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Indirect competitive immunoassay for the detection of fungicide Thiabendazole in whole orange samples by Surface Plasmon Resonance

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A highly sensitive and specific SPR-based competitive immunoassay for the detection of Thiabendazole (TBZ) has been developed. An indirect format where a TBZ-protein conjugate is immobilized onto gold surfaces has been selected. Under the optimal conditions, a LOD of 0.67 nM (0.13 µg L^{-1}) and an IC_{50} of 3.2 nM (0.64 µg L^{-1}) have been achieved which are comparable to the values obtained by conventional ELISA. Analysis of real samples has been attempted by first evaluating the influence of complex matrix samples coming from whole oranges and secondly measuring samples containing TBZ previously evaluated by chromatographic methods. A methanolic extraction procedure followed by a simple dilution in assay buffer has proven to be sufficient to measure orange samples using the developed immunoassay with an excellent recovery percentage. The sensitivity and the feasibility of measuring whole orange samples demonstrate the effectiveness and robustness of the SPR biosensor, which can be useful for the determination of TBZ in food at concentrations below the Maximum Residue Levels (MRL) established by the European legislation.

Introduction

Pesticide residues considered to be of toxicological significance may remain in food, agricultural commodities, or animal feeds long time after application. In order to ensure consumer safety, the European Commission (EC) fixes the maximum residue levels (MRLs) as the highest levels of pesticide residues that are legally tolerated in/on food or feed. In the EU, Regulation (EC) No 396/2005 lists the MRLs for 315 fresh products.[1] Conventional methods for the analysis of pesticides include gas chromatography (GC) and high performance liquid chromatography (HPLC). They allow determining the presence of several compounds of the same or related families simultaneously and usually with acceptable to good sensitivity. However, specialized equipment with extensive maintenance and skilled personnel is always necessary. Moreover, sample pretreatment usually involves several steps of extraction, concentration and purification, which prolongs the process. Overall, this results in time-consuming and high-cost techniques,[3, 4] which usually leads to outsource the analyses. As a consequence, new technologies and instruments are continuously demanded in order to simplify and speed up the analyses at affordable cost which in turn increases the efficiency in the surveillance programs. Antibody-based methodologies have already shown remarkable progress in this field, and many immunoassays have been developed for the determination of pesticide residues in fruit and vegetables.[5, 6] The use of antibodies has enabled quantitative measurements of small traces of analyte following easy and fast procedures which often require simple sample preparation. Moreover, their implementation in sensor systems (i.e. biosensors) has given rise to analytical tools which can provide fast, sensitive and reliable on-site measurements at low cost.[7] Several immunosensors have been applied in the environmental field for pesticide analysis.[8, 9] Among these, optical-based biosensors and in particular, Surface Plasmon Resonance (SPR) biosensors play an outstanding role due to their ability to determine the presence of target compounds in a direct configuration, in real time, without the use of labels and with high levels of sensitivity. These platforms have already been used for the detection of some pesticides,[10-13] usually under standard buffer conditions. Although food matrices have been assessed, in most cases this has been limited to fortified samples to evaluate the performance of the assay. On the other hand, fewer examples can be found where real samples are evaluated. From those, most attempts are focused on quantification of pesticides in natural waters [14-16] and scarce cases are based on the detection of food.[17]

Thiabendazole (TBZ, see Figure 1) is a systemic benzimidazolic fungicide and is one of the most frequently used to control postharvest diseases caused by fungi in fruits and vegetables. Due to its widespread use, TBZ constantly appears as one of the most detected pesticides in agro-food products in Europe and USA. TBZ residues are normally found in citrus, apples, pears and bananas, as also in their processed derived juices. Thiabendazole MRLs for raw fruit and vegetables range from 0.05 to 15 mg Kg^{-1} depending on the product. In particular,

for the aforementioned fruits the MRL is 5 mg Kg^{-1} which is also considered as the legal threshold for their processed products, such as the juices.

