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Development of immunoaffinity columns for pyraclostrobin extraction from fruit juices and analysis by liquid chromatography with UV detection

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ABSTRACT

Pyraclostrobin belongs to a new generation of fungicides widely used to preserve high valuable crops. In the present study, three monoclonal antibodies with different affinities to this modern strobilurin have been evaluated for their usefulness in the production of immunoaffinity columns suitable for the solid-phase extraction, concentration, and clean-up of residues from food commodities. Different immunosorbents were produced and characterized in terms of antibody immobilization efficiency, immunosorbent binding capacity, optimum elution conditions, and reusability. Covalent coupling of the antibodies to Sepharose–CNBr gel took place with high yield (over 90%), whereas the immunosorbent efficacy to retain the analyte (from 28 to 68%) was shown to depend on the amount and type of antibody immobilized on the support. As a matter of fact, columns prepared with the monoclonal antibody PYs5#14 were able to selectively bound up to 53 μg of pyraclostrobin per gram of beads. Acetonitrile solutions were preferred over methanolic ones for analyte elution, and some immunosorbents could be reused at least 4–6 times provided that the amount of pyraclostrobin and the volume of sample did not overload the column. Effectiveness of the selected immunoaffinity column was evidenced by the development of an extraction procedure for pyraclostrobin residues from fruit juices and further determination by high-performance liquid chromatography with UV detection. A concentration factor of 50 times was achieved with the developed immunoaffinity column, which eventually resulted in a limit of quantification of 0.01 mg L^{-1}. Finally, quantitative recoveries were obtained on apple juice and red grape must samples spiked with pyraclostrobin from 0.01 to 1 mg L^{-1}.

1. Introduction

Pyraclostrobin (methyl 2-[1-(4-chlorophenyl)pyrazol-3-yloxymethyl]-*N*-methoxycarbanilate; PY) (Fig. 1) is a fungicide of the strobilurin family that shows a novel mode of action based on the inhibition of mitochondrial respiration by binding at the Qo site of cytochrome b [1,2]. With estimated global annual sales at $950 million, PY is currently one of the most relevant agrochemicals commercialized by BASF. Alone or in combination with other fungicides, mainly boscalid and different conazoles, PY is able to efficiently combat fungal diseases affecting high-value crops, including grapes, strawberries, peppers, tomatoes, and cereals [3]. In a recent report published by the European Food Safety Authority (EFSA), residues of PY were detected in 3.8% of the fruit and vegetable surveillance samples analyzed in the 27 EU Member States, Norway and Iceland [4]. The European Community Regulation no. 750/2010 establishes maximum residue levels (MRLs) for PY in a wide range of food commodities, ranging from 0.02 mg kg^{-1} (lower limit of analytical determination) to 10 mg kg^{-1} (lamb's lettuce) [5].

Analytical methodologies employed for the determination of PY residues usually involve separation techniques such as HPLC and GC, preferably coupled to MS detectors to increase both selectivity and sensitivity. Table 1 summarizes the procedures found in the scientific literature dealing with the determination of PY residues in fruits and vegetables. Basically, all of them are based on an extraction with organic solvents, like ethyl acetate–hexane [6], acetone [7], or ethyl acetate [8,9], and a direct chromatographic measurement. Solid-phase extraction (SPE) for sample clean-up with standard solid supports such as octadecyl-silica (C_{18}), primary secondary amine (PSA), or graphitized carbon black (GCB) has been shown to improve method sensitivity [10–13]. Ultrasonic extraction (USE) coupled to a clean-up step based on gel permeation chromatography (GPC) [14] or to stir bar sorptive extraction (SBSE) [15], has also been employed for the analysis of PY residues in food. Recently, solid-phase microextraction (SPME) has been used for

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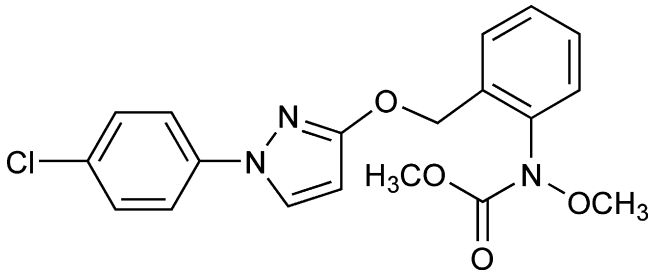

Fig. 1. Molecular structure of pyraclostrobin.

the analysis of PY traces using polydimethylsiloxane, divinylbenzene, polyacrylate, or carboxen fibers, with excellent sensitivity [16,17].

