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ORIGINAL PAPER

Direct surface plasmon resonance immunosensing of pyraclostrobin residues in untreated fruit juices

E. Mauriz · C. García-Fernández · J. V. Mercader · A. Abad-Fuentes · A. M. Escuela · L. M. Lechuga

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Abstract A surface plasmon resonance (SPR) immunoassay for on-line detection of the strobilurin fungicide pyraclostrobin in untreated fruit juices is presented. The analysis of pyraclostrobin residues is accomplished in apple, grape, and cranberry samples by monitoring the recognition events occurring separately in a two-channel home-made SPR biosensor. Covalent coupling of the analyte derivative results in a reversible method, enabling more than 80 measurements on the same sensor surface. Optimization of the immunoassay conditions provides limits of detection as low as 0.16 μg L^{-1}. The selectivity and reproducibility of the analysis is ensured by studying both non-specific interactions with unrelated compounds and inter-assay coefficients of variation. Excellent recovery ranging from 98 to 103 % was achieved by a simple 1:5 dilution of fruit juice with assay buffer before the analysis. The lack of previous cleaning and homogenization procedures reduces the analysis time of a single food sample to only 25 min, including the regeneration cycle.

Keywords SPR · Biosensor · Immunoassay · Pyraclostrobin · Food analysis

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E. Mauriz (✉) · C. García-Fernández
Institute of Food Science and Technology (ICTAL),
University of León,
24007 León, Spain
e-mail: elba.mauriz@unileon.es

J. V. Mercader · A. Abad-Fuentes
Institute of Agrochemistry and Food Technology,
Spanish National Research Council,
46100 Burjassot, València, Spain

A. M. Escuela
IUMA, Institute for Applied Microelectronics,
University of Las Palmas GC,
Las Palmas GC 35001, Spain

L. M. Lechuga
Research Center on Nanoscience and Nanotechnology CIN2 (CSIC) and CIBER-BBN,
Campus UAB,
08193 Bellaterra, Barcelona, Spain

Introduction

Ecosystems and humans are exposed to a large number of organic and inorganic chemicals from agricultural and non-agricultural sources. Monitoring of pesticides which remain in food is a key aspect of protecting public health from risks posed by the residues present in crops or animal products. Environmental and agricultural policies, particularly in industrialized countries, demand the monitoring of potentially harmful chemicals likely to be found in food and water. The European Union (EU) has provided a framework to ensure the continuous monitoring of pollutants by enforcing drinking water and food legislation (Water Framework Directive 2000/60/EC; Regulation 178/2002/EC of General Food Law Regulation) [1, 2]. Similarly, the Environmental Protection Agency (EPA) has established legal instruments to promote drinking water quality and food safety by enacting The Safe Drinking Water Act (SDWA) and The Food Quality Protection Act (FQPA). The purpose of mandatory monitoring programs is to protect public health from pesticide risks by regulating public water supply and all stages of food and/or feed production and distribution.

In particular, the intended use of pyraclostrobin to treat a variety of crops has been enforced by recent European recommendations. Pyraclostrobin is a new active substance with fungicidal properties that belongs to the group of the strobilurins. Pyraclostrobin has eradicative, curative, and protective action against fungi both on the plant surface

and within the tissues of a wide range of dicotyledonous and monocotyledonous species. The biochemical mode of action is inhibition of mitochrondrial respiration but it also affects plant physiology acting as a plant-growth regulator. In the last decade, pyraclostrobin sales have increased steadily because of its effectiveness in the control of fungal diseases and its use to promote plant growth.

In European Union legislation, pyraclostrobin was first included in Annex I of Directive 91/414/EEC [3] by Commission Directive 2004/30/EC [4], for use only as a fungicide, and, in accordance with Directive 2009/25/EC [5], was regulated as a plant-growth regulator. Maximum residue levels (MRLs) are established in Annexes II and III of Regulation (EC) No 396/2005 [6] and have been submitted thereafter to subsequent modifications by the European Food Safety Authority (EFSA). The existing MRLs derived from EFSA proposals for various crops have been implemented in successive EU Regulations (no. 459/2010; no. 750/2010; no. 508/2011, and no. 978/2011). EFSA concludes that limits of quantification (LOQ) for pyraclostrobin residues in food of plant origin by validated analytical methods are set in the range 0.01–3 $mg kg^{-1}$, depending on the food product [7].

Methods for enforcement of pyraclostrobin residues in commodities of plant and animal origin have almost entirely relied upon high-performance liquid or gas chromatography coupled with single or tandem mass spectrometry (HPLC–MS–MS; GC–MS–MS) and ultraviolet (HPLC–UV) detection [8, 9]. Despite the inherent advantages of these methods in terms of accuracy and sensitivity, analysis involves time-consuming and laborious pretreatment procedures that restrict their use as on-site monitoring tools. For instance, the extraction of pyraclostrobin from fruit and vegetables requires the use of either organic solvents, for example ethyl acetate, hexane, acetonitrile, or acetone [10, 11] or solid-phase extraction and microextraction (SPE, SPME) before chromatographic determination to extract or clean the extracts [12–16]. Recently, immunoaffinity chromatography (IAC) has been successfully applied to pyraclostrobin detection by exploiting the affinity and selectivity provided by antibodies [17]. Nevertheless, these methods neither improve nor reduce the time and cost of the analysis.