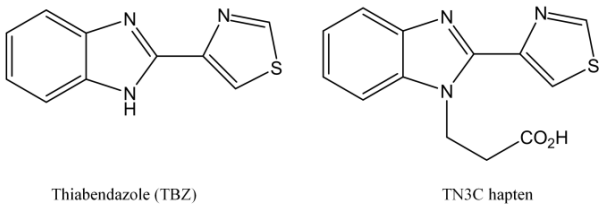

Fig. 1 Structures of Thiabendazole and TN3C hapten

TBZ-treated commodities are usually analyzed by conventional chromatographic techniques, such as HPLC-MS,[18-20] HPLC-UV,[21] capillary electrophoresis,[22] micellar electrokinetic chromatography (MEKC) coupled with a previous step of solid phase extraction (SPE),[23] and more recently using molecular imprinted polymers in solid phase microextraction (SPME) processes.[24] Sample cleanup is usually the bottleneck for the development of fast and efficient analysis and is a requirement for these aforementioned techniques. Some progress has been made in this regard, with methodologies like QuEChERS [25] which speed up the process, but immunochemical methods are still the best alternative to skip this step. Monoclonal antibodies have already been produced against TBZ [26, 27] which has led to several specific ELISAs with excellent sensitivity. The assays have been applied to the detection of TBZ in fruit juices and potatoes.[27-30] Recently also a strip-based immunoassay has been developed [31] and spiked juices samples have been analyzed. In all cases, sensitivity was below the MRLs set for this pesticide. However, up to our knowledge, no work has been yet reported based on the detection of TBZ using a label-free biosensing platform such as an SPR sensor. In this work we develop an indirect competitive immunoassay method for the detection of TBZ based on specific monoclonal antibodies. The conditions of the assay have been optimized to reach sensitivities that comfortably meet the requirements from the European Legislation. Moreover, the assay allows TBZ detection in whole grinded oranges, including external peel. As aforementioned, the developed immunoassay can provide food operators with a fast, low-cost screening device for the control and monitoring of TBZ in fruits.

Experimental

Chemical and Immunoreagents

Common reagents (i.e. 16-mercaptohexadecanoic acid (MHDA), 11-mercaptoundecanol (MUD), ethanolamine hydrochloride, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC), Tween 20 and inorganic salts for buffer preparation (i.e. PBS (phosphate buffer saline) and MES (2-(*N*-morpholino)ethanesulfonic acid)) were purchased from Sigma-Aldrich (Spain). Organic solvents for gold surface cleaning (trichloroethylene, acetone and ethanol) and piranha solution components (H_2SO_4 and H_2O_2) were supplied by Panreac (Spain). Superblock® was provided by Pierce (IL, US).

Standard for Thiabendazole (TBZ) was purchased from Riedel-de Haën (Seelze, Germany). A 1 mM stock solution of TBZ was prepared in dry *N*,*N*-dimethylformamide (DMF) and stored at -20ºC. Working standards were freshly prepared from concentrated stock by sequential dilution in PBST(0.002) or PBST(0.05) buffer (PBS 10 mM Phosphate 135 mM NaCl with 0.002% or 0.05% of Tween 20, respectively).

Thiabendazole hapten TN3C (see Figure 1), bovine serum albumin (BSA) conjugate (TN3C-BSA) and monoclonal antibody (MAb) LIB-TN3C13 specific for TBZ were previously prepared by the Immunotechnology Group (Universitat Politècnica de València) as previously reported.[27] TN3C hapten introduces a spacer arm at the N1 position of the TBZ structure with a carboxylic group at its end, which is used to be covalently bound to BSA protein and obtain the coating surface antigen. Hapten density for the as-prepared TN3C-BSA (hapten-to-protein molar ratio) was found to be 19. A stock solution of 1 mg mL^{-1} in PBS was stored at -20ºC and working dilutions were freshly prepared when needed. Monoclonal antibody was purified by $(NH_4)_2SO_4$ immunoprecipitation. A stock solution of 1 mg mL^{-1} in PBS was prepared and stored at 4ºC. Working dilutions were also freshly prepared when needed.

SPR Biosensor

SPR measurements were performed with a homemade portable SPR biosensor device using gold surfaces (1 cm x 1 cm x 0.3 mm, with 2 nm Cr and 45 nm Au from Ssens bv, Hengelo, Netherlands). The sensor employs a Kretschmann configuration and incorporates two flow cells (300 nL each) for independent analysis. A polarized light of 670 nm is divided by a beamsplitter into two equal beams and directed onto the gold-coated sensing surface, enabling the measurement of two independent samples. Real-time monitoring is done by detecting changes in the intensity of the reflected light at a fixed angle of incidence, which is correlated to changes in mass on the surface due to binding events. The device incorporates all the optics, electronics and fluidics components necessary to operate autonomously. A continuous flow is constantly delivered to the sensor surface at a speed of 20 µl min^{-1} and samples (250 µL) can be simultaneously injected by means of two injection valves. For this immunoassay, analysis of a sample takes 15 min, and 25 min when considering also the bioactive surface regeneration.

Gold Surface Biofunctionalization

Gold surfaces were first cleaned following a standard protocol.[16] Briefly, chips were consecutively immersed in trichloroethylene, acetone, ethanol, water, piranha solution (H_2SO_4/H_2O_2, 3:1) and again in water (for each solvent, an incubation step for 1 min at 60ºC and a sonication step for 1 min more were done, except for piranha, which was 30s at room temperature with no sonication). Finally the chips were dried with N_2. A mixed self-assembled monolayer (SAM) was formed by incubating the chips overnight at room temperature with a solution of thiols MHDA:MUD (ratio 1:50, total concentration of thiols of 250 µM in ethanol). The chips were then thoroughly rinsed with ethanol, dried with N_2 and mounted in the device. The bioactive surface was prepared by covalently immobilizing the antigen conjugate TN3C-BSA onto the chip surface through the amino residues of the accessible lysines present in the BSA and the carboxylic groups of the surface (formation of an amide bond). The entire process was

