, 10, 18, 25, 50, 200, and 400 μg/mL. The artificially fined wine was left under stirring for 15 min and then was kept quiescent for 20 min at room temperature; no precipitation was observed, thus assuring that the added proteins had completely dissolved into the wine matrix. Aliquots of 2.5 mL of each fortified wine sample were subjected to purification by SEC, and 3.5 mL of 10 mM sodium acetate buffer was passed through the disposable cartridge, collected, and filtered before injection.

Wine sample purification and pre-concentration

For pre-concentration experiments, several wine matrices, both white and roseè, were considered and treated as follows: 3-mL wine aliquots were fortified with EW stock solution to reach the following concentration levels: 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5, 10, and 20 μg/mL. Aliquots of 2.5 mL were withdrawn from each fortified wine sample and were subjected to purification by SEC; 3.5 mL of 10 mM sodium acetate buffer was passed through the disposable cartridge, collected, and subsequently centrifuged for 10 min at 4, 000 rpm, in ultrafiltration (UF) tubes with 10-kDa cutoff membranes, achieving a tenfold pre-concentration factor. A final volume of 350 μL was collected and filtered before injection.

Comparison with a confirmatory LC-MS method

The SPR response was tested against a LC-MS method developed in our laboratories. With this aim, larger amounts of artificially contaminated wines were prepared by adding to wine variable amounts of EW to cover the concentration range 0.25–10 μg/mL. Parallel experiments were performed with the same sample split in two aliquots, one undergoing the optimized purification strategy followed by SPR immunoassay detection, while the other aliquot was subjected to ad hoc developed protocols described elsewhere for sensitive, either LC-HR-MS or LC-SRM, detection [17, 28].

Results and discussion

Taking advantages from the high molecular weight of the marker under investigation, a single-step, direct assay was designed for the detection of OVA selected as protein marker to trace allergenic fining agents present as residues in clarified white and roseé wines.

Assay development

Optimization of the ligand immobilization conditions

A rabbit polyclonal anti-ovalbumin antibody (poly-anti-OVA-Ab) was selected as bio-specific ligand for the immunoassay, which was covalently immobilized on dextran-coated sensor chips (CM5) through a well-established amine coupling procedure [31]. As a first step, a pre-concentration assay aimed at pH scouting was performed in a single channel detection mode, in order to identify the appropriate immobilization pH for the selected antibody (IgG, pI≈5–6). With this aim, the poly-anti-OVA-Ab stock solution was diluted to a fixed concentration in nine different buffers at variable pH comprised in the range 4.0–7.2 and injected onto fresh unactivated CM5 chip. According to our results, sodium acetate buffer (10 mM) at pH 4.8 showed to provide the best compromise between pre-concentration amount and immobilization reproducibility.

In these conditions, the poly-anti-OVA-Ab was immobilized onto the sensing channel surface FC_1 by a three-step functionalization procedure: (i) activation of the chip surface by a mixture of NHS/EDC, (ii) covalent binding of the antibody to the surface, and (iii) deactivation of any remaining active esters by ethanolamine [31]. Analogously, the reference channel was functionalized with a negative antibody for subtraction of signal arising from bulk effects and non-specific binding.

Optimization of the antigen–antibody interaction and regeneration conditions

After production of the active sensing surface, the investigation was focused onto the evaluation of the analyte–receptor surface-mediated interactions. If solution-based antigen–antibody recognition occurs typically at physiological pH, surface-mediated interactions may differ significantly as a consequence of the mass transport mechanism of analyte flowing from the solution bulk towards the dextran matrix. In order to achieve a reliable, semiquantitative information from the immunoassay, the interaction mechanism should be kept under mass transport limit; thus, the rate of mass transport becomes independent from analyte-ligand binding affinity and directly proportional to the analyte concentration [32]. In this frame, a surface pre-concentration mechanism driven by electrostatic attraction plays a crucial role, and customized experiments were designed in order to evaluate the optimized conditions to promote efficient antigen–antibody interaction.

First, a specific experiment was tailored to investigate the optimal pH of the OVA standard solutions to be analyzed. With this aim, OVA stock solution was diluted to a fixed concentration, in several buffers with variable pH within the range 4–6; the relevant differential response, ΔRU_{1-2}, showed a significant change of the specific signal recorded as a function of the analyte buffered pH, thus confirming that the surface pre-concentration mechanism driven by electrostatic attraction affected detection sensitivity. By plotting the amount of OVA bound to specific poly-anti-OVA-Ab against the pH, it appeared that the highest analytical signal was obtained by using 10 mM acetate buffer at pH 4.8 (Fig. 1A) as a consequence of the maximum analyte surface pre-concentration and specific binding with the immobilized ligand. At lower pH values (4.0, 4.4), denaturation of the analyte and/or ligand might occur, resulting in the decrease of specific biorecognition yield. On the other hand, at higher pH values (5.2–6.0), even if the native conformation was retained, a less efficient surface pre-concentration hampered the analyte–antibody interaction.