This limitation may be overcome by using alternative analytical techniques capable of reducing the assay time while maintaining sensitivity similar to that of conventional methods. In this sense, use of optical biosensors is extremely promising, because of their rapid, specific, and sensitive response with the possibility of detecting multiple analytes in a single run. Specifically, this paper focuses on the determination of pyraclostrobin residues in fruit juice samples by use of an SPR biosensor based on an immunoassay. Aspects of analytical performance including immobilization, specificity, and reproducibility of measurements are considered. Likewise, special attention has been paid to optimizing assay sensitivity for both buffer and food matrices. In particular, this work tries to respond to recent environmental legislation by using technological advances that refine existing analytical strategies.

Experimental

Chemicals and immunoreagents

Chemicals for functionalization of the sensor surface were from Sigma–Aldrich (Steinheim, Germany): mercaptoundecanoic acid (purity 99 %); *N*-hydroxysuccinimide (NHS, purity 98 %), and *N*-(3 dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC). The blocking agent ethanolamine hydrochloride, the surfactant Tween 20, and the organic solvent trichloroethylene (purity 99.5 %) were also supplied by Sigma–Aldrich (Steinheim, Germany). Other organic solvents used for cleaning gold films—ethanol (absolute) and acetone (purissimum)—and potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate, used for the preparation of 1× PBS 10 mmol L^{-1}, pH 7.35, were from Panreac (Barcelona, Spain). Sodium chloride and sulfuric acid (purity 95–97 %) were from Merck (Darmstadt, Germany), and hydrogen peroxide (approx. 30 % aqueous solution) was from Prolabo (Fontenay sous Bois, France). Gold-coated substrates 10 mm^2 used as sensor surfaces were purchased from Ssens (Hengelo, Netherlands). The matching oil for coupling to the SPR system was from Panreac (Barcelona, Spain). Milli-Q water supplied by a Millipore gradient A apparatus was used for preparing buffer and other working solutions.

Pyraclostrobin and boscalid standards were supplied by the Institute of Agrochemistry and Food Technology (Valencia, Spain). Analytical standards were prepared as 10 mmol L^{-1} stock solutions in dry dimethylformamide (DMF) and stored at −20 °C. Working standards were prepared daily by serial dilution with PBST (PBS: 10 $mmol L^{-1}$ phosphate-buffered saline solution, pH 7.35, containing 0.05 % of the surfactant Tween- 20). Production and characterization of the immunoreagents were carried out at the Institute of Agrochemistry and Food Technology (Valencia, Spain). The synthesis of pyraclostrobin hapten Pys5 and the production of BSA-Pys5, BSA-PYa6, and OVA-Pys5 conjugates have been described elsewhere [18]. The synthesis and generation of Pyo5 hapten, BSA-Pyo5 conjugate, and anti-pyraclostrobin monoclonal antibody (MAb) have also been described elsewhere [19]. Anti-pyraclostrobin monoclonal antibodies MAbPys5#11 and MAbPys5#13 were produced from BSA conjugates of the hapten PYs5 and anti-boscalid MAb-BLa5#115 used for the specificity studies were generated by following well-established procedures

for hybridoma generation (specific details will be published elsewhere).

SPR instrumentation

The configuration characteristics of the home-made SPR device have already been described in previous publications [20]. Briefly, a two-channel SPR instrument working in the Kretschmann configuration enables the simultaneous monitoring of binding events occurring separately in its two channels (Fig. 1). The particular design of the SPR device makes possible either the analysis of two independent measurements happening at the same time in each sensing channel or the use of one as a reference. Therefore, parallel and simultaneous detection of two analytes can be monitored in real-time. Monitoring of biomolecular interactions is achieved by measuring changes in refractive index at a fixed angle of incidence. The SPR signal is detected as variations of the reflected light intensity by use of a multielement photodiode; the signal is amplified and converted to a digital signal. Samples are passed over the sensing surface by use of a fully automated liquid-handling system consisting of two flow cells (300 nL), a peristaltic pump, and two diaphragm pumps with their corresponding injection valves. A continuous flow of an assay buffer is maintained at a constant speed of 25 μL min^{-1} for monitoring binding and a flow-rate of 45 μL min^{-1} is used for regenerating the immunoreactive surface. The sensitivity and low time of