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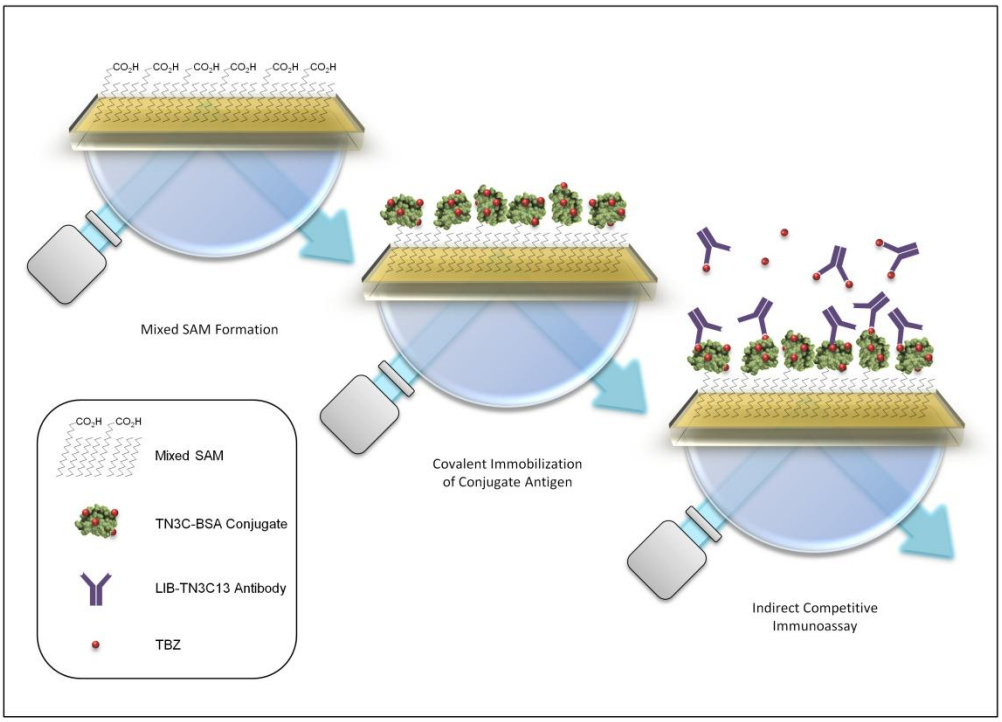

Fig.2 Scheme representing the three steps in the development of the indirect competitive immunoassay over the sensor surface: (a) formation of a mixed SAM; (b) biofuncionalization with the conjugate antigen covalently linked to the SAM; (c) indirect assay where a competition between conjugate, free analyte and free antibody takes place

performed in-flow. The carboxylic groups were first activated as carbodiimide esters by injecting a solution of EDC and NHS (EDC 0.2 M / NHS 0.05 M) in MES buffer (100 mM, 500 mM NaCl, pH= 5.5). Subsequently, a solution of TN3C-BSA (20 µg mL^{-1} in 10 mM PBS pH 7.5) was injected. The remaining unreacted activated groups were then blocked by delivering an aqueous solution of 1M ethanolamine.

Immunoassay format and optimization of protocol

The selected detection principle was based on an indirect competitive immunoassay. In this format, a fixed concentration of antibodies is incubated with samples containing different analyte concentrations which are then flowed over the antigen coated surface. To perform the inhibition assay, samples containing thiabendazole at different concentrations (from 500 nM to 0.5 nM) and specific antibody LIB-TN3C13 at a fixed concentration (1 µg mL^{-1}) were incubated for 20 min and then injected into the system. A calibration curve of the analyte (from 500 nM to 0.5 nM) was prepared from a stock concentrated solution of TBZ (1 mM in DMF) by serial dilution in the appropriate buffer, PBST(0.002) or PBST(0.05).

Experimental parameters (i.e. antigen concentration, antibody concentration, assay buffer and regeneration conditions) were studied by monitoring the response of the SPR sensor in order to obtain the optimal signal for antibody (in the absence of TBZ) as well as the best sensitivity (for the inhibition assays in the presence of TBZ). Reusability of surface was accomplished by disrupting antibody-hapten interaction using a solution of 100 mM NaOH. Selectivity of the assay was confirmed by measuring the signal of nonspecific antibodies on the immobilized TN3C-BSA and the signal of the LIB-TN3C13 antibody on nonspecific immobilized antigen. Sensitivity of the method can be extracted from a sigmoidal calibration curve, by representing the observed signal versus the logarithm of the analyte concentration.[32] The points of the resulting curve include solutions which only contain antibody and no analyte (zero concentration), solutions with analyte concentrations which saturate the antibodies and solutions with variable intermediate analyte concentrations. Sigmoidal curves were fitted to a four parameter equation according to the following formula:

$$Y = B + \frac{T - B}{1 + 10^{p(logIC50 - \log C)}}$$

where Y is the SPR signal (variation of reflectivity at a fixed angle, ΔRpp), T is the maximum signal (zero concentration of analyte), B is the asymptotic minimum (background signal) , $IC50$ is the concentration which produces 50% of maximum signal, C is the concentration , and p is the slope of the sigmoidal