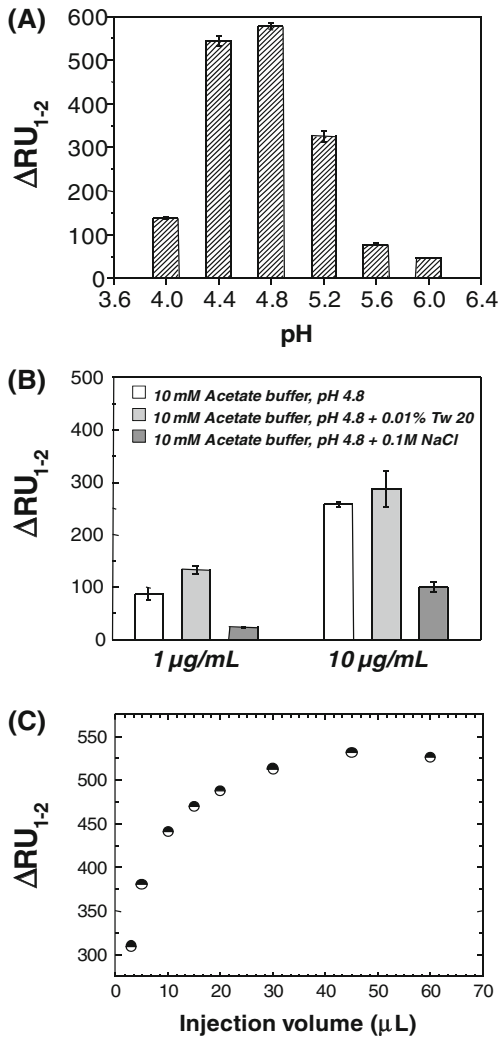

Fig. 1 Optimization of the antigen–antibody interaction conditions. (**A**) Effect of buffer pH: variation of the differential response (ΔRU_{1-2}) upon specific binding of ovalbumin (OVA) diluted in buffered solutions to same concentration but different pH values. (**B**) Effect of ionic strength and surfactant addition: averaged response of OVA injections 1 and 10 μg/mL, diluted either in (i) sodium acetate buffer at pH 4.8, I=0.01 (*white columns*), or (ii) sodium acetate buffer at pH 4.8 added with 0.01 % Tween 20 (*light gray columns*), or (iii) sodium acetate buffer at pH 4.8 added with 0.1 M NaCl (*gray columns*). (**C**) Optimization of the injection volume (constant flow rate 10 μL/min)

Besides pH optimization, the effect of ionic strength and surfactant addition on the bio-specific surface-mediated interaction was also investigated. Iterations of 1 and 10 μg/mL OVA solution injections were performed, diluting OVA stock solution either (i) in sodium acetate buffer with pH 4.8 and low ionic strength, I=0.01, or (ii) in sodium acetate buffer with pH 4.8 added with 0.01 % Tween 20, or (iii) in sodium acetate buffer with pH 4.8 added with 0.1 M NaCl. The reference-subtracted responses obtained under the three conditions tested at both concentrations are reported in panel B of Fig. 1. The histograms clearly showed that the addition of sodium chloride in the sample solution induced a signal suppression. This trend can be likely attributed to the well-known ability of strong electrolytes to interfere with specific antigen–antibody recognition and in addition to the screening effect of the sodium cations which, coupling with the residual negative charges of the carboxymethylated moieties of the matrix surface, reduced the electrostatic pre-concentration of OVA onto the sensing area. Although the addition of a surfactant produced a slight increase of the specific signal, a lower reproducibility was noticed. In conclusion, acetate buffer at the lowest ionic strength (I=0.01, pH 4.8) was confirmed to be the best compromise for a good analyte response. However, given the peculiar pH selected, a deeper attention was paid to the evaluation and the management of potential non-specific binding of positively charged analyte and matrix components due to electrostatic interaction. From this, the need to carry out multichannel measurements with fully functionalized reference channel in which a comparable amount of a generic rabbit polyclonal antibody (referred to as negative antibody) was immobilized; for any acquisition presented hereafter, a real-time baseline correction was applied allowing the subtraction of any potential non-specific binding to the analytical signal.

In addition, given the significant difference in refractive index between sample and running buffers and the wide analyte concentration range under investigation, it was preferred to place the report points for measuring analyte response shortly after the end of the sample injection. The validity of this choice was supported by the slow dissociation rate of the antigen–antibody complex, confirmed by the very slow drop off of the sensorgram after the injection end.

As for regeneration conditions, the action of both high pH and high ionic strength were exploited to destroy the antigen–antibody complex without significantly impairing the antibody biological affinity. With this aim, short pulses of a sodium hydroxide and chloride mixture were flushed onto the sensor to induce surface regeneration. Injection time was modulated from 20 to 60 s depending on the amount of bound analyte, the final goal being the full recovery of the baseline in both sensing and reference channels.