Nowadays, the development of new SPE sorbents and their application to the extraction of the target analyte from food commodities or to the clean-up of extracts prior to the chromatographic determination of pesticide residues is a subject of great interest. Over the last years, new materials have been studied to replace the standard supports, such as molecular imprinted polymers [18], polymer composites [19], carbon nanotubes [20], carbon nanoparticles [21], or immunoaffinity supports [22,23]. Immunoaffinity chromatography (IAC) columns take advantage of the extreme affinity and specificity usually displayed by antibodies to provide a sort of SPE method that enables the selective extraction of the target analyte even from complex matrices. Several strategies and supports have been considered for the in-house production of IAC columns, yet Sepharose gels, activated with cyanogen bromide (CNBr) or *N*-hydroxysuccinimide, dominate the field because of their performance, availability, and simplicity of the procedure for covalent coupling of the selected antibody to the gel. Unlike mycotoxins, vitamins, and veterinary drugs [23], to the best of our knowledge there are not commercially available IAC columns for pesticides, although examples have been published for carbofuran [24], phenylurea herbicides [25], triazines [26], thifluzamide [27], imazalil [28], and bioallethrin [29], to name a few.

Due to the large difference in molecular weight between pesticides and antibodies, common IAC columns display binding capacities for the target in the nanogram to microgram range, depending on bed size and on the origin, purity, and affinity of the immobilized antibody. As previously reported, an optimum antibody for the production of IAC columns should display a moderate affinity to its antigen – high enough to retain the analyte, but at the same time, low enough to easily elute it under mild conditions [30,31]. The aim of this study was the evaluation of several monoclonal antibodies (mAbs) showing different affinities to PY for the production of IAC columns valuable for efficiently and selectively entrap the analyte from relevant matrices. Remarkably, the immunosorbents herein described are the first ever reported for this relevant agrochemical. Following complete characterization of a number of supports prepared under different conditions, the most efficient column was combined with HPLC–UV for the determination of PY in fortified apple juice and grape must.

2. Experimental

2.1. Reagents and instrumentation

Anti-PY mAbs used in the preparation of IAC columns were produced in our laboratory following well-established procedures for hybridoma generation [32]. Immunizations were carried out with BSA conjugates of a hapten that was functionalized at the chlorophenyl ring of the target fungicide. Particular details will be published elsewhere. Following ammonium sulfate precipitation, mAbs were purified with HiTrap protein G affinity columns from GE Healthcare Biosciences (Uppsala, Sweden). Amicon Ultra 30K NMWL centrifugal filter devices obtained from Millipore (Billerica, MA, USA) were employed in the dialysis and concentration of antibody solutions. CNBr-activated Sepharose 4B (GE Healthcare Biosciences) was employed as solid support. Polypropylene SPE tubes of 3 mL volume with polyethylene frits (20 μm porosity) and caps were obtained from Scharlab (Barcelona, Spain). Bond Elut C_{18} SPE cartridges (500 mg, 3 mL) were obtained from Varian (Lake Forest, CA, USA). QuEChERS method was carried out using DisQuE dispersive sample preparation form Waters (Milford, MA, USA).

Chromatographic determinations were performed with a Hitachi HPLC (Schaumburg, IL, USA) equipped with a L-2130 pump, a L-4500 diode array detector, and a 7125 Rheodyne injector (Cotati, CA, USA) fitted with a 50 μL sample loop. The separation was carried out in a LiChrospher 100 RP-18 analytical column (250 mm × 4.0 mm, 5.0 μm) from Merck (Darmstadt, Germany).

Enzyme-linked immunosorbent assay (ELISA) determinations of PY were performed with Costar flat-bottom high-binding polystyrene ELISA plates from Corning (Corning, NY, USA). Plates were washed with an ELx405 microplate washer and the absorbance was read with a PowerWave HT, both from BioTek Instruments (Winooski, VT, USA). PY, azoxystrobin and boscalid pure standards, as well as other fungicides used in cross-reactivity studies, were purchased from Fluka (Seelze, Germany), and stock solutions were prepared in *N*,*N*-dimethylformamide (DMF) and

Table 1
Published procedures for the determination of pyraclostrobin in food commodities.