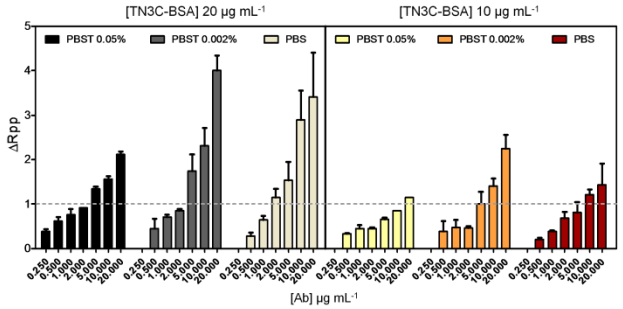

Fig. 3 Non-competitive indirect assays showing SPR response as a function of antigen concentration (10 and 20 µg mL-1) and antibody concentration (0.25, 0.5, 1, 2, 5, 10 and 20 µg mL-1) at three buffer conditions. Each point represents the mean ± SD of at least three measurements

curve at the inflection point. Representative standard inhibition curves were obtained by averaging at least three individual curves

Real sample treatment and evaluation

For analysis of real samples, whole oranges were cut, chopped, ground and stored as a homogenized mixture at -20ºC before analysis. Two blank samples (B1 and B2) with no TBZ and three positive samples with TBZ (M1, M2, and M3), were used for the study being all of them previously analyzed by an accredited laboratory using a reference HPLC-MS/MS. Extraction of TBZ was performed by sonicating the homogenate with MeOH for 1 h (4 mL g^{-1}). The solution was filtered through a 0.45 µm microporous syringe filter and then stored at 4ºC before use.

The methanolic solution from B1 and B2 was diluted in PBST(0.05) at different dilution factors to evaluate the matrix effect on the SPR assay. M1, M2 and M3 samples were diluted (from 1/40 to 1/250-fold times) in PBST(0.05). The concentration was determined by interpolating from the standard calibration curve fitting.

Results and Discussion

Optimization of SPR-based immunoassay

The choice of the best immunoassay configuration is highly dependent on the properties of the target and the purpose of the assay. Molecular weight is a key factor, as SPR sensing depends on the mass deposited on the transducer surface. For small molecules such as TBZ (MW = 201 Da), the direct approach, where antibody is immobilized on the surface and target molecules are flowed through the cell and directly detected, is less appropriate. For this reason, and also considering the final goal of analyzing real samples as oranges or similar fruits, with the inherent complexity of this processed food, an indirect approach is more suitable (see Figure 2) and was therefore selected. In this format, an antigen is covalently immobilized on the surface of the sensor and the antibody is injected into the device. The antigen consists of an analogous compound of the analyte conjugated to a carrier molecule, usually a protein. A fixed concentration of the specific antibody is incubated with the analyte at different concentrations. This way, the lower the analyte concentration, the higher the amount of free antibody available to interact with the antigen surface is and, therefore, the higher the sensor response. This strategy is often preferred since it is a more stable and robust approach. It allows higher number of measurements and regeneration cycles, contrary to the direct immobilization of the antibody, which may imply its modification and the loss of activity. Moreover, the aim of injecting samples coming from complex matrices might deteriorate the active surface faster, being more advisable to have the surface covered with the antigen rather than the antibody, which is essentially more sensitive to aggressive environments.

TN3C hapten conjugated to free Lys groups of BSA was used as antigen (TN3C-BSA conjugate). Hapten density of the conjugate was determined to be 19. Considering that BSA possesses around 30-35 accessible Lys in its structure (i.e., accessible -NH_2 groups, commonly used for covalent linkage), this density leaves enough free available groups to react with an appropriate functionalized surface. In particular a surface incorporating carboxylic groups was generated by creating a mixed SAM consisting of 16-mercaptohexadecanoic acid (MHDA) and 11-mercaptoundecanol (MUA). The free amino group in TN3C-BSA antigen reacted with the carboxylic groups establishing an amide bond by means of the carbodiimide linkage.

Several immunoassay conditions were evaluated in order to obtain a reproducible biofunctionalized surface which at the same time yielded competitive immunoassays with high sensitivity. The most relevant factors which can affect the final sensitivity are usually the concentration of antigen, the concentration of antibody and the immunoassay buffer. Two concentrations of TN3C-BSA (10 and 20 µg mL^{-1}) were studied. For each case, a set of different antibody concentrations were injected (0.25 to 20 µg mL^{-1}) in three different incubation buffers, PBS, PBST(0.002) or PBST(0.05) (see Figure 3). As expected, results showed an overall higher response against same antibody concentration for those surfaces with higher amount of antigen immobilized on the surface. However, the influence of Tween was less obvious. Usually, when this detergent is added at low concentrations, it prevents nonspecific interactions and also improves the reproducibility by reducing the dispersion among replicates. This can be observed in Figure 3 where the standard deviation is significantly higher for PBS. When comparing the use of 0.002% and 0.05% of Tween, an overall decrease in the signal for larger Tween percentages could be observed, although the reproducibility was better. Thus, initially both conditions were selected to perform calibration curves. An antibody concentration of 1 µg mL^{-1} was selected, which guaranteed a sufficient maximum signal (i.e. variation in reflectivity of ΔRpp~1) under non-saturation conditions of immunoreagents. Higher concentrations of antibody, despite rendering higher response, usually tend to diminish the overall sensitivity of the assay[33]: a less amount of antibody usually permits a more efficient competition between the free TBZ and the immobilized antigen for the binding to the antibody, thereby improving the overall sensitivity.