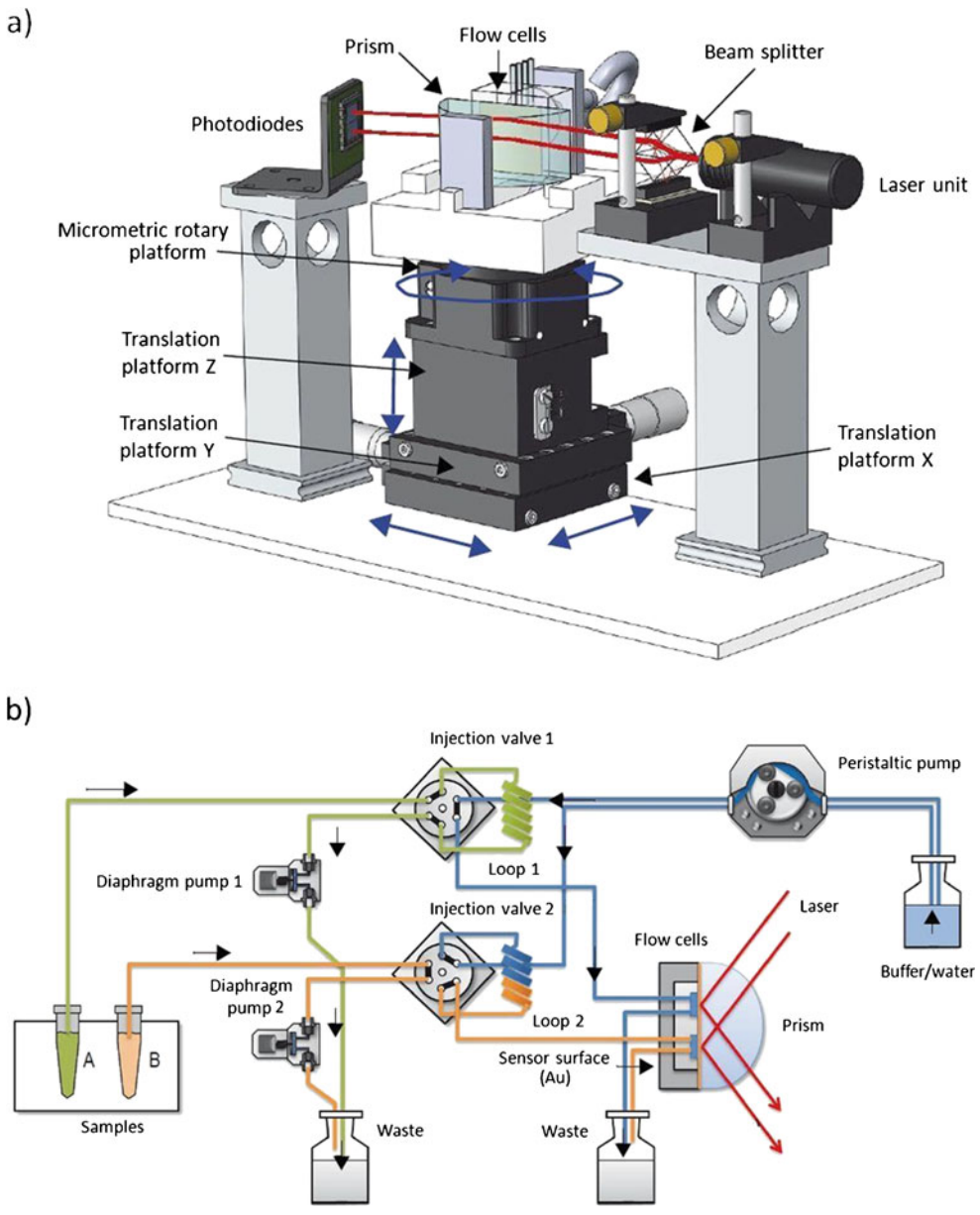

Fig. 1 Schematic representation of the SPR device: (**a**) set-up and (**b**) microfluidics

response of the analysis is achieved by selecting the appropriate flow-rate [21]. A whole assay cycle comprising sample injection and regeneration is complete in 25 min.

Immunosurface preparation and functionalization

Gold-coated substrates ($10\times10\times1$ mm^3) were comprehensively cleansed before being used as sensing surfaces. The cleaning procedure comprised sequential immersion in organic solvents (trichloroethylene, acetone, and ethanol) followed by rinsing for 15 min in an ultrasonic bath. Finally the sensor chip is dipped in freshly prepared piranha solution (H_2SO_4–H_2O_2, 3:1) then ultrasonicated in distilled water for 15 min. Cleaned and dry gold-coated sensor surfaces were coupled to the prism of the SPR platform by use of a matching oil of the same refractive index (n=1.52).

The biomolecules were immobilized by formation of self-assembled monolayers (SAMs). Amine groups of the recognition element interact with carboxyl terminal groups of the alkanethiol monolayer through a carbodiimide linkage. The whole immobilization process from surface alkanethiol functionalization to the covalent binding of the immunoreactive compound is monitored in real-time by passing the corresponding solutions over the sensor surface at a constant speed. A continuous flow of 1× PBS 0.05 % Tween 20 (PBST) was maintained throughout the immobilization procedure. First, a densely packed monolayer was formed by pumping mercaptoundecanoic acid (MUD), 0.05 mmol L^{-1} in ethanol, over the gold sensing layer. Activation of alkanethiol carboxyl groups to a stable intermediate (*N*-hydroxysuccinimide ester) was achieved by use of a mixed solution of EDC and NHS (0.2 and 0.05 mol L^{-1}, respectively, in distilled water). At this juncture, the modified sensor surface is readily available for the amine groups of the protein–hapten conjugate used as recognition element. Therefore, solutions containing 10 μg mL^{-1} BSA-Pys5, BSA-Pyo5, BSA-PYa6, or OVA-Pys5 in PBST were covalently bound to the functionalized surface. Undesirable effects because of non-specific binding and formation of conjugate multilayers were counteracted by using a solution of ethanolamine 1 mol L^{-1}, pH 8.5 as blocking agent so that all remaining unreacted NHS esters could be deactivated. Finally a solution of 0.1 mol L^{-1} HCl was injected to complement ethanolamine deactivation by weakening electrostatic interactions between the immobilized biomolecule and the sensing surface and consequently elute any remaining non-covalently bound conjugates (Fig. 2).

Immunoassay detection format

Measurements of pyraclostrobin were performed in triplicate using inhibition binding assays in which samples containing a fixed concentration of monoclonal antibody were mixed with analyte solutions at serial dilutions. After a short incubation step, only the antibody that remained free in the mixture might couple the BSA–hapten conjugate immobilized at the sensor surface. The antibody binding is limited by the presence of pyraclostrobin in the sample, therefore as pyraclostrobin concentration increases the SPR signal diminishes.