Sample	Extraction	Clean-up	Determination	Recovery (%)	LOQ (ppb)	Ref.
Grape, wine	Ethyl acetate–hexane	–	HPLC–UV	95–104	200	[6]
Lettuce, orange, apple, cabbage, grape, wheat flour	Acetone	LLE (dichloromethane-light petroleum)	HPLC–MS–MS	93–96	10	[7]
Wheat	Ethyl acetate	–	HPLC–MS–MS	88–96	1–4	[8]
Strawberry	Ethyl acetate	–	HPLC–MS–MS	92	20	[9]
Grape, wine	Ethyl acetate–hexane	SPE (PSA-GCB)	GC–MS–MS	59–147	5–7	[10]
Honeybees	Acetonitrile–water	SPE (PSA-C_{18}-GCB)	GC–MS–MS	–	–	[11]
Wine	Acetonitrile–NaCl–$MgSO_4$	SPE (PSA-GCB)	HPLC–MS–MS	70–120	0.1–10	[12]
Grapes	MSPD (C_{18}-silica)	–	GC–MS	88–95	9	[13]
Fruit, vegetable, beverage, cereal, nuts, meat, egg, milk	USE (ethyl acetate–cyclohexane)	GPC	GC–MS	60–120	2–15	[14]
Pear, lemon, grape	USE (ethanol)	SBSE	HPLC–UV	92–100	25	[15]
Baby food	SPME	–	GC–MS	106	0.4	[16]
Mango	SPME	–	GC–MS	74–116	17	[17]

C_{18}, octadecyl silica; GC, gas chromatography; GCB, graphitized carbon black; HPLC, high-performance liquid chromatography; LLE, liquid–liquid extraction; LOQ, limit of quantification; MS, mass-spectrometry; MS–MS, tandem mass spectrometry; MSPD, matrix solid-phase dispersion; SBSE, stir bar sorptive extraction; SPE, solid-phase extraction; SPME, solid-phase microextraction; PSA, primary secondary amine; USE, ultrasonic extraction; UV, ultraviolet detection.

kept at −20 °C in amber glass vials. Methanol and acetonitrile HPLC grade were obtained from Scharlab.

2.2. Competitive ELISA determination of pyraclostrobin

An ELISA methodology was employed for the quantification of PY during the characterization of IAC columns [32]. Ninety-six-well polystyrene ELISA plates were coated with 100 μL of a 1 μg mL^{-1} mAb PYo5#31 solution in 50 mM carbonate–bicarbonate buffer (pH 9.6) and plates were incubated overnight at room temperature. Coated plates were washed four times with 150 mM NaCl containing 0.05% (v/v) Tween 20 and received, afterwards, 50 μL per well of standard or sample in water plus 50 μL per well of a 20 ng mL^{-1} HRP–PYo5 tracer solution in 20 mM sodium phosphate buffer (pH 7.4) with 280 mM NaCl and 0.05% (v/v) Tween 20. The immunological reaction took place during 1 h at room temperature, and plates were washed again as described. Finally, retained peroxidase activity was determined by addition of 100 μL per well of freshly prepared 2 mg mL^{-1} *o*-phenylenediamine (Sigma, Madrid, Spain) and 0.012% (v/v) H_2O_2 in 25 mM citrate, 62 mM sodium phosphate buffer (pH 5.4). The enzymatic reaction was stopped after 10 min at room temperature by the addition of 100 μL per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Standard curves (0.02–1000 nM) were prepared in deionized water from a 1000 μM PY stock solution in DMF. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Antibody affinity was calculated as the concentration of analyte at the inflection point of the fitted competitive curve, typically corresponding to a 50% reduction (IC_{50}) of the maximum absorbance (A_{max}) if the background signal approaches to zero.

2.3. Production of IACs

Affinity-purified mAbs were long-term stored as ammonium sulfate precipitates, so a previous dialysis step with Amicon 30 K centrifugal filters was performed. Antibodies were dissolved in coupling buffer (100 mM sodium bicarbonate, pH 8.4, with 0.5 M NaCl) at a concentration of 10 g L^{-1} and stored at 4 °C for short-term storage.