Regeneration of the bioactive surface was also studied. Acid solutions (HCl at different concentrations and Glycine 100 mM pH 3) and basic solutions (NaOH at different concentrations) were tested. A NaOH 100 mM solution injected for 60 s turned out to be the most effective regeneration solution, completely

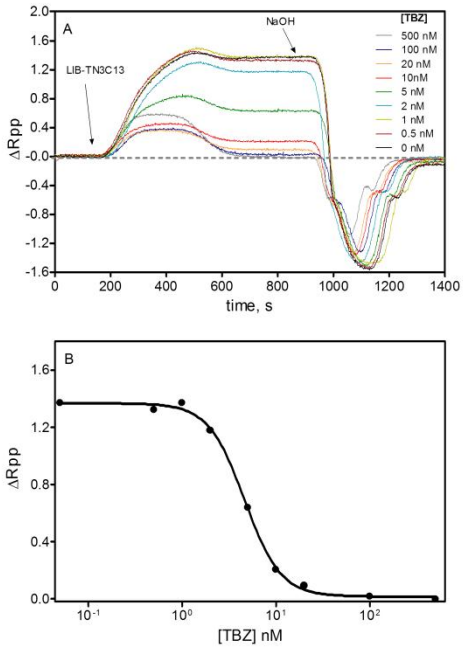

Fig. 4 Real time competitive immunoassay for TBZ. (A) Sensograms indicating the interaction of free unbound antibody at different TBZ concentrations and the subsequent regeneration step. (B) Inhibition calibration curve obtained from the stabilized signal obtained for each TBZ concentration

removing the bound antibody. These conditions ensured the reusability of the surface for more than 100 cycles without significantly decreasing the maximum response, therefore maintaining binding activity (data not shown). The stability in the maximum signal (i.e. less than a 14% of CV of the maximum signal for antibody in the absence of TBZ considering around 100 cycles) confirmed the non-apparent damage of the immobilized antigen or the surface itself. Overall, considering the flow rate and including both the interaction and the regeneration steps, a whole cycle could be completed in less than 25 minutes. Figure 4 shows representative real time sensograms including the interaction and the regeneration step, for different TBZ concentrations, and the corresponding inhibition curve generated from a set of 9 different TBZ concentrations. Another pesticide, carbaryl, was evaluated under the optimized immunoassay conditions at high concentration (i.e. 500 nM) and no inhibition was observed. Also when non-specific antibodies were tested with the antigen-coated surface or specific antibodies with a different antigen coating the surface, no signal was observed in either case, (ΔRpp=0). These experiments were indicative of high specificity of the assay.

In summary experimental parameters for the optimized assay in buffer conditions were: 20 µg mL^{-1} of antigenTN3C-BSA, 1 µg mL^{-1} of monoclonal antibodyLIB-TN3C13, a preincubation time of 20 min and a range of analyte concentration from 500 to 0.5 nM (100 ng mL^{-1} to 0.1 ng mL^{-1}). As can be seen in Figure 5 almost identical calibration curves were obtained regardless the percentage of Tween in the assay buffer. The main features of both assays are summarized in Table 1. The achieved sensitivity was comparable to the one obtained by conventional ELISA using the same immunoreagents,[27] with an IC_{50} of 0.13 µg L^{-1} (0.2 µg L^{-1} in the case of the ELISA). A significantly narrower

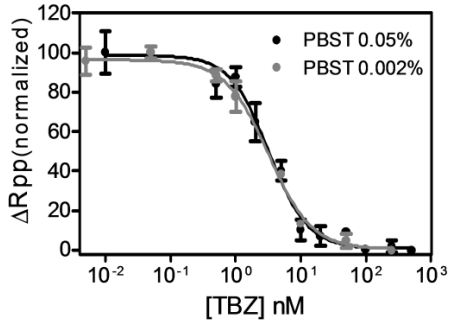

Fig. 5 Standard calibration curves of TBZ under two different buffer conditions. Each point represents the mean ± SD of at least three measurements and they are fitted to a four-parameter logistic equation

dynamic range and higher LOD was obtained with the SPR resulting from a slightly higher slope in the current fitting compared with the ELISA (1.46 vs 1.09). Despite these small differences, the sensitivity limits are far below the established MRLs for TBZ (between 50 µg L^{-1} and 15 mgL^{-1}) and also for those products with no assigned MRLs (<10 µg L^{-1}).[34]