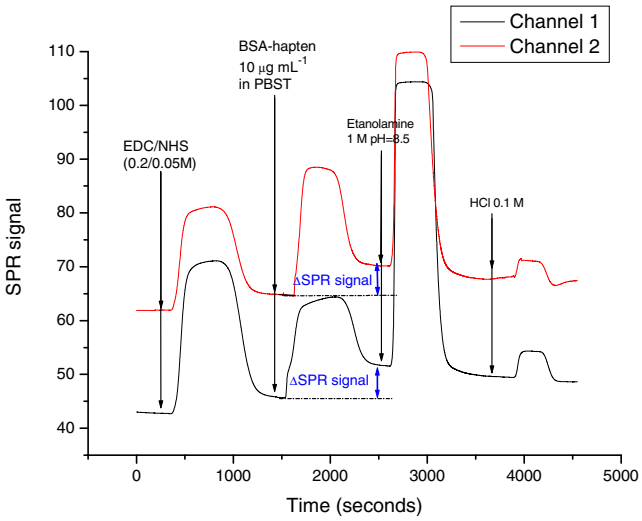

Fig. 2 Monitoring of the SPR signal during the process of immobilization of BSA–hapten conjugates, from activation of the mercaptoundecanoic acid functionalized surface to blocking of the sensing surface

In particular, standard dilutions of pyraclostrobin were prepared in the 0.0038–3.87 μg L^{-1} range in PBST from a stock solution of 3.87 g L^{-1} in DMF. For immunoassay optimization monoclonal antibodies (MAbPys5#11, MAbPys5#13, MAbPyo5#31) at fixed concentrations were mixed with pyraclostrobin dilutions. Before the analysis, the mixture was incubated for 10 min at room temperature. Binding between free antibodies and the immobilized BSA–hapten conjugate and regeneration of the immunosurface were monitored in real-time. The immunobinding event was disrupted by using 0.1 mol L^{-1} HCl as regeneration agent.

The immunosensor response obtained by measuring serial dilutions of mixed pyraclostrobin–antibody samples was first normalized by expressing the SPR signal (SPR_{signal}) of each standard point as the percentage of the maximum response [$100\times(SPR_{signal}/SPR_{signal,max})$] and then converted to normalized standard curves by plotting reflectance (V) against the logarithm of pyraclostrobin concentration. The experimental data were fitted to a four-parameter logistic equation:

$$y = \left\{D + (A - D) \Big/ \left[1 + (x/C)^B\right]\right\}$$

where A is the asymptotic maximum (maximum SPR signal in the absence of analyte, SPR_{max}), B is the curve slope at

the inflection point (related to the analyte concentration giving 50 % inhibition of SPR_{max}: C, I_{50}), and D is the asymptotic minimum (background signal).

SPR analysis in fruit juice samples

Apple, grape, and cranberry juice were obtained from a local supermarket. Before analysis, fruit juice samples were diluted 1:5 with PBST. Recovery experiments were conducted by artificial fortification of fruit juice samples with pyraclostrobin at different concentrations. From a 3.87 gL^{-1} stock solution, fruit juice samples under study were spiked with pyraclostrobin at six concentrations ranging from 0.038 to 3.878 μgL^{-1}.

Results and discussion

Optimization of conjugate immobilization

Fabrication of biosensors involves coupling of the biological sensing element to the surface of the transducer in a simple and reproducible manner. As described above, hapten–carrier conjugates were covalently attached to the sensing surface previously functionalized with a mercaptoundecanoic acid monolayer. For carboxyl-terminated thiols, the ideal immobilization procedure involves formation of a carbodiimide bond that couples amine groups of the protein to carboxylic acids of the alkanethiol monolayer. This linkage confers stability to the assay while providing well-ordered and controlled structures. The reliability of our conjugate-coated format was ensured by evaluating the effect of different analytical conditions on the immunoassay performance.

Previous publications have already reported that conjugates with BSA as carrier protein have higher hapten to protein molar ratios than those produced with OVA proteins. Distribution of free hapten binding sites over an alkanethiol-activated sensor surface ensures that BSA–hapten conjugate binding rates are higher, with less antibody concentration, as reported elsewhere [22]. Therefore, BSA conjugates attached to different types of hapten were used first. The binding capacity of three pyraclostrobin monoclonal antibodies were evaluated against these two types of conjugate-coated immunosurfaces.

Immunoassay conditions, for example assay buffer concentration, pH, and flow rates, were maintained constant throughout the analysis. Non-specific interactions could be overcome by including Tween 20 detergent in the PBS buffer in working solutions at the concentration normally used in ELISA formats (0.05 %).

The SPR immunoreaction responses for monoclonal antibodies MAbPys5#11, MAbPys5#13, and MAbPyo5#31 at 0.45, 0.25, and 0.5 μgmL^{-1}, respectively, in PBST were evaluated against conjugate-coated surfaces with either BSA-Pys5 or BSA-Pyo5 at 10 μgmL^{-1} in PBST buffer. Several differences were found depending on the type of immobilized hapten. For BSA-PYs5, similar SPR response was observed when testing MAb generated from the same immunizing hapten, i.e. for the homologous hapten. In contrast, lower binding rates were observed after testing MAbPyo5#31, obtained from a heterologous hapten. Likewise, the SPR immunoreaction response for BSA-Pyo5 coated surfaces diminished for MAbPys5#11 and MAbPys5#13 antibodies whereas good binding rates were achieved for the antibody corresponding to the homologous hapten. These results were confirmed by evaluating the SPR response of MAbPys5#11, MAbPys5#13, and MAbPyo5#31 monoclonal antibodies over BSA-PYa6 immunosurfaces. In this particular case, antibody–hapten binding rates were lower for all the antibodies tested, indicating the effect of the immobilized hapten on the immunoreaction under SPR immunoassay conditions. Finally, the effect of immobilized OVA–hapten conjugates was confirmed by monitoring the SPR response of MAbPys5#11 over an OVA-Pys5 coated surface. The low binding rate showed the unsuitability of using OVA conjugates in SPR immobilization over alkanethiol activated monolayers.