Antibodies were covalently bound to the Sepharose gel essentially following the procedure recommended by the supplier. Thus, 0.15 g of CNBr-activated Sepharose beads, corresponding to an approximate 0.5 mL of swollen gel, were weighted inside a prefritted 3 mL polypropylene tube and suspended in HCl 1 mM. The gel was washed with 30 mL HCl 1 mM, and then with 5 mL coupling buffer. The support was mixed with a particular amount of antibody dissolved in 1 mL coupling buffer, and gently stirred for 2 h at room temperature. The antibody–Sepharose gel was washed again with 5 mL coupling buffer, and the coupling efficiency of the antibody to the column was estimated by UV absorption at 280 nm as the difference between the total amount of mAb initially loaded and the amount of mAb found in the eluted and washing solutions [30]. Then, the gel was suspended in 2 mL Tris–HCl buffer (0.1 M, pH 8.0) and gently stirred for 2 h at room temperature in order to block the unreacted CNBr-activated sites. After that, the gel was washed with 3 cycles of 5 mL acetate buffer (0.1 M, pH 4.0, 0.5 M NaCl) and 5 mL Tris–HCl buffer (0.1 M, pH 8.0, 0.5 M NaCl). Then, a polyethylene frit was placed on the top of the gel and the column was stored at 4 °C in PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.4). In the case of long-term storage, 0.01% (w/v) sodium azide was added to the columns as bacteriostatic agent. Control columns were also produced using the same procedure without antibody and with a mAb that was specific for an unrelated target [35].

2.4. Characterization of immunoaffinity columns

2.4.1. Elution of pyraclostrobin

Columns containing 16 mg of antibody per gram of Sepharose beads were employed in order to establish the optimal conditions for the quantitative elution of the analyte. After loading with 1 mL of a 1 μg mL^{-1} PY solution in PBS, columns were eluted with five 1-mL fractions of deionized water, methanol (1, 10, 25, 50 and 75%, v/v, in water), or acetonitrile (1, 10, 25, 50 and 75%, v/v, in water). Solvent fractions were separately collected in different tubes and they were measured by competitive ELISA. This study was carried out twice.

2.4.2. Immunosorbent capacity

For every mAb, IAC columns were prepared at four different antibody–Sepharose ratios: 4, 8, 16 and 25 mg g^{-1}. Supports were preconditioned with 5 mL PBS at an approximate flow of 0.5 mL min^{-1} (gravity flow). The maximum amount of analyte retained by the immobilized antibodies was determined as follows. Twenty millilitres of 0.5 mg L^{-1} PY standard in PBS were passed through every column (a total PY amount of 10 μg). Gels were washed with 5 mL of 10% (v/v) acetonitrile in water and the elution of the retained PY was carried out with 5 mL of 75% (v/v) acetonitrile in water. Quantification of PY in the elution volume was performed by competitive ELISA. Two replicates were carried out in this study.

2.4.3. Reusability

The capacity of the immunosorbents to withstand repeated uses was evaluated with columns containing 16 mg g^{-1} of mAb. Analyte recoveries were determined after loading the columns with 2 mL of a solution containing 100 μg L^{-1} of PY (0.2 μg), then washing with 10% acetonitrile (5 mL), and finally eluting with 75% acetonitrile (2 mL). Gels were regenerated with 5 mL PBS and re-used for a new cycle. The process was repeated 12 times, and after each cycle the concentration of PY in the loading, washing, and eluted fractions was measured by competitive ELISA.

2.5. HPLC–UV determination of pyraclostrobin

IAC columns were acclimatized at room temperature and preconditioned with 5 mL PBS. Commercial grape and apple juices were spiked with PY, directly percolated through the bed, and eluted by gravity. In the case of high-pulp juices, a previous filtering step should be required. A washing step with 5 mL of 10% acetonitrile was performed to eliminate matrix compounds, and PY was eluted with 2 mL of 75% acetonitrile. If columns were expected to be used again, they were washed with PBS (5 mL) and stored at 4 °C.

The mobile phase for HPLC separation was 80% (v/v) acetonitrile in water at a flow rate of 1.0 mL min^{-1}, and the sample injection volume was 50 μL. UV spectrometric detection was performed from 200 to 300 nm, with a spectral bandwidth of 4 nm and a spectral interval of 400 ms. The D-7000 HPLC System Manager Software from Hitachi was employed to generate the mean chromatogram at 276 nm and to calculate the corresponding peak area. Retention times of 3.1, 3.5 and 4.8 min were obtained for azoxystrobin, boscalid and PY, respectively. Recovery studies were done in triplicate.