Evaluation of SPR performance with orange samples and real sample analysis

Thiabendazole's wide use has entailed its constant detection in many fruits and vegetables. In the case of citrus fruits, the fungicide is found at high concentrations in the peel, which is then absorbed and transferred to the inner parts. In order to assess and monitor the concentrations of TBZ in postharvests treatments, the analysis of the whole fruit including the peel is more accurate compared to the case where only the pulp or the extracted juice is analyzed. Orange peel has an essentially hydrophobic nature. This increases the complexity of the analysis, since a homogenate has to be obtained from the whole fruit, requiring an extraction step typically carried out with organic solvents. For chromatographic analyses, this must be followed by a cleanup and concentration of the sample. These latter steps can be avoided with immunochemical techniques, by applying a simple dilution factor with assay buffer. Nevertheless, this is highly dependent on the sensitivity and other factors of the assay, such as robustness and stability, and also on the nature of the sample (i.e. potential interferences originated in the extraction).

For this reason it was necessary to determine the influence in the SPR-based immunoassay of the so-called matrix effect coming from substances found in the oranges before analyzing real samples. Accordingly, oranges collected from local markets in the Valencia metropolitan area were processed to finally obtain a homogenate. A parallel process of extraction and cleanup based on a dispersive solid-phase QuEChERS extraction was followed prior to the determination of the levels of TBZ by standardized HPLC-MS/MS multiresidue accredited method.[35, 36] Those oranges which tested negative to TBZ (B1 and B2) were further used to establish the matrix effect on the SPR-immunoassay. Sample preparation for immunoassays is usually simpler than for chromatographic methods. In many occasions an extraction and/or dilution of the sample with assay media may be enough to minimize any significant effect. As previously described, in the

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Table 1 Influence of Tween 20 on the features of the immunoassay

	LOD (IC_{90})	IC_{50}	Working Range (IC_{80}-IC_{20})
PBST 0.002%	0.61 nM (0.13 µg L^{-1})	3.2 nM (0.64 µg L^{-1})	1.13-9.35 nM (0.23-1.88 µg L^{-1})
PBST 0.05	0.67 nM (0.13 µg L^{-1})	3.2 nM (0.64 µg L^{-1})	1.21-8.5 nM (0.24-1.7 µg L^{-1})
ELISA[27]	0.1 nM (0.02 µg L^{-1})	1 nM (0.2 µg L^{-1})	0.25-4.5 nM (0.05-0.9 µg L^{-1})

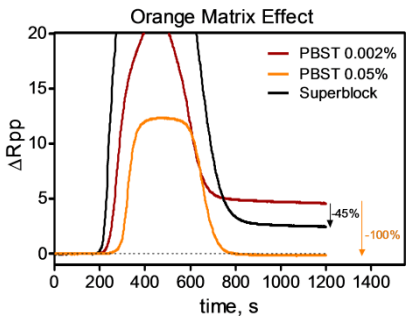

Fig. 6 SPR sensograms showing the signal resulting from orange extracts diluted 1/20 fold in different buffers. Reduction of non-specific signal from the matrix is achieved with PBST 0.05% as dilution buffer

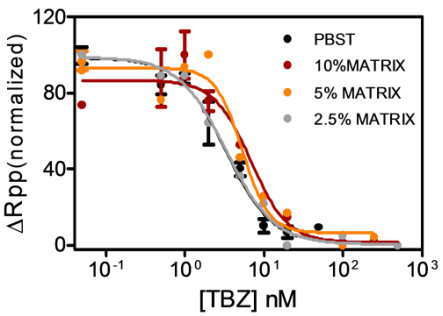

Fig. 7 Influence of the amount of orange methanolic extract on the calibration curve. A 2.5% of matrix (1/40 fold dilution in PBST 0.05%) ensures the elimination of any effect coming from the matrix altering the assay. Each point represents the mean ± SD of at least three replicates and they are fitted to a four-parameter logistic equation

Table 2 Influence of orange matrix on the features of the immunoassay

	LOD (IC_{90})	IC_{50}	Working Range (IC_{80}-IC_{20})
PBST 0.05% Tween	0.67 nM (0.13 µg L^{-1})	3.20 nM (0.64 µg L^{-1})	1.21-8.51 nM (0.24-1.71 µg L^{-1})
+ 10% Matrix	0.58 nM (0.11 µg L^{-1})	6.85 nM (1.33 µg L^{-1})	1.85-13.61 nM (0.35-2.64 µg L^{-1})
+ 5% Matrix	1.51 nM (0.29 µg L^{-1})	5.30 nM (1.02 µg L^{-1})	2.73-10.27 nM (0.53-1.99 µg L^{-1})
+ 2.5% Matrix	0.68 nM (0.13 µg L^{-1})	3.33 nM (0.65 µg L^{-1})	1.23-8.75 nM (0.24-1.69 µg L^{-1})