On the basis of these results, immobilization with BSA-Pys5 was chosen for MAbPys5#11 and MAbPys5#13 immunobinding, whereas MAbPYo5#31 was used for BSA-Pyo5 conjugate coated surfaces.

Assay sensitivity

The sensitivity of SPR immunoassays was estimated by analysis of pyraclostrobin at seven different concentrations from 0.0038 to 3.87 μgL^{-1} in PBST. Standard solutions were mixed with the corresponding MAb, depending on the conjugate immobilized over the functionalized surface, i.e., MAbPys5#11 and MAbPys5#13 on BSA-Pys5 immunosurfaces and MAbPYo5#31 for BSA-Pyo5 sensing substrates. Samples were injected into the flow cells after a pre-incubation step of 10 min. Mean standard curves were obtained by averaging triplicate normalized measurements for each pyraclostrobin concentration. The limit of detection (LOD) was experimentally determined as the analyte concentration causing 10 % inhibition of the maximum SPR signal. The linear working range was defined by the analyte concentrations causing 20 and 80 % inhibition of the maximum SPR signal, respectively.

To provide a mean standard curve with the highest sensitivity, the effect of antibody concentration on assay sensitivity was studied by comparing MAbPys5#13 at concentrations of 0.5 and 0.25 μgmL^{-1} (Fig. 3a). The dynamic range corresponding to 0.5 μgmL^{-1} was between 0.23 and 0.99 μgL^{-1}, with an I_{50} value of 0.52 μgL^{-1} and an LOD of 0.16 μgL^{-1}. For MAb at 0.25 μgmL^{-1}, the pyraclostrobin

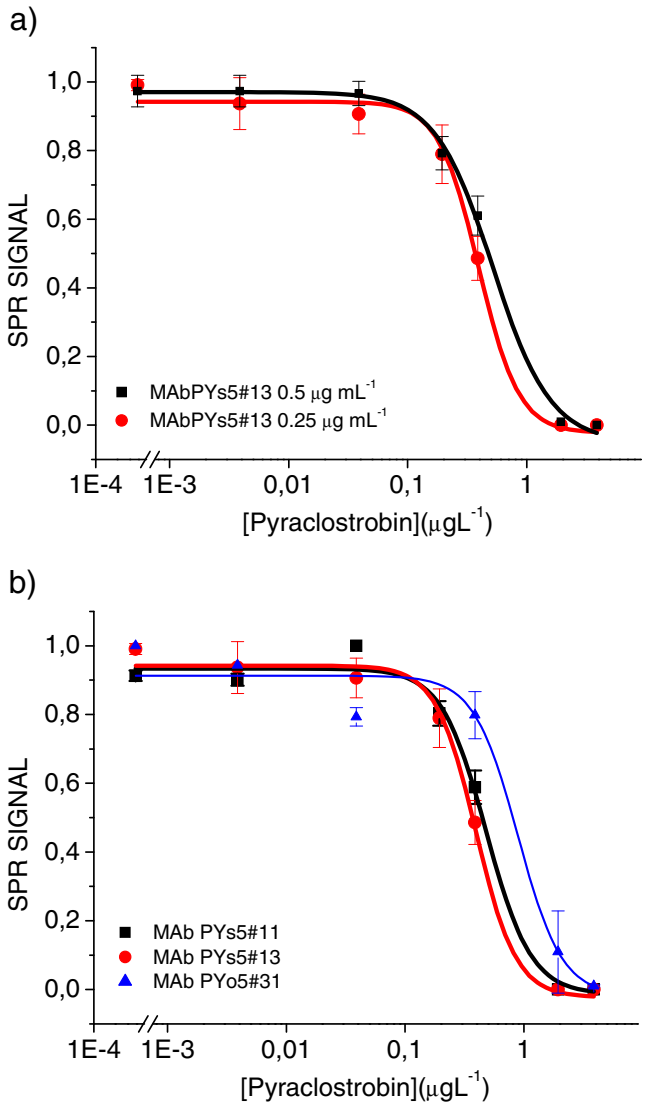

Fig. 3 Standard calibration curves for: (**a**) optimization of monoclonal antibody concentration and (**b**) selection of monoclonal antibody according to specificity and sensitivity

detection limit was 0.16 $\mu g L^{-1}$, with an I_{50} value of 0.39 $\mu g L^{-1}$ and a 0.23–0.63 $\mu g L^{-1}$ working range. Although differences are negligible, the most sensitive SPR immunoassay which required the lowest reagent expense was achieved at 0.25 $\mu g mL^{-1}$. Following these results, the optimum concentration for the other two antibodies was selected taking into account the lowest concentration giving the highest SPR signal needed to monitor immunobinding.

Immunochemical determinations of pyraclostrobin for MAbPys5#11 and MabPyo5#31 were carried out by using 0.45 and 0.5 $\mu g mL^{-1}$ concentrations, respectively. Standard calibration curves revealed significant differences depending on the immobilization format and the type of MAb used (Fig. 3b). Analytical data, expressed as I_{50}, LOD, and working ranges are shown in Table 1. The assay sensitivity for

Table 1 Optimization of SPR immunoassay by comparing variability and sensitivity values for different immobilization formats

Pyraclostrobin concentration ($\mu g L^{-1}$)	Monoclonal antibody		
	MAbPys5#11	MAbPys5#13	MAbPyo5#31
0	1.66	1.61	0
0.00388	1.97	8.06	0
0.03878	0	6.37	3.41
0.19391	4.47	10.78	–
0.38782	8.26	13.19	8.57
1.16346	0	0	109.07
1.9391	0	0	30.50
Mean	2.33	5.72	25.26
I_{50} ($\mu g L^{-1}$)	0.44	0.37	0.89
LOD (10 % inhibition concentration, $\mu g L^{-1}$)	0.19	0.16	0.35
Working range (I_{80}–I_{20}, $\mu g L^{-1}$)	0.26–0.86	0.22–0.65	0.49–1.54

MAbPyo5#31 in the BSA-Pyo5 immobilization format was slightly worse than for the other monoclonal antibodies tested. On the other hand, similar sensitivity results were obtained irrespective of the antibody for BSA-Pys5-coated surfaces. This meant that either MAbPYS5#11 or MAb-PYS5#11 could be used in the optimized immunoassay format. However data for assay variability, for example relative standard deviation (RSD), were slightly better for MAbPys5#11. For this reason MAbPys5#11 was chosen for further studies with fruit juice matrices.

Comparison of sensitivity values obtained by SPR immunoassays and ELISA formats for the same haptens in terms of LOD and I_{50} values revealed some differences. Limits of detection were similar for SPR and colorimetric ELISAs [18, 19] whereas I_{50} values were approximately one order of magnitude lower in SPR immunoassays. The better sensitivity of the ELISA format could be attributed to the stability and well-ordered structure of the conjugate-coated surface for ensuring immunoreactions and preventing non-specific interactions.

Therefore, under optimum conditions, the SPR immunoassay for pyraclostrobin determination includes:

- immobilization of hapten derivative BSA-Pys5 (10 $\mu g mL^{-1}$) on a carboxyl-terminated mercaptoundecanoic acid monolayer functionalized by a carbodiimide bond; and
- performance of binding inhibition tests with MAb-Pys5#11 monoclonal antibody at a concentration of 0.45 $\mu g mL^{-1}$ in assay buffer (PBST) and serial dilutions of pyraclostrobin standards in PBST.

Analytical calibration values are shown in Table 2. All measurements, from formation of the alkanethiol monolayer

Table 2 Analytical calibration values for pyraclostrobin determination under optimized conditions

Analytical calibration values; I_{50}±SD (μgL^{-1})	0.44±0.028
LOD ± SD (10 % inhibition concentration, μgL^{-1})	0.19±0.068
Working range ± SD (μgL^{-1})	0.26±0.056–0.86±0.16

to immunoreactions, are monitored in real-time under a continuous flow of 1× PBS, pH 7.35, with 0.05 % Tween 20, at a constant rate of 25 μL min^{-1}.

Specificity and reproducibility of SPR immunoassays

Interference of unrelated compounds

The selectivity of the immunoassay to recognize pyraclostrobin specifically was evaluated by studying possible non-specific interactions with unrelated compounds, for example non-specific antibodies and other pesticides. Because the affinity and specificity of pyraclostrobin monoclonal antibodies have been previously described in ELISA assays [18, 19], the purpose of this work was to achieve selectivity of the conjugate sensing layer by exposing the sensor surface to a flow of unrelated immunoreagents of different molecular weight. The capability of the functionalized SAM to recognize only the complementary reagents was proved by determination of another pesticide (boscalid), and the interference of high-molecular-weight compounds was assessed by monitoring the absence of response to monoclonal antibody MAbBla5#11. First, a solution containing anti-boscalid monoclonal antibody MAb-BLa5#115 was passed over the conjugate-coated surface. The SPR signal did not increase, the baseline was maintained, and no immunoreaction response was monitored at the sensing surface.

For analysis of interferences with low-molecular-weight analytes, the pesticide boscalid, present in most commercial presentations as a premixed formulation with pyraclostrobin, was selected for the study. Boscalid and pyraclostrobin mixtures at six different concentrations (0.038–3.878 μgL^{-1}) were prepared by serial dilution in PBST and mixed with MAbPys5#11 at a fixed concentration of 0.45 μgmL^{-1}. Final solutions were incubated for 10 min and injected over BSA-Pys5-coated surfaces. Measurements were performed in triplicate and the data obtained were analyzed as described above. As Fig. 4 shows, no significant difference was observed with regard to the optimized binding inhibition tests performed in the absence of boscalid. These results confirmed that pyraclostrobin detection is not affected by the presence of non-specific interactants in the same sample, thus indicating the feasibility of the immunosensing surface and the selectivity of the immunoassay.

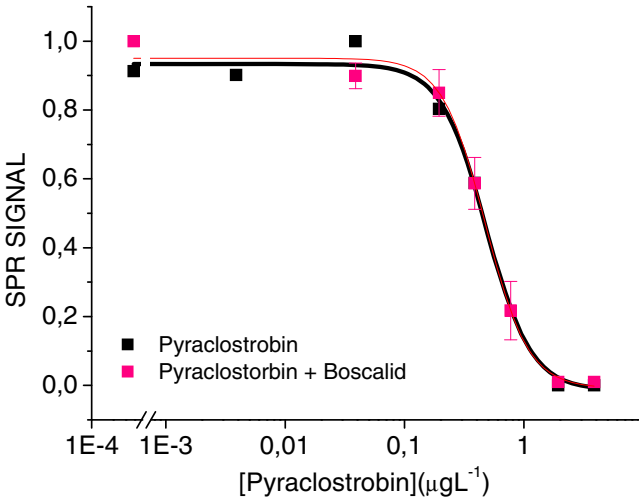

Fig. 4 Standard calibration curve for interference of the pesticide boscalid with the SPR immunoassay

Variability and reusability

In addition to specificity, other method characteristics, for example reproducibility, must be checked to ensure the repeatability and accuracy of measurements. With this objective, inter-assay variability studies were performed by comparing the coefficients of variation and I_{50} values obtained on different days and over distinct functionalized surfaces.

The variation of RSD values between calibration curves carried out on different days was used to determine inter-day variation. Measurements were performed by injecting daily prepared samples from seven-point standard curves mixed with the same fixed concentration of antibody. Analysis of the calibration curves showed coefficients of variation as low as 2.33 % (Table 1). Inter-assay variation was calculated from assay sensitivity values and coefficients of variation from standard calibration curves obtained for conjugate-coated surfaces immobilized on different days. The difference between I_{50} values was minimum (0.44 and 0.47 μg L^{-1}) and RSD were in the range 2.3–3.53.

Different gold substrates were compared by estimation of sensitivity from standard curves obtained in triplicate under optimized immunoassay conditions. Assay sensitivity was similar, irrespective of the gold slide used, with I_{50} values ranging between 0.44 and 0.53 μgL^{-1}. These variability values are indicative of the suitability of this method, in terms of precision and accuracy, for detection of pyraclostrobin without loss of assay sensitivity.

The main advantage of biosensors over other immunoanalytical methods is the stability under regeneration conditions. Therefore, possible alteration of the physicochemical properties of the immobilized conjugate after sensor surface regeneration must be prevented to enable re-use of the sensing surface. The effect of the regeneration agent on the

hapten conjugate-immobilized surface was studied throughout the assay, from placing of the gold substrate on the SPR platform to the end of the analysis. To disrupt associations between immobilized haptens and antibodies, solutions of HCl (0.1 molL^{-1}) were passed over the immunosensor surface. The binding capacity of the hapten–protein carrier conjugate was evaluated by monitoring both the drop in SPR signal after the immunoreaction and the loss of assay sensitivity. Nevertheless, the decrease in SPR signal does not entirely determine the immunosensor response, because all measurements are normalized by the blank signal of each seven-point standard calibration curve, affecting equally the values of the blank and the sample. The only limitation is to maintain a minimum allowable signal for the blank (analyte-free) sample that creates clear differentiation between diminishing analyte concentrations. Taking into account these considerations, the same immunosurface could be re-

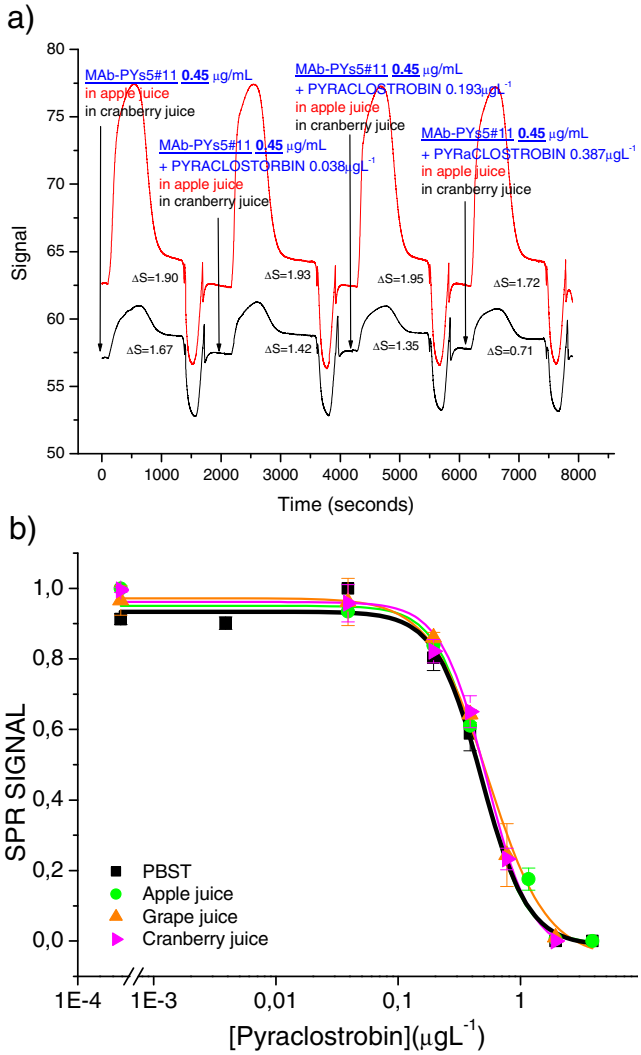

Fig. 5 Evaluation of SPR response to fruit juice tests by monitoring (**a**) signal variation along measurements and (**b**) calibration curves for spiked samples of apple, grape and cranberry juices

Table 3 Recovery of pyraclostrobin from spiked apple, grape, and cranberry juices

Fruit juice	Pyraclostrobin added (µgL^{-1})	Pyraclostrobin recovered (µgL^{-1})	Recovery (%)	CV (%)
Apple	0.0387	0.039	100.6	1
	0.193	0.197	101.6	1
	0.387	0.389	100.3	2
	1.163	1.15	98.8	17
	3.878	3.82	98.5	1
Grape	0.0387	0.0384	99	7
	0.193	0.195	100.6	2
	0.387	0.384	99	2
	0.776	0.764	98.5	18
	1.939	1.954	100.8	1
Cranberry	0.0387	0.0384	99	5
	0.193	0.197	102.1	4
	0.387	0.388	100	7
	0.776	0.764	98.5	13
	1.939	1.982	102.2	1

used for 82 measurements of pyraclostrobin with the same assay sensitivity. In this period the SPR signal decreased by only 10.9 % after 40 days of continuous operation.

Analysis in fruit juice samples

The strobilurin fungicides, including pyraclostrobin, are relatively new in agricultural applications. Pyraclostrobin is a chemical product used as a pesticide to kill fungi (mildews, molds, etc.) in a wide variety of fruits and vegetables. Hazards to humans from pyraclostrobin exposure after aerial application include eye injury and skin irritation. Exposure can also occur as a result of eating fruit or vegetables that contain pyraclostrobin residues. According to some reports 64 % of fresh fruit and vegetables and 59 % of processed fruit and vegetables contained detectable pesticide residues [23]. Because pyraclostrobin is used to control fungi by foliar application, monitoring of pyraclostrobin residues in fruit commodities is of major importance.

The use of pyraclostrobin is recommended to treat botrytis fungus in pome fruit, table grapes, and some small fruits, for example berries. Therefore, apple, grape, and cranberry juices seem to be good target commodities for screening for pyraclostrobin residues.

The effect of these pulp-free fruit juices on pyraclostrobin determination was studied by simply diluting 1:5 with PBST assay buffer. Fruit juice samples were spiked with six pyraclostrobin concentrations in the range 0.0038–3.878 µgL^{-1}. Pyraclostrobin detection in fruit juices was performed in triplicate by binding inhibition assays with MABPYs5#11 at 0.45 µgmL^{-1}. Spiked fruit samples for each pyraclostrobin

concentration were mixed with the MAb in 1× PBST giving a 1:10 final dilution of fruit juice in PBST compared with the solution used to maintain the continuous flow over the SPR sensing surface. The change in refractive index between solutions gave a small shift in the intensity of reflected light that did not interfere in pyraclostrobin measurements, because the SPR response corresponds to the hapten–antibody association with respect to the baseline before injection (Fig. 5a).

Analysis of the assay sensitivity values showed that interference of fruit matrices with SPR response was insignificant (Fig. 5b). Pyraclostrobin was detected with similar sensitivity in PBST (0.19 $\mu g L^{-1}$) and in diluted fruit juice (0.16–0.20 $\mu g L^{-1}$). Limits of detection were below the maximum residue levels imposed by the European Union and other food safety organizations for quantification of pyraclostrobin in apple (0.3 ppm), grape (1 ppm), and cranberries (3 ppm). Sensitivity values obtained from fruit juice samples were all at parts per billion levels, irrespective of the fruit juice tested. For apple, grape, and cranberry fruit juices, concentrations inhibiting 50 % of the blank signal were, respectively, 0.54, 0.52, and 0.52 $\mu g L^{-1}$. Similarly, limits of detection were broadly below maximum residue levels for apple, grape and cranberry juices. The interference of food matrices was also studied by measurement of percentage recovery to accurately determine the presence of pyraclostrobin in fruit juice samples. As shown in Table 3, mean recovery data for apple, grape, and cranberry juices were between 98 and 103 %. The excellent results show both the low variability and the negligible effect of the fruit matrix.

A small number of publications have already described the use of SPR biosensors for analyte measurements in fruit juices, most of them for microorganisms [24, 25]. However, to our knowledge, this is the first SPR-based immunoassay applied to pyraclostrobin determinations in this type of food commodity. Furthermore, pyraclostrobin is detected at extremely high sensitivity without pretreatment of the samples.

Conclusions

This paper reports an SPR-based immunoassay for detection of pyraclostrobin, a new active compound with fungicidal properties, in fruit juice samples. Pyraclostrobin was monitored at ultrasensitive detection levels in untreated fruit juices (LOD=0.16–0.20 $\mu g L^{-1}$) without the help of time-consuming precleaning and extraction procedures. All measurements were carried out in real-time in only 25 min, including the immunoreaction and regeneration of the biosensing surface.

The covalent immobilization of a BSA–hapten conjugate to a previously functionalized surface by use of an alkanethiol self-assembled monolayer provided both the stability of the biological sensing element after regeneration and the reproducibility of the analysis. One of the main advantages of linking the conjugate to the gold substrate by SAMs was the possibility of reusing the same sensing surface for more than 80 assay cycles without significant differences in assay sensitivity. The robustness of the hapten–protein conjugate modified surface under the regeneration conditions was demonstrated by low inter-assay coefficients of variation throughout the analysis (2.3–3.53 %).

Optimization of the immunochemical conditions rendered in the selection of conjugate-coated surfaces that yielded higher sensitivity in pyraclostrobin determination. The SPR response was examined in PBST buffer and apple, grape, and cranberry juices. Standard calibration curves for fruit commodities were prepared as for control samples except for previous 1:5 dilution with PBST buffer; the diluted sample was then injected directly on to the sensing surfaces. No significant differences were found in terms of assay sensitivity and recovery values between fruit juices and PBST samples. Limits of detection were more than two orders of magnitude below current maximum permitted residue levels in the corresponding food commodities (apple=0.16 $\mu g L^{-1}$; grape=0.20 $\mu g L^{-1}$; cranberry=0.18 $\mu g L^{-1}$). Mean percentage recovery was in the 98–103 % range for all the juices tested.

The main advance of this SPR immunoassay is to enable direct detection of the fungicide pyraclostrobin in fruit juices avoiding the laborious pretreatment procedures of chromatographic methods. The acceptance of SPR biosensing in environmental monitoring and food-quality analysis makes possible its potential application to the comprehensive surveillance and early determination of food contaminants. Therefore, our immunoanalytical method complies with EU and EPA recommendations for responding to potential risks by using technological advances that refine existing strategies of analysis. Further work will deal with the application of this method to real food samples containing pyraclostrobin at certified concentrations.

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