Optimization of the injection time

Finally, the time of analyte–ligand interaction was evaluated by tuning the injection time/volume. Several injections with increasing volume of OVA standard solution at constant flow were performed ranging from 3 to 60 μL (corresponding to 18–180 s injection time) and the relevant analytical signals reported in Fig. 1C. An injection volume of 30 μL (3 min) was chosen as good compromise between a good detection sensitivity and low analysis time and sample consumption.

Assay evaluation

Selectivity, reproducibility, and sensor lifetime

In view of immunoassay application to the analysis of real matrices, the sensor selectivity and contribution of non-specific binding (NSB) to the analytical signal were assessed. General approaches consider the inclusion of surfactant agents, such as Tween 20, to the running buffer in order to reduce the NSB. In addition, in this case, given the choice of an acidic sample buffer, further non-specific interactions are likely to occur due to the net negative charge resulting from unactivated carboxyl groups of CM5 chip surface that may induce electrostatic interaction with positively charged matrix components. Potentially, both the analyte itself and other matrix components can absorb onto the sensor surface by non-specific interactions, such as hydrophobic and/or electrostatic, resulting in a change of the refractive index, thus the need for a dual channel measurement with real-time reference correction. In order to assess the assay selectivity, to keep the non-specific absorption under control in each measurement and preserve the long-term stability of the sensor chip from the gradual fouling, the composition of the running buffer was adjusted in terms of ionic strength and surfactant percentage, and the SPR response after injections of non-specific proteins was recorded. In particular, consecutive injections of 10 μg/mL standard solutions of OVA, EW, whole egg (WhE), lysozyme (LYS), ovotransferrin (OVT), bovine serum albumin (BSA), caseinate (CNate), and skim milk powder (SM) were performed, employing a phosphate running buffer (10 mM, pH 7.4) added with both a surfactant (0.01 % Tween 20) and a strong electrolyte (0.1 M NaCl). Results obtained are reported in Fig. 2A. By comparing the analytical signal recorded for OVA as purified protein with that referred to the typical OVA containing fining agents EW and WhE, an OVA-related trend was pointed out in the different egg-based powders screened. As for non-specific proteins and/or protein mixtures, the analysis of single channel sensorgrams indicated a low non-specific absorption of the tested proteins in both the reference and sensing surfaces. The real-time subtraction of reference channel resulted in a non-specific signal always lower than 4 %, except for ovotrasferrin (<9 %) displaying some cross-reactivity towards the anti-OVA Ab and then proved to recognize, although in minor part, another egg allergen characterizing egg white powder. In conclusion, under the selected assay conditions, the analytical signal can be however considered highly selective.

The intra-day and inter-day precision of the assay, expressed as the percent coefficient of variation in response (CV_{resp}) at a fixed concentration, was also evaluated for ten consecutive measurements, iterated over 1 week of continuous operation; in particular, precision was tested on freshly prepared chip (t=0) and after one, two, and seven operation days. The assay provided a CV_{resp} ranging from 2 to 6 % within the same day; the mean values obtained on different days were compared by a one-way ANOVA test, turning out to be not significantly different at 95 % confidence level. As a consequence, the four data sets (t=0, 1, 2, and 7 days) were averaged providing an inter-day CV_{resp} of 8 %.

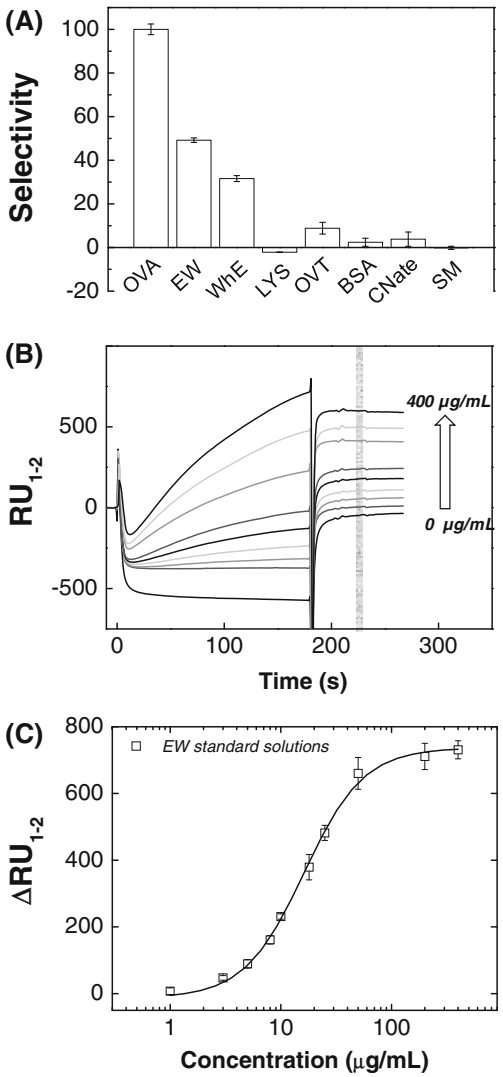

Fig. 2 (**A**) Assessment of the assay selectivity by injection of several proteins and protein mixtures: ovalbumin (*OVA*), egg white (*EW*), whole egg (*WhE*), lysozyme (*LYS*), ovotransferrin (*OVT*), bovine serum albumin (*BSA*), caseinate (*CNate*), and skim milk (*SM*). Typical reference-subtracted sensorgrams (**B**) and relevant calibration curve (**C**) recorded upon injections of egg white standard solutions with variable concentrations (range 0–400 μg/mL, diluted in 10 mM sodium acetate buffer, pH 4.8); the *gray strip*, located 30 s after the end of injection, highlights where the report points were placed

The lifetime of the biosensor chip was ultimately investigated by injecting a fresh OVA standard solution at fixed concentration onto the same sensor chip over a period of 2 months of continuous operation. After the end of the second month (equivalent to approximately 500 total injections), a signal decrease of about 40 % was recorded.

Dynamic range and sensitivity

The dynamic range of the developed assay was investigated by using the previously optimized conditions. Analysis of OVA standard solutions in the concentration range 0.5–200 μg/mL were performed; by plotting the averaged differential response against the analyte concentration, a calibration curve was obtained with a sigmoidal trend under semi-logarithmic scale, the relevant fitting parameters summarized in Table 1. Blank measurements by direct injection of buffer solution were also performed in order to calculate the LOD and the LLOQ of the assay (see "Experimental section" for further details), and quite challenging values were achieved: 0.6 and 2 μg/mL, respectively. The range 2–20 μg/mL was assessed as operating range for the OVA assay.

After its optimization performed on the single protein standard solutions, the method was tested for the analysis of EW, i.e., the fining agent typically employed for wine clarification. Typical sensorgrams recorded after EW solution injections at variable concentrations (range 0–400 μg/mL) are reported in Fig. 2B. The gray strip in the same figure highlights the sensorgram region where the increase in resonance units, correlating to the amount of bound analyte, was measured [30]. Finally, a calibration curve of EW stock solutions was built up by plotting averaged signals against the different concentration levels tested; panel C of Fig. 2 showed a typical curve obtained for the fining agent EW. The experimental data set was fitted by a sigmoidal equation providing a LOD and LLOQ of 0.7 and 4 μg/mL, respectively (see Table 1).

Immunoassay applicability to wine matrixes

After the preliminary investigations carried out in order to optimize the method, the applicability of the devised biosensor to real matrices and the relevant assay performances were tested in artificially contaminated wines. The whole workflow used for sample handling is schematized as route A in Fig. 3. First, two different wines were purchased from a local retailer (a white wine produced from Falanghina grape variety and a roseé wine produced by a mixture of grapes Negroamaro and Malvasia Nera, hereafter referred to as Salice Salentino), and both matrices were fortified with variable amounts of EW (final concentration range in wine is 1–300 μg/mL). After a fixed incubation time to facilitate egg protein interaction with matrix components, the wines were subjected to a very short sample pre-treatment by SEC and microfiltration, and the resulting samples were directly injected onto the sensor surface. The disposable columns provided a fast and efficient tool to remove low molecular weight matrix components, mainly polyphenols (with cutoff 5 kDa), which could hamper the detection performances by gradual surface fouling, with the additional advantage to adjust both the pH and the ionic strength of the sample, as requested by the immunoassay (see previous section). The total running time for each sample including the purification, filtration, and injection was only 15 min. Calibration curves were built up for each type of wine by plotting averaged signals obtained with different concentration levels. As an example, a typical calibration curve of Salice Salentino roseè wine spiked with EW and purified by SEC was shown in Fig. 4.

Although the matrix proved to influence the detection, a good dynamic range was achieved for both wine varieties, and the specific fitting parameters are reported in Table 1. Blank allergen-free wine samples were analyzed and the recorded analytical signals employed to calculate LOD and LLOQ values. In particular, Falanghina wine allowed a dynamic range of 4–30 μg/mL with a LOD of 1 μg/mL, and Salice Salentino roseé wine allowed a dynamic range of 1.4–50 μg/mL with a LOD of 0.7 μg/mL, thus confirming the feasibility of a rapid label-free detection of egg proteins in real wine matrices.

Table 1 Summary of the main features achieved by the biosensor under development

Assay features		OVA	EW		
		Standard solutions	Standard solutions	Wine matrices SEC only	
				White: Falanghina	Roseé: Salice Salentino
Operating range (μg/mL)		2–20	4–50	4–30	1.4–50
Fitting Parameters	X^2	600	190	160	50
$y = A_2 - \frac{A_2 - A_1}{1 + \left(\frac{x}{x_0}\right)^p}$	A_2	510±20	730±11	311±10	294±8
	A_1	0.7±30	9±12	2±23	−46±19
	x_0	4.3±0.4	16.9±0.7	5.9±0.8	7.9±1.0
	p	1.7±0.3	1.74±0.11	1.4±0.3	1.0±0.1
LOD (μg/mL)		0.6	0.7	1	0.7
LLOQ (μg/mL)		2	4	4	1.4

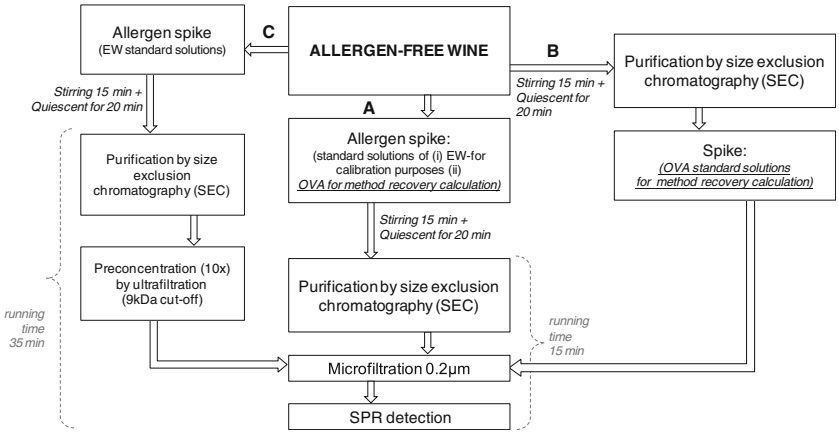

Fig. 3 Representation of the workflow developed for wine matrix handling and calculation of method recovery

Analytical method recovery

Investigations on the recovery of the whole analytical procedure were accomplished by detecting the presence of OVA in the aforementioned wines artificially fortified with the purified protein. Ad hoc experiments were designed, in which the analytical response obtained by injection of an OVA-fortified wine sample subjected to the purification procedure (route A in Fig. 3) was correlated to that recorded for an allergen-free wine sample processed in the same way and fortified with an amount of allergenic protein assuming 100 % recovery (route B in Fig. 3). From the percentage ratio of the differential response shifts, method recovery was calculated (% $Rec = \Delta RU_{1-2}(A)/\Delta RU_{1-2}(B) \times 100$).

Recoveries were found to slightly change according to the wine matrix ranging from 74±8 % for white wine to 55±12 % for roseè wine. These values showed some analyte lost during sample purification and analysis which might be due to different contributions: (i) physical lost of analyte during transfer and handling by adsorption, (ii) masking effect by matrix components which hamper the biological recognition of the specific antigen epitope, and (iii) OVA denaturation and consequent failure in the specific recognition by the antibody which was raised against the native protein. Given the proof-of-concept nature of the present work, the relative contributions of these phenomena were not further investigated.

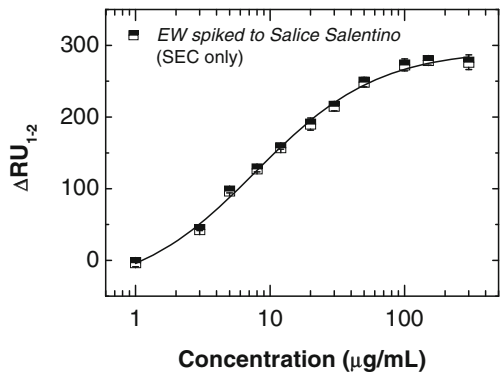

Fig. 4 Typical calibration curve of Salice Salentino roseè wine spiked with EW after size exclusion chromatography sample purification (*route A* of Fig. 3)

Method validation by comparison with LC-MS method

For comparative purposes, the SPR-based immunoassay was compared with a LC-MS-based method in order to assess the reliability of the semi-quantitative information provided by the devised biosensor. At this aim, a previously developed protocol based on the combination between SEC followed by UF of protein-containing wines, and tryptic digestion of the dialyzed wine extracts was employed [17]. The enzymatic digest after chromatographic separation was analyzed by two MS platforms allowing *selective reaction monitoring* MS/MS (SRM) and *full-scan* high-resolution MS (HR-MS) analytical approaches previously assessed in our laboratories to provide reliable, quantitative, and confirmative information [17, 28]. Parallel experiments were performed in which a set of artificially fortified wine samples was split into two aliquots: one subjected to SEC and microfiltration purification procedure followed by SPR-based detection and the other subjected to the aforementioned protocol, detailed elsewhere [17], followed by either SRM or HR-MS detection [28]. Five-point calibration curves were performed in the concentration range between 0.25 and 10 µg/mL. The peptides GGLE PINFQTAADQAR (844.423) and LTEWTSSNVMEER (799.362) were highlighted as best markers for ovalbumin providing the most intense electrospray ionization (ESI)-MS response and, in particular, the former (m/z=844.423) was chosen as a quantitative marker for the protein under investigation.

Peak areas from the chromatographic traces were plotted as a function of the EW concentration, and the LOD values were calculated as the minimum concentration of added fining agent that can be detected, at S/N=3 (the standard deviation of the calibration line intercept was used in this case as an estimate of noise). An LOD of 0.16 µg/mL was achieved with LC-HR-MS acquisition and 0.2 µg/mL with SRM acquisition,

which compared to the 1-μg/mL LOD value previously obtained with the devised biosensor proved a quite lower sensitivity of the proposed biosensing approach than LC-MS methods. Still, plotting the SPR response in terms of shift in differential resonance units versus the chromatographic peak areas, a good linear correlation was obtained in the concentration range 1–8 μg/mL. In Fig. 5, correlation curves of SPR signal with both SRM (panel A) and HR-MS (panel B) were reported.

Comparison with a confirmatory technique, such as MS, proved the relevance and the reliability of the SPR immunoassay-based approach for the analyses of egg white traces in wines, confirming biosensors as promising tools for the rapid, semiquantitative screening of allergenic contamination in fined wines.

Challenging the limit of detection towards OIV performance criteria

The first datasets collected with two different wine types used as model matrices suggested the huge potentiality of the method under investigation still with some important constrains, in terms of sensitivity. In particular, basing on the performance criteria issued by OIV for routine control of potentially allergenic fining agents in wine, limits of detection and quantification of ≤0.25 and 0.5 mg/L, respectively, are required. Both confirmatory techniques, such as LC-MS, and rapid diagnostic tools such as commercially available ELISA kit, already hit these goals.

Table 2 Summary of the sensitivity achieved by the devised biosensor under optimized sample pre-treatment

Wine type	Wine grape variety	Operating range (μg/mL)	LOD (μg/mL)	LLOQ (μg/mL)
White	Falanghina	0.3–5	0.15	0.3
	Trebbiano	0.3–10	0.16	0.3
	Sannio	0.09–2.5	0.03	0.09
	Chardonnay	0.17–5	0.03	0.17
	Terre Siciliane	0.11–5	0.03	0.11
Roseé	Mixed Negramaro and Malvasia Nera	0.3–5	0.2	0.3
	Bombino Nero	0.08–2.5	0.04	0.08
	Primitivo	0.5–40	0.2	0.5

As for SPR-based biosensing, an optimization of the analytical procedure and in particular of the sample pre-treatment was investigated, the final goal being to lower the LOD down to values comparable with LC-MS, thus meeting the issued criteria.

A new set of artificially contaminated wines at lower spike levels (0.03–10 μg/mL) was prepared, and a pre-enrichment step was introduced in the sample preparation procedure. The

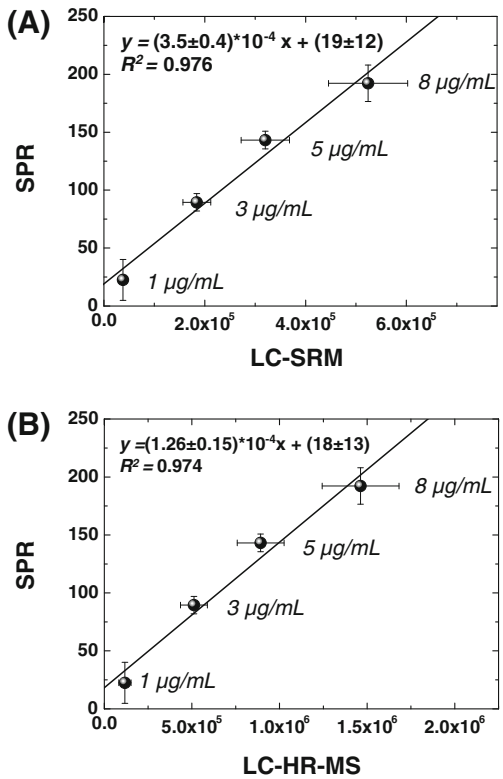

Fig. 5 Correlation curves of SPR response with (**A**) selective reaction monitoring MS/MS (*SRM*) and (**B**) full-scan high-resolution MS (*HR-MS*) analytical approaches

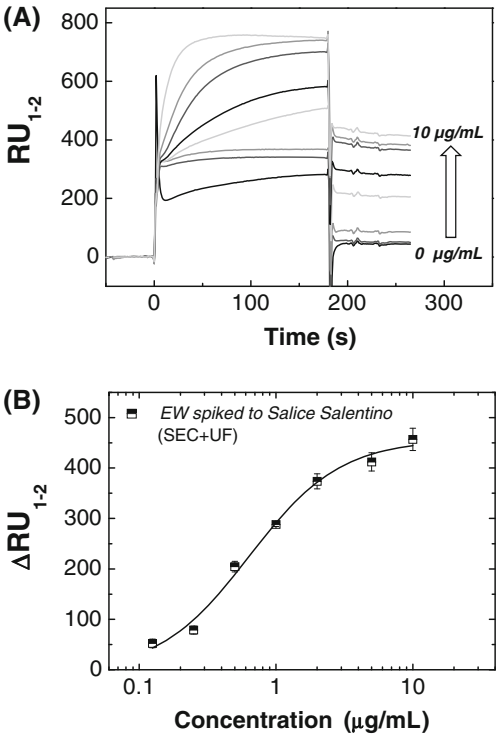

Fig. 6 Typical sensorgrams (**A**) and relevant calibration curve (**B**) of Salice Salentino roseè wine spiked with EW after combined size exclusion chromatography and ultrafiltration sample purification/enrichment (*route C* of Fig. 3)

elute volume collected after SEC purification (3.5 mL) was tenfold enriched onto UF devices with 10-kDa cutoff filters, before its injection into the SPR system (route C in Fig. 3).

The implementation of such step allowed to lower LODs for both white and roseé wines down to more challenging limits, thus making the biosensor suitable for tracing egg allergen residues in fined wines. Several wine samples and types were tested in order to assess the detection reliability and matrix effect on the final sensitivity; the assay features obtained are reported in Table 2.

The performance levels were confirmed in all selected matrices with a slight fluctuation in the detection limits ranging between 0.03 and 0.2 μg/mL for LODs and between 0.08 and 0.5 for LLOQs, depending on the wine sample; apparently, no correlation between sensitivity and wine type (either white or roseé) can be highlighted.

For the sake of clarity, typical differential sensorgrams and relevant calibration curve of Salice Salentino roseè wine spiked with EW after SEC and UF are shown in Fig. 6.

Finally, the recovery of the new devised analytical procedure was evaluated and observed to be equal to 76±2 % (for white wine) and 55±3 % (for roseè wine): as expected, the introduction of a further step in the preparation protocol enabled a general improvement in terms of sensitivity high enough to meet the OIV requirements.

Conclusions

In the present paper, the assessment of feasibility and reliability of an SPR-based biosensor for the fast screening of fining agent residuals in the finished wine product was provided opening new perspectives for wine producers that might spot the real risk associated to the use of allergenic proteins of animal origin. The SPR-based transduction was proved to be a valid alternative to the most common ELISA kits dominating the market, thanks to the rapidity of the total analysis time. Additional advantages lie in the potential to speed up and simplify the sample handling, in conjunction with the feasibility to automate and miniaturize the transduction system for a fast, in situ, semiquantitative monitoring of the contamination levels.

Acknowledgments This research was funded by the Project S.I.Mi.S.A.: Innovative tools for the improvement of food safety: prevention, control and correction - P.O.N. Ricerca e competitività 2007–2013 per le Regioni della Convergenza Codice Progetto PON02_00657_00186_3417512/1." Obiettivo Operativo: "Reti per il rafforzamento del potenziale scientifico-tecnologico delle Regioni della Convergenza." The work was also partly funded by the Italian Ministry of Economy and Finance to the National Research Council for the project "Innovazione e Sviluppo del Mezzogiorno - Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare – Legge n.191/2009."

References

1. Castillo-Sánchez JJ, Mejuto JC, Garrido J, García-Falcón S (2006) Influence of wine-making protocol and fining agents on the evolution of the anthocyanin content, colour and general organoleptic quality of Vinhão wines. Food Chem 97:130–136
2. Sauvage FX, Bach B, Moutounet M, Vernhet A (2010) Proteins in white wines: thermo-sensitivity and differential adsorption by bentonite. Food Chem 118:26–34
3. Kirschner S, Belloni B, Kugler C, Ring J, Brockow K (2009) Allergenicity of wine containing processing aids: a double-blind, placebo-controlled food challenge. J Investig Allergol Clin Immunol 19:210–217
4. EFSA Panel on Dietetic Products, Nutrition and Allergies (2011) Scientific opinion related to a notification from the International Organisation of Vine and Wine (OIV) on casein/caseinate/milk products to be used in the manufacture of wine as clarification processing aids pursuant to Article 6, Paragraph 11 of Directive 2000/13/EC—for permanent exemption from labeling. EFSA J 9:2384–2397
5. European Commission (2007) Commission Directive 2007/68/EC. Off J Eur Union L130:11–14
6. European Commission (2010) Commission Regulation (EU) no 1266/2010. Off J Eur Union L347:27–28
7. European Commission (2012) Commission Implementing Regulation (EU) no 579/2012. Off J Eur Union L171:4–7
8. OIV (2012) Criteria for the quantification of potentially allergenic residues of fining agent proteins in wine. OIV-OENO 427–2010 modified by OIV–Comex 502–2012. Available at: http://www.oiv.int/oiv/info/enmethodesinternationalesvin
9. Monaci L, Visconti A (2009) Mass spectrometry-based proteomics methods for analysis of food allergens. Tr Anal Chem 28:581–591
10. Johnson P, Baumgartner S, Aldick T, Bessant C, Giosafatto V, Heick J, Mamone G, O'Connor G, Poms R, Popping B, Reuter A, Ulberth F, Watson A, Monaci L, Mills ENC (2011) Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. J AOAC Int 94:1026–1033
11. Cryar A, Pritchard C, Burkitt W, Walker M, O'Connor G, Burns TD, Quaglia M (2013) Towards absolute quantification of allergenic proteins in food—lysozyme in wine as a model system for metrologically traceable mass spectrometric methods and certified reference materials. J AOAC Int 96:1350–1361
12. Monaci L, Pilolli R, De Angelis E, Godula M, Visconti A (2014) Multi-allergen detection in food by micro-HPLC coupled to a dual cell linear ion trap mass spectrometer. J Chromatogr A 1358:136–144
13. Cereda A, Kravchuk AV, D'Amato A, Bachi A, Righetti PG (2010) Proteomics of wine additives: mining for the invisible via combinatorial peptide ligand libraries. J Proteom 73:1732–1739
14. Mattarozzi M, Milioli M, Bignardi C, Elviri L, Corradini C, Careri M (2014) Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine. Food Control 38:82–87
15. Monaci L, Losito I, Palmisano F, Visconti A (2011) Reliable detection of milk allergens in food using a high-resolution stand-alone mass spectrometer. J AOAC Int 94:1034–1042
16. Monaci L, Losito I, Godula M, Visconti A (2011) Towards the quantification of milk allergens in caseinate fined white wines using HPLC coupled with single stage Orbitrap mass spectrometry. Food Addit Contam 28:1304–1314
17. Monaci L, Losito I, De Angelis E, Pilolli R, Visconti A (2013) Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry. Rapid Commun Mass Spectrom 27:2009–2018
18. Tolin S, Pasini G, Curioni A, Arrigoni G, Masi A, Mainente F, Simonato B (2012) Mass spectrometry detection of egg proteins in red wines treated with egg white. Food Control 23:87–94

19. Tolin S, Pasini G, Simonato G, Mainente F, Arrigoni G (2012) Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens. Food Control 28:321–326
20. Monaci L, Visconti A (2010) Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. Tr Food Sci Techn 21:272–283
21. Cucu T, Jacxsens L, De Meulenaer B (2013) Analysis to support allergen risk management: which way to go? J Agric Food Chem 61:5624–5633
22. Pilolli R, Monaci L, Visconti A (2013) Advances in biosensor development based on integrating nanotechnology and applied to food-allergen management. Tr Anal Chem 47:12–26
23. Yman IM, Eriksson A, Johansson AM, Hellenas KE (2006) Food allergen detection with biosensor immunoassays. J AOAC Int 89: 856–861
24. Rebe Raz S, Liu H, Norde W, Bremer MGEG (2010) Food allergens profiling with an imaging surface plasmon resonance-based biosensor. Anal Chem 82:8485–8491
25. Sener G, Uzun L, Say R, Denizli A (2011) Use of molecular imprinted nanoparticles as biorecognition element on surface plasmon resonance sensor. Sens Actuat B 160:791–799
26. Subramanian P, Lesniewski A, Kaminska I, Vlandas A, Vasilescu A, Niedziolka-Jonsson J, Pichonat E, Happy H, Boukherroub R, Szunerits S (2013) Lysozyme detection on aptamer functionalized graphene-coated SPR interfaces. Biosens Bioelectron 15:239–243
27. Vasilescu A, Gaspar S, Mihai I, Tache A, Litescu SC (2013) Development of a label-free aptasensor for monitoring the self-association of lysozyme. Analyst 138:3530–3537
28. Pilolli R, De Angelis E, Godula M, Visconti A, Monaci L (2014) Orbitrap™ monostage MS versus hybrid linear ion trap MS: application to multi-allergen screening in wine. J Mass Spectrom 49: 1254–1263
29. Biacore® assay handbook, 29-0194-00, edition AA, chapter 7. http://www.biophysics.bioc.cam.ac.uk/wp-content/uploads/2011/02/Biacore_assay_handbook.pdf. Accessed on January 9th, 2015
30. Biacore® concentration analysis handbook, edition December 2001, version AA. http://www.biophysics.bioc.cam.ac.uk/wp-content/uploads/2011/02/Conc_Handbook.pdf. Accessed on January 9th, 2015
31. Biacore® X instrument handbook, edition January 2001, version AB, chapter 5, 1–6
32. Myszka DG, He X, Dembo M, Morton TA, Goldstein B (1998) Extending the range of rate constants available from BIACORE: interpreting mass transport-influenced binding data. Biophysical J 75:583–594