case of TBZ analysis from several fruit's peel,[6] a simple extraction with MeOH was selected for the analysis of samples using conventional immunoassays. MeOH was the optimal choice given its organic nature and its miscibility with H_2O; in addition it may have a minimal influence on the antibody-antigen interaction if the dilution is appropriate. As a comparative, a 5-fold dilution was initially selected (assuming therefore a high concentration of methanolic matrix of 20%) which, considering the range of TBZ concentration usually found in citrus and the sensitivity of the assay, would be far from the required dilution to fit the dynamic range of the SPR-based immunoassay. The methanolic solution was diluted in three different buffers (PBST(0.002), PBST(0.05) and Superblock®) and directly injected in the flow cell to observe the behavior of the components present in the matrix. No antibody was added in order to evaluate the signal coming exclusively from the solution. As can be seen in Figure 6, interference from the matrix remained bound to the surface, rendering positive signals for both PBST(0.002) and Superblock®. From these results the amount of Tween in the dilution buffer seemed to be especially important in order to remove the interference signal coming from substances extracted during the sonication process. While 0.002% of Tween led to a high non-specific adsorption (with a ΔRpp of 5, which is considerably higher than the signal resultant of injecting the specific antibody in buffer media, therefore producing a complete mask of specific signal) this could be reduced by 45% using Superblock® instead of PBST. This buffer is widely used for its effectiveness to reduce non-specific adsorptions, but also in this case, it was unsuccessful. However, PBST(0.05) ensured a completely minimization of adsorption to the surface. In light of these results, PBST(0.05) was finally selected to go further with the experiments for the detection of TBZ in oranges.

Besides the adsorption of components of the matrix on the bioactive surface, which can lead to false positives in label-free mass-depending sensing techniques, the matrix can also induce some degree of interference on the antigen-antibody interaction,

Table 3 Results from determination of TBZ in whole orange samples by SPR and HPLC[a]

Sample	HPLC, μg L^{-1}	SPR immunosensor, μg L^{-1}	Recovery %
1	371	391.2±52.7	105.2
2	261	288.6±49.1	110.9
3	337	354.1±68.8	105.1

[a] Each value represents the mean ± SD of at least five measurements.

which, eventually, will lead to an alteration of the final sensitivity. In order to study this, standard curves prepared in PBST(0.05) with variable ratios of orange extract were injected and evaluated by SPR. As shown in Figure 7 and Table 2, a significant effect on the sensitivity could be observed when a higher percentage of matrix was added to the buffer (i.e. 10% and 5%). When the percentage was 2.5% both standard and matrix calibration curve showed similar IC_{50} and LOD. Thus, extracts from orange samples should be diluted at least 40-fold prior to accurately analyze its TBZ content by the SPR immunoassay.

Following these findings three real samples were analyzed, M1, M2 and M3, whose TBZ concentration was previously determined by HPLC. Several dilutions of each methanolic extract were prepared with the assay buffer applying a single-step dilution factor and then analyzed by SPR.

Only those diluted samples that could be measured within the dynamic range of the assay were considered. Samples were measured at least five times in three different days, and an average recovery slightly above 100% was obtained in all cases (see Table 3). Despite this slight overestimation, which is found within the range accepted in an analytical methodology, the data indicate a good correlation with HPLC confirming the feasibility of the developed label-free assay to analyze TBZ pesticide in whole fruits.

Conclusions

A rapid and highly sensitive SPR-based immunoassay for the detection of TBZ in oranges has been developed. An indirect competitive format with the antigen immobilized on the surface has been selected. Several parameters have been optimized in order to obtain a reproducible and stable assay, with a LOD of 0.16 μg L^{-1}, an IC_{50} of 0.64 μg L^{-1} and a dynamic range between 0.24-1.7 μg L^{-1}. The achieved sensitivity comfortably allows detection of the pesticide well below the established MRL values for all the listed fruits. A complete assay cycle, including regeneration, is accomplished in 25 min. The bioactive surface with the immobilized antigen has proven to be reusable and remains homogenous upon regeneration for more than 100 cycles on average, avoiding potential denaturation and loss of recognition by specific antibodies even under harsher conditions derived from the analysis of complex real samples. Evaluation performed with real orange samples indicates that MeOH extraction and further dilution seem to be an adequate sample pretreatment to analyze TBZ in the whole fruit (with recoveries around 100%). Potential matrix effects from oranges are removed by carefully selecting the most appropriate assay buffer. The described methodology allows an overall fast and selective detection of TBZ in real citrus samples and is in good agreement with conventional chromatographic methods. The robustness, reproducibility and sensitivity of the assay, together with its capability of analyzing complex samples such as whole oranges (including peel) profiles this label-free sensing platform as an attractive alternative to more time-consuming chromatographic techniques, both as a quantitative or fast screening method to be implemented in surveillance programs.