2.6. Reference extraction methods

A SPE procedure using C_{18} cartridges and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) [33] were employed to compare the performance of the proposed IAC columns. C_{18} cartridges were pre-conditioned with 2 mL acetonitrile and 2 mL deionized water at gravity flow. Then, 2 mL of spiked sample was loaded on the cartridge and matrix compounds were removed with

two additional millilitres of deionized water. Pyraclostrobin was eluted from the column using 2 mL acetonitrile and analyzed by HPLC–UV as explained in Section 2.5.

For sample extraction by the QuEChERS procedure, 15 mL of fortified fruit juice was introduced in a 50 mL polypropylene centrifuge tube with 1.5 g of sodium acetate, 6 g of magnesium sulfate and 15 mL of 1% acetic acid in acetonitrile. Tube was agitated using a vortex shaker and then centrifuged at 3500 rpm for 5 min. One millilitre of the organic fraction was cleaned-up with 50 mg PSA and 150 g anhydrous magnesium sulfate, and it was 0.2 μm filtered before HPLC–UV analysis.

3. Results and discussion

3.1. Antibody selection

This study capitalizes on the availability of a collection of anti-PY mAbs generated in our laboratory following standard hybridoma technology (unpublished results). A set of ten antibodies were characterized in terms of affinity to PY and avidity to the enzyme tracer. From this study, three mAbs (PYs5#14, PYs5#21 and PYs5#33) were selected for the preparation of IAC columns. These antibodies were chosen because they greatly differed in their binding properties (Table 2). Thus, mAb PYs5#14 exhibited a 30-fold higher affinity to PY than the two other mAbs (PYs5#21 and PYs5#33). In addition, mAb PYs5#14 displayed a notable avidity to the enzyme tracer HRP-PYs5. On the contrary, mAbs PYs5#21 and #33 poorly bound the bioconjugate. In particular, mAb PYs5#33 required as much as 1000 ng mL^{-1} of tracer to generate a sufficient maximum signal in competitive ELISA, whereas mAb PYs5#21 needed an intermediate concentration (100 ng mL^{-1}). Cross-reactivity studies with other strobilurins (kresoxim–methyl, trifloxystrobin, azoxystrobin, dimoxystrobin, fluoxastrobin, metominostrobin, picoxystrobin) and other common fungicides (boscalid, fenhexamid, mepanipyrim, pyrimethanil, procimidone, tolylfluanid, cyazofamid, tebuconazole, fludioxonil, vinclozolin, and cyprodinil) showed that all three mAbs were highly selective to PY, with cross-reactivity percentages lower than 0.1% in all cases.

3.2. Immunosorbent characterization

IAC columns produced with the aforementioned antibodies were adequately characterized in terms of coupling efficiency, elution conditions, immunosorbent binding capacity, and reusability. All these aspects are discussed below.

3.2.1. Coupling efficiency

Antibodies are highly valuable and scarce reagents. With few exceptions [31,34], home-made IAC columns are most commonly produced with a single antibody at a fixed antibody-to-bed ratio, either following manufacturer's guidelines or attending to the amount of available antibody. Herein, we produced a series of immunosorbents at four coupling ratios with three different mAbs. The antibody concentration was measured in the eluted and washing fractions after covalent immobilization to the support had occurred, and this value was compared with that of the initial antibody solution. Antibodies were almost quantitatively immobilized to the gel, which confirmed the high performance of the CNBr-activated Sepharose for the production of IAC columns. Even at the highest amount of antibody that was tested (25 mg g^{-1}), coupling efficiencies were mostly higher than 90%.

Table 2
Curve parameters obtained by competitive ELISA with the selected antibodies.

Antibody[a]	[Tracer] (μg L^{-1})	A_{max}	IC_{50} (nM)
PYs5#14	10	1.02	1
PYs5#21	1000	0.67	34
PYs5#33	100	0.65	39

[a] Coating concentration was 1 mg L^{-1}.

3.2.2. Optimum elution conditions

The desirable conditions for an adequate elution of a particular analyte from IAC columns are: (i) to recover the analyte with a quantitative yield; (ii) to use small volumes of eluent; (iii) to obtain a clean extract with minimum matrix interferