

Challenging the Limit of Detection for Egg Allergen Detection in Red Wines by Surface Plasmon Resonance Biosensor

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Abstract The improvement of a surface plasmon resonance (SPR)-based immunoassay for the detection of traces of egg-based fining agents in red wines is herein described. The latter represents an extension of a previously developed direct assay targeted to the detection of ovalbumin (OVA) as marker of the presence of egg white powder residues, a typical fining agent utilized by the winery industry. In this paper, a suitable pre-treatment procedure was optimized for the sensitive detection of OVA at sub-ppm levels in red wines fortified with egg-white powder, by using an immunoassay proved to be reliable for both white and rosé wines. A red wine from Chianti grapes selected as reference matrix was artificially contaminated with egg-white powder before undergoing different sample purification. Several purification strategies were investigated and tested in order to challenge the limit of detection (LOD) obtained with the methods currently in use for egg allergen detection. Finally, the optimized two-step pre-treatment, combining polyvinylpyrrolidone-based purification and size exclusion chromatography, enabled to achieve an LOD in red wine as low as 0.2 µg/mL. The optimized SPR-based method met the method performance criteria issued by the International Organization of Vine and Wine (OIV) concerning the minimum sensitivity required for the analyses of potentially allergenic fining agent proteins in wines, confirming the biosensor as promising tool to monitor the residual contamination level of fined red wines.

Keywords Surface plasmon resonance · Biosensor · Allergen · Ovalbumin · Egg white · Red wine

Introduction

The clarification or fining of wine removes undesired substances (mainly proteins, phenols, and tannins), which would spoil the wine and cause bitterness and astringency. Egg-derived products having optimal fining properties are utilized in various commercial preparations for processing aids in winemaking thanks to the ability to interact and promote precipitation of wine polyphenols and other undesirable compounds (Castillo-Sánchez et al. 2006). However, any residues of egg white proteins remaining in the wine could represent a risk for allergic consumers. Fining proteins should be removed by decantation or filtration steps (Lacorn et al. 2011) and eventual further residuals by a secondary fining procedure, using inorganic agents, such as bentonite (Sauvage et al. 2010). Although, some investigations apparently proved that such removal steps might lead to non-allergenic end-products (Kirschner et al. 2009), the risk for wine allergic consumers cannot be completely excluded as already highlighted in the opinions issued by the European Food Safety Agency (EFSA 2011). As from July 1, 2012, the European Union obliges European wine producers to indicate the use of allergenic aids of animal origin whenever added for fining purposes (European Commission 2010, 2012).

Within this European legislative frame, the development of reliable and sensitive analytical approaches for the detection of egg proteins in foods and beverages might open new perspectives for the producers that might spot the real risk associated to certain categories of products where allergens are likely to remain as residues. No official analytical method for the determination of fining agent proteins are prescribed,

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till now, the only reference being the article 120g of the European regulation 1234 of 2007 concerning analytical methods to detect wine composition (European Commission 2007). In the latter, a direct reference to the International Organization of Vine and Wine (OIV) resolution was stated, who in the resolution 427-2010 (OIV 2010) modified by the OIV/COMEX 502-2012 (OIV 2012) set up the analytical requirements to be fulfilled by methods under development.

As confirmatory technique, mass spectrometry has established its pivotal role to analyze residual fining agents (Monaci and Visconti 2009). Typical bottom-up proteomic approach was successfully applied to the detection of residual egg and milk proteins in wines, either as such or after being artificially fortified with milk- or egg-related fining agents, i.e., caseinate or egg white powders (Cereda et al. 2010, Losito et al. 2013, Mattarozzi et al. 2014, Monaci et al. 2011a, b, 2013, Pilolli et al. 2014, Tolin et al. 2012a, b). Complementary to confirmatory techniques, rapid diagnostic tools are increasingly being requested from producers, in order to evaluate with a fast semi-quantitative preliminary screening, the actual risk of the end-products for allergic consumers (Monaci and Visconti 2010; Lebesi et al. 2010). The most commonly used rapid methods for routine monitoring are based on enzyme-linked immunosorbent assays (ELISAs) in a 96-well plate format (Cucu et al. 2013). For the specific case of allergenic residue detection in fined wines, several ELISA kits are already commercially available, and recently, some collaborative tests were performed to validate the relevant performances according to the OIV resolution (Restani et al. 2014; Lacorn et al. 2014). In particular, Restani et al. reported in 2014 an interlaboratory collaborative trial on ELISA kit tailored to the detection of either caseinate or egg white-based fining agents in wines. The authors proved the fulfillment of recommended criteria, with detection and quantification limits, calculated by blank determination, as low as 0.0564 and 0.1578 $\mu\text{g/mL}$, respectively, for egg white detection in red wines. Similarly, Lacorn et al. reported on a further collaborative test for whole egg detection in red wines with a limit of detection (LOD) of 0.32 $\mu\text{g/mL}$.

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 (Pilolli et al. 2013). Typically, optical biosensors are based on measurement of differences in the surface properties of a sensor chip induced by the specific recognition of the analyte to the bioactive sensing layer. Among the various optical transduction mechanisms, surface plasmon resonance (SPR)-based technique plays an important role.

Few examples were reported on SPR-based systems applied to the detection of egg proteins. In 2006 (Yman et al. 2006), a sandwich immunoassay for the detection of conalbumin was for the first time developed and applied to the detection of residual amounts of egg in pasta

due to cross-contamination in shared production lines. Rebe Raz et al. in 2010 developed an imaging SPR biosensor for the multiplex detection of food allergens (egg included), in cookies and dark chocolate. Further recent examples report the development of lysozyme biosensors using molecular imprinted nanoparticles (Sener et al. 2011) or specific aptamers (Subramanian et al. 2013; Vasilescu et al. 2013) as a biorecognition element. Recently, we reported on the development of the first SPR-based biosensor tailored to the fast detection of residual egg-based fining agents in white and roseé wines (Pilolli et al. 2015). A direct assay against ovalbumin (OVA), the most abundant protein in egg white powder, was designed basing on the use of polyclonal anti-OVA antibody as bio-specific receptor. The fine-tuning of the assay conditions enabled to obtain a sensitive response at sub-ppm levels with minimal sample pre-treatment required.

In this short communication, we present an update of the previous method, customized for the analysis of red wines. An artificially fortified red wine from Chianti grapes was selected as reference matrix, and different sample purification strategies were investigated. As a result, a two-step procedure was optimized and proved to meet the sensitivity specified by the OIV.

Finally, a comparison of sensitivities achieved with the proposed SPR-based approach and a liquid chromatography mass spectrometry (LC-MS) method developed in our laboratories was performed.

Materials and Methods

Reagents

Egg white, acetic acid, sodium hydroxide, sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, Tween 20, and poly(vinylpyrrolidone) (PVPP) 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. 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- β 2microglobulin polyclonal antibody (neg-Ab), and PD-10 desalting columns were purchased from GE Healthcare Life Sciences.

SPR Detection

SPR detection was performed by a Biacore® X apparatus (GE Healthcare Life Sciences) using Biacore CM5 sensor chips (carboxymethylated dextran matrix) for all experiments. HBS-EP buffer solution was used as running buffer for immobilization of ligand on the sensor chips. Details about the apparatus, the sensor chip functionalization, and the immunoassay experimental parameters were described elsewhere (Pilolli et al. 2015). A real-time baseline correction was automatically performed during each measurement, and differential response between the two channels was referred to as $RU_{1-2} = RU_{FC1} - RU_{FC2}$. The analytical signal was given by the resonance unit shift (ΔRU_{1-2}) measured at a fixed time (30 s) after the end of the injection. All SPR measurements were carried out at 25 °C with a flow rate of 10 μ L/min.

All buffer solutions employed were preliminary filtered through 0.20- μ m cellulose acetate filters, whereas all wine samples were subjected to centrifugation at 10,000 rpm for 10 min.

Stock Solutions and Egg White-Fortified Wines

Stock solutions of egg white (EW) used for wine fortification were freshly prepared by dissolving 1 and 5 mg of powder, respectively, in 1 mL of ammonium bicarbonate 50 mM.

The red wine produced by Chianti grape varieties was purchased from a local retailer and used as reference wine matrix to check the applicability of the developed method. For calibration purposes, typically, 3-mL wine aliquots were fortified with EW stock solution (1000 or 5000 μ g/mL) to reach concentration levels of either 30 or 300 μ g/mL, depending on the specific experiment. After the EW addition, the wine was left under stirring for 15 min and then 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.

Optimization of the PVPP-Based Purification Protocol for Reduction of Polyphenol Content

Both the PVPP/wine ratio and the exposure time to PVPP microparticles were separately taken into consideration. One-milliliter aliquots of EW 30 μ g/mL spiked wine was incubated with 40 mg of PVPP under vigorous stirring for variable time: 5, 10, 15, 25, 40, 60, 120, and 180 min. After centrifugation for 15 min at 4000 rpm, the supernatant of each sample was collected and diluted 1:5 and 1:10 in sodium acetate buffer pH 4.8 to a final buffer concentration of 10 mM. Each sample at the two dilution levels was injected three times.

Analogously, 1-mL aliquots of EW 30 μ g/mL spiked wine was incubated for 120 min with variable amounts of PVPP: 0, 1, 5, 8, 11, 15, 25, 40, 70, and 120 mg under vigorous stirring. After centrifugation for 15 min at 4000 rpm, each supernatant was collected and diluted 1:5 and 1:10 in sodium acetate buffer pH 4.8 to a final buffer concentration of 10 mM. Each sample at the two dilution levels was injected three times.

Comparison of Sample Pre-Treatment Protocols

Different sample preparation procedures were itemized in the following:

- (A) Size exclusion chromatography (SEC): following previous results obtained with white and roseé wines (Pilolli et al. 2015), 2.5-mL aliquots of blank and EW fortified (300 μ g/mL) wine samples were loaded directly onto PD-10 disposable cartridges and eluted with 3.5 mL of 10 mM sodium acetate buffer pH 4.8.
- (B) PVPP-based purification followed by 1:5 dilution: 1-mL aliquots of blank and EW fortified (30 μ g/mL) wines were incubated for 120 min with 70 mg of PVPP and after centrifugation for 15 min at 4000 rpm, the supernatant of each sample was collected. Finally, the samples were diluted 1:5 in sodium acetate buffer pH 4.8 to a final buffer concentration of 10 mM before injection.
- (C) PVPP-based purification followed by SEC: 4-mL aliquots of blank and EW-fortified (30 μ g/mL) wines were incubated for 120 min with 70 mg of PVPP and after centrifugation for 15 min at 4000 rpm, the supernatant of each sample was collected; 2.5 mL of the supernatant was loaded directly onto PD-10 disposable cartridges and eluted with 3.5 mL of 10 mM sodium acetate buffer pH 4.8.

Calibration curves were built up for each procedure (A, B, and C) by monitoring differential response variation as a function of the EW concentration in wines sample. For each data set lower concentration levels were prepared by serial dilution of the purified contaminated wine (at 300 μ g/mL for protocol A and at 30 μ g/mL for protocols B and C) and the purified blank sample to simulate the intermediate spike levels. The results (averaged upon three injections) were fitted by a sigmoidal equation in semi-logarithmic scale: $y = Rhi - \frac{Rhi - Rlo}{1 + (\frac{C_{conc}}{A})^x}$. Moreover, ten replicates of blank determinations were averaged for each calibration curve ($y_0 \pm s_{y_0}$). The LOD and lower limit of quantification (LLOQ) were extrapolated from the sigmoidal curve and defined as the concentration at which the signal was $y_0 + 3s_{y_0}$ and $y_0 + 10s_{y_0}$, respectively. The LOD and LLOQ achieved were used as parameters for the selection of the best sample pre-treatment.

Comparison of the Biosensor Analytical Performances with an LC-MS Method

The SPR response sensitivity was tested against a LC-MS method developed in our laboratories. With this aim, larger amounts of artificially contaminated wines were prepared by spiking variable amounts of EW to cover the concentration range 0.25–10 $\mu\text{g/mL}$ (five concentration levels). Parallel experiments were performed with the same sample split in two aliquots, one undergoing the optimized purification strategy (procedure C) followed by SPR immunoassay detection, while the other aliquot was subjected to ad hoc-developed protocol based on incubation with PVPP, purification by size exclusion chromatography, and pre-concentration by ultrafiltration with 9-kDa cutoff membranes. The resulting set of samples was subjected to enzymatic digestion prior to injection and detection, according to conditions described elsewhere (Pilolli et al. 2014).

Results and Discussion

In our previous investigation, the feasibility of a SPR-based biosensor for a fast detection of residual egg-based fining agents in white and roseé wines was proved. A single-step direct immunoassay was designed against ovalbumin, as the most abundant protein in EW powder, the common formulation employed for fining purposes in winemaking procedure. The duly optimization of the assay parameters enabled to obtain a sensitive response in a short analysis time meeting the performance criteria issued by the OIV ($\text{LOD}=0.25 \mu\text{g/mL}$, $\text{LOQ}=0.5 \mu\text{g/mL}$) in terms of sensitivity, for immunoassay under development aimed at the detection of allergenic proteins in wines.

In this short communication, the focus was placed on red wine matrices, where egg white powder is most commonly preferred to other proteins of animal origin for fining purposes. Allergen-free red wine from Chianti grapes provided by a local retailer was selected as reference matrix.

As a first step, the same sample pre-treatment previously developed, based on the rapid purification by SEC was tested for the detection of ovalbumin in egg white-fortified red wine samples (procedure (A) in the experimental section). Fortified wine samples with different EW contamination levels were produced by serial dilution, covering the concentration range 1–300 $\mu\text{g/mL}$, and the resulting samples were directly injected on the sensor surface. A calibration curve was built up by repeated injection of each concentration level, and the resulting variation in the differential response was plotted as a function of the spike level (see Fig. 1); the fitting parameters are reported in Table 1. Under the same conditions successfully selected for white and roseé wines (Pilolli et al. 2015),

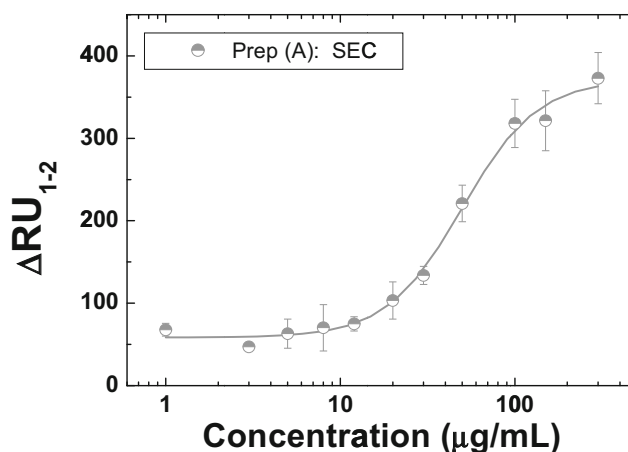


Fig. 1 Calibration curves obtained after repeated injections of Chianti wine spiked with egg white in the range 1–300 $\mu\text{g/mL}$ after purification by procedure A

the biosensor showed a poor sensitivity once tested on a more complex matrix like red wine.

Blank allergen-free wine samples were analyzed resulting in a quite high analytical response $58 \pm 7 \text{ RU}$, likely ascribed to the undesirable fouling of the sensor surface, by various matrix components, as also confirmed by a progressive drift in the baseline RU value, indication of an incomplete regeneration of the sensor surface. By extrapolation of the fitting sigmoidal curve (see [materials and methods](#) section for details), the limit of detection and quantification were calculated, found equal to 13 and 30 $\mu\text{g/mL}$, respectively.

As an evidence, the application of the immunoassay to a more complex matrix required a new evaluation of the most suitable sample pre-treatment procedure, the final goal being to reduce the interferences from matrix components which impaired the achievement of the required sensitivity and reliability in the detection of EW.

Optimization of PVPP-Based Purification Protocol

Given the potential of water-insoluble PVPP to interact with polyphenols via H bonds, a PVPP-based purification step was taken into consideration for our purpose. In particular, two sets of experiments were designed in which the exposure time to PVPP and the polymer/wine ratio were separately optimized.

First, aliquots of Chianti wine artificially contaminated with the same concentration level of EW (30 $\mu\text{g/mL}$) were prepared. A fixed amount of PVPP-insoluble microparticles was added, and various incubation times were evaluated ranging from 5 to 180 min. All supernatants were collected and as can be seen in Fig. 2a, wine aliquots showed a significant color change compared to the untreated wine sample (labeled as “ref” in the picture), clearly indicating the reduction of polyphenols content; however, no apparent color difference was noted by visual inspection of the samples as a function of the PVPP incubation time.

Table 1 Summary of the main features achieved with the devised biosensor for each procedure tested

Assay features		Sample pre-treatment procedures		
		(A) SEC	(B) PVPP (70 mg/mL, $t = 2$ h) + Dilution 1:5	(C) PVPP (70 mg/mL, $t = 2$ h) + SEC
Operating range ($\mu\text{g/mL}$)		30–300	0.7–20	0.3–20
Fitting parameters	χ^2	110	20	10
	Rhi	371 ± 13	280 ± 30	227 ± 8
	Rlo	-58 ± 5	-5 ± 4	-5 ± 4
	A_1	50 ± 4	6.5 ± 1.1	2.67 ± 0.18
	A_2	2.0 ± 0.2	1.20 ± 0.17	1.09 ± 0.09
Blank (RU)		58 ± 7	10.0 ± 1.2	3.5 ± 1.1
LOD ($\mu\text{g/mL}$)		13	0.4	0.2
LLOQ ($\mu\text{g/mL}$)		30	0.7	0.3

After dilution of 1:5 and 1:10 in a suitable buffer solution, the samples were analyzed in three replicates and the averaged differential response was calculated and reported in Fig. 2c. The first experimental trend, by testing both dilutions, indicated a direct proportionality of the specific analytical signal, recorded after the target recognition, with the PVPP incubation time, until reaching a plateau after 120-min incubation time. A likely explanation of this trend could be the progressive hydration of the PVPP microparticles over time, resulting in the improvement of the PVPP-polyphenols interaction efficiency.

The second parameter investigated for the optimization of the PVPP-based purification was the PVPP/wine ratio with

variable amounts of PVPP-insoluble microparticles in the range 0–120 mg/mL. The appearance of the collected supernatants for the different ratios investigated is reported in Fig. 2b. The picture clearly showed a gradual decrease in the color intensity of the wine sample as a function of the increasing amount of polymer added. In this case, the analytical signal showed a quite unpredictable trend with two different regions: the first with an inverse proportionality between PVPP amount and analytical response (from 0 to 15 mg_{PVPP}/mL_{wine}) and the second where the signal appeared to be directly proportional to the PVPP amount (20–120 mg_{PVPP}/mL_{wine}). However, deeper observations revealed that at low PVPP/wine ratio, an undesirable fouling of the sensor surface

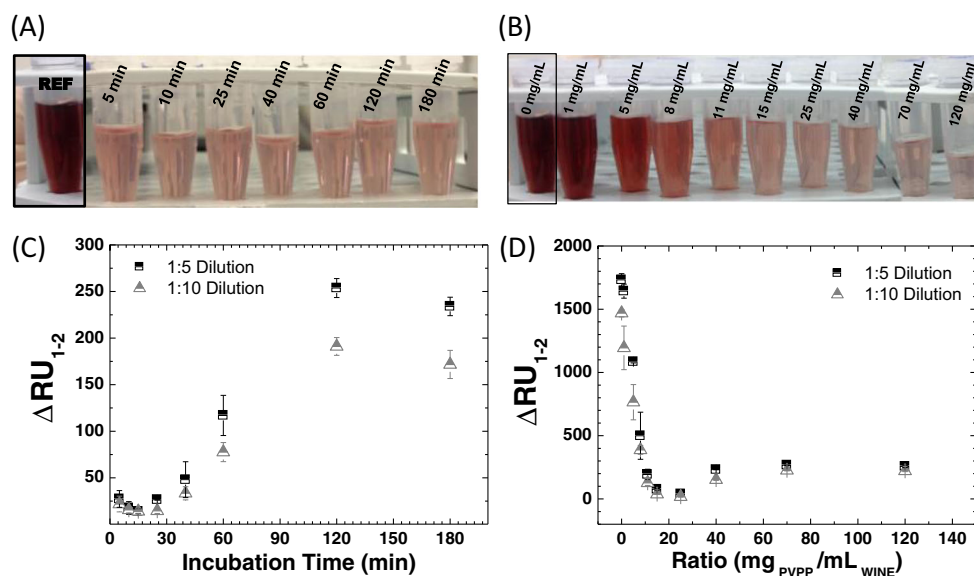


Fig. 2 Optimization of the poly(vinylpyrrolidone) (PVPP)-based purification. Picture of wine samples spiked with egg white (30 $\mu\text{g/mL}$) resulting from wine purification with PVPP microparticles (a) added at 40 $\text{mg}_{\text{PVPP}}/\text{mL}_{\text{wine}}$ after variable exposure time from 5 to 180 min and (b) resulting from wine purification for 120 min with variable amount of

PVPP microparticles 0–120 $\text{mg}_{\text{PVPP}}/\text{mL}_{\text{wine}}$. Comparison of recorded differential responses for samples subjected to PVPP-based purification as a function of the exposure time (c) and as a function of ratio $\text{mg}_{\text{PVPP}}/\text{mL}_{\text{wine}}$ (d). Wine samples were injected into SPR system after dilution (1:5 and 1:10) in sodium acetate buffer at pH 4.8

occurred, which blocked the active binding sites and harsher regeneration conditions were required thus causing a significant decrease of the chip lifetime. For these reasons, only the second region of the optimization plot was taken into account, selecting the ratio $70 \text{ mg}_{\text{PVPP}}/\text{mL}_{\text{wine}}$ and by fixing 2-h incubation time as best conditions for further experiments.

The optimized PVPP-based purification procedure indicated as procedure (B) in the experimental section was then tested on a set of artificially contaminated wine samples at variable EW concentration levels in order to assess the correlation between differential resonance response and analyte concentration, as well as biosensor sensitivity. All samples were diluted 1:5 in sodium acetate buffer before injection. An operating range of almost two orders of magnitude was assessed from 0.7 to $20 \mu\text{g}/\text{mL}$. The relevant calibration curve was built up and reported in Fig. 3a, and the fitting parameters obtained after sigmoidal interpolation were indicated in Table 1. Compared with results achieved with procedure (A), based only on size exclusion chromatography, the PVPP-based pre-treatment followed by a simple dilution of the sample under investigation allowed a significant improvement of the overall sensitivity lowering the limit of detection from 13 to $0.4 \mu\text{g}/\text{mL}$. This achievement can be justified by a significant reduction of the matrix component interferences as proved by the lower blank signal recorded upon allergen-free wine sample injection ($10.0 \pm 1.2 \text{ RU}$). Still, the sensitivity achieved under these conditions was not sufficient to meet the performance criteria defined by the OIV for analytical methods devised for the detection of fining agent proteins in wines. Therefore, a further improvement of the pre-treatment procedure was required.

In particular, procedures (A) and (B) were combined into procedure (C) consisting of a two-step-procedure: first, blank and artificially contaminated (EW $30 \mu\text{g}/\text{mL}$) wine samples were incubated with PVPP (by using the optimized conditions, $70 \text{ mg}_{\text{PVPP}}/\text{mL}_{\text{wine}}$ for 2 h) in order to induce the co-precipitation of matrix polyphenols which interfered with the sensing surface, and second, the supernatant was further purified by size exclusion chromatography aiming at removing other low molecular weight components arising from the matrix. Such purified samples were used to prepare the full calibration curve by serial dilution, and the whole set of samples was injected onto the sensor chip. As a first evidence, an improvement of the sample purification scheme, following the more efficient removal of the matrix components, was proved by the remarked reduction of the allergen-free blank signal ($3.5 \pm 1.1 \text{ RU}$). A good correlation of the differential analytical response with the EW concentration level was assessed over the whole range under investigation, and the quality of the recorded sensorgrams was highlighted in Fig. 3b. By extrapolation of the fitting sigmoidal curve (Fig. 3c), the limit of detection and lower limit of quantification were calculated and found equal to 0.2 and $0.3 \mu\text{g}/\text{mL}$,

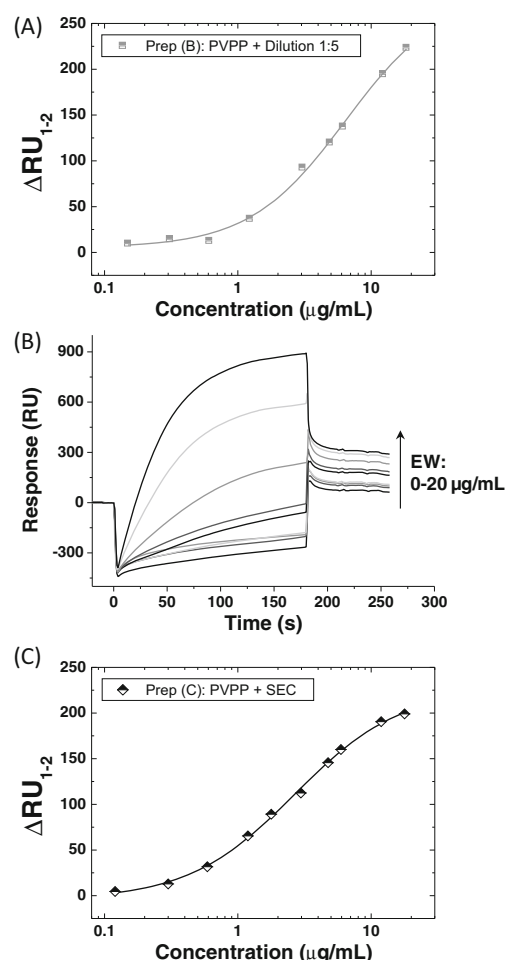


Fig. 3 (a) Calibration curve obtained after sample purification by procedure B of Chianti wine spiked with egg white in the range 0.12–20 $\mu\text{g}/\text{mL}$. (b) Typical sensorgrams recorded in the sensing channel for Chianti wine samples spiked with egg white and purified by procedure C (PVPP + SEC). (c) Calibration curve obtained after sample purification by procedure C of Chianti wine spiked with egg white in the range 0.12–20 $\mu\text{g}/\text{mL}$

respectively. Therefore, the implementation of the two-step procedure (C) made our SPR-based immunoassay a suitable and sensitive detection tool for tracing eventual egg allergen in fined red wines.

After optimization, recovery studies of the whole analytical procedure were also accomplished through ad hoc experiments in which the analytical response obtained by injection of an EW-fortified wine sample ($30 \mu\text{g}/\text{mL}$) subjected to the purification procedure was correlated to that recorded for an allergen-free wine sample processed in the same way and fortified with an amount of allergenic ingredient assuming 100 % recovery (Pilolli et al. 2015). From the percentage ratio of such differential response shifts, method recovery was calculated and found equal to $54 \pm 3 \%$ (evaluated on three replicate experiments). The recovery value of the whole analytical procedure was quite low, likely because the PVPP could interact also with egg white proteins inducing co-precipitation; still,

the efficiency in the removal of matrix-interfering components was high enough to not only compensate for ovalbumin depletion but also to enable the general improvement in terms of detection sensitivity.

As a final step, the sensitivity achieved in the proposed SPR-based detection was compared with a LC-MS-based method in order to assess the reliability of the quantitative information provided by the devised biosensor. At this aim, an analytical approach based on high-resolution MS (HR-MS) approach was used, previously developed in our laboratories, where specific tryptic peptides were selected as quantitative markers. The method was based on the combination of PVPP-based purification, SEC, and ultrafiltration (UF) of protein-containing wines, tryptic digestion of the dialyzed wine extracts, and LC/HR-MS analysis of tryptic digests. Parallel experiments were performed in which a set of artificially fortified red wine samples were divided into two aliquots: one subjected to the optimized purification procedure (procedure C) followed by biosensor detection and the other subjected to previously developed protocols described elsewhere, followed by LC/HR-MS. Five-point calibrations were performed in the concentration range between 0.25 and 10 $\mu\text{g/mL}$. The peptides GGLEPINFQTAADQAR (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 quantitative marker for the protein under investigation.

Peak areas from the extracted peptide ion chromatograms 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 0.1 $\mu\text{g/mL}$ was achieved with LC/HR-MS compared to the LOD of 0.2 $\mu\text{g/mL}$, here assessed with the devised biosensor.

Sensitivity comparison with a confirmatory technique, such as MS, and compliance with the OIV recommendation proved the relevance and the potential of the SPR immunoassay-based approach for the analyses of egg white traces in red wines, confirming biosensors as promising alternative to ELISA kit for the quantitative screening of allergenic contamination in fined red wines.

Conclusions

The optimization of a SPR-based method for the sensitive detection of residual egg-fining agents in red wines was described. Following previous results, a single-step direct immunoassay against ovalbumin was chosen as format, due to its prevalence in egg white powder, typically utilized by the winery industry for fining purposes. An artificially fortified red

wine from Chianti grapes was selected as reference matrix, and the duly optimization of the purification strategies enabled to accomplish the reliable and sensitive detection of residual egg allergen traces. In particular, the implementation of a two-step pre-treatment combining polyvinylpyrrolidone-based purification and size exclusion chromatography significantly improved the limit of detection, as low as 0.2 $\mu\text{g/mL}$, with a LLOQ of 0.3 $\mu\text{g/mL}$. These features allowed to meet the sensitivity required by the international Organization of Vine and Wine for immunoassay under development ($\text{LOD} = 0.25 \mu\text{g/mL}$, $\text{LOQ} = 0.5 \mu\text{g/mL}$) and provided performances very close to that achieved with the confirmatory LC-MS method, which involved longer sample preparation procedure, more expensive instrumentation, and skilled personnel.

Compliance with Ethical Standards This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of Interest R. Pilolli declares that she has no conflict of interest. L. Monaci declares that she has no conflict of interest.

Informed Consent Not applicable.

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