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1. "Factsheet: New Rules on pesticide residues in food", 2008, **European Comission, September 2008**.
2. EU pesticides database. Available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm. Last visited, January 2012.
3. H. C. Liang and M. T. Hay, Water Environ. Res., 2011, **83**, 956-982.
4. C. M. Torres, Y. Picó and J. Mañes, J. Chromatogr. A, 1996, **754**, 301-331.
5. V. S. Morozova, A. I. Levashova and S. A. Eremin, J. Anal. Chem., 2005, **60**, 202-217.
6. A. Dankwardt, in Encyclopedia of Analytical Chemistry, John Wiley & Sons, Ltd, 2006.
7. S. Rodriguez-Mozaz, M. Lopez de Alda and D. Barceló, Anal. Bioanal. Chem., 2006, **386**, 1025-1041.
8. C. R. Suri, R. Manoj and V. Grish, Crit. Rev. Biotechnol., 2002, **22**, 15-32.
9. X. Jiang, D. Li, X. Xu, Y. Ying, Y. Li, Z. Ye and J. Wang, Biosens. Bioelectron., 2008, **23**, 1577-1587.
10. K. V. Gobi, H. Tanaka, Y. Shoyama and N. Miura, Sens. Act. B, 2005, **111–112**, 562-571.
11. E. Mauriz, A. Calle, J. J. Manclus, A. Montoya, A. M. Escuela, J. R. Sendra and L. M. Lechuga, Sens. Act. B, 2006, **118**, 399-407.
12. M.-F. Gouzy, M. Keß and P. M. Krämer, Biosens. Bioelectron., 2009, **24**, 1563-1568.
13. S. J. Kim, K. V. Gobi, H. Tanaka, Y. Shoyama and N. Miura, Sens. Act. B, 2008, **130**, 281-289.
14. M. Farre, E. Martinez, J. Ramon, A. Navarro, J. Radjenovic, E. Mauriz, L. Lechuga, M. P. Marco and D. Barcelo, Anal. Bioanal. Chem., 2007, **388**, 207-214.
15. E. Mauriz, A. Calle, A. Abad, A. Montoya, A. Hildebrandt, D. Barcelo and L. M. Lechuga, Biosens. Bioelectron., 2006, **21**, 2129-2136.
16. E. Mauriz, A. Calle, L. M. Lechuga, J. Quintana, A. Montoya and J. J. Manclús, Anal. Chim. Acta, 2006, **561**, 40-47.
17. M. Petz, Monatshefte Fur Chemie, 2009, **140**, 953-964.
18. M. Fernandez, Y. Pico and J. Manes, Chromatographia, 2001, **54**, 302-308.
19. M. Fernandez, Y. Pico and J. Manes, Food Add. Contam., 2001, **18**, 615-624.

20. M. Fernandez, R. Rodriguez, Y. Pico and J. Manes, J. Chromatogr. A, 2001, **912**, 301-310.
21. S. J. Tuan, H. M. Tsai, S. M. Hsu and H. P. Li, J. Food Drug Anal., 2009, **17**, 163-177.
22. D. T. Eash and R. J. Bushway, J Liq Chromatogr R T, 2000, **23**, 261-272.
23. R. Rodriguez, Y. Pico, G. Font and J. Manes, J. Chromatogr. A, 2001, **924**, 387-396.
24. F. Barahona, E. Turiel and A. Martin-Esteban, Anal. Chim. Acta, 2011, **694**, 83-89.
25. S. J. Lehotay, K. A. Son, H. Kwon, U. Koesukwiwat, W. S. Fu, K. Mastovska, E. Hoh and N. Leepipatpiboon, J. Chromatogr. A, 2010, **1217**, 2548-2560.
26. D. L. Brandon, R. G. Binder, A. H. Bates and W. C. Montague, J. Agr. Food Chem., 1992, **40**, 1722-1726.
27. A. Abad, J. J. Manclus, M. J. Moreno and A. Montoya, J. AOAC Int., 2001, **84**, 156-161.
28. D. L. Brandon, R. G. Binder, R. E. Wilson and W. C. Montague, J. Agr. Food Chem., 1993, **41**, 996-999.
29. R. J. Bushway, D. L. Brandon, A. H. Bates, L. Li, K. A. Larkin and B. S. Young, J. Agr. Food Chem., 1995, **43**, 1407-1412.
30. R. J. Bushway, K. Larkin and B. Perkins, Food. Agr. Immunol., 1997, **9**, 249-255.
31. M. Blazkova, P. Rauch and L. Fukal, Biosens. Bioelectron., 2010, **25**, 2122-2128.
32. M.-C. Hennion, Analusis, 1998, **26**, 149-155.
33. M.-P. Marco, S. Gee and B. D. Hammock, TrAC, Trends Anal. Chem., 1995, **14**, 415-425.
34. "Regulation (EC) No. 396/2005 of the European parliament and of the council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC". Official Journal of the European Union, 2005.
35. S. J. Lehotay, A. de Kok, M. Hiemstra and P. van Bodegraven, J. AOAC Int., 2005, **88**, 595-614.
36. M. Anastassiades, S. J. Lehotay, D. Stajnbaher and F. J. Schenck, J. AOAC Int., 2003, **86**, 412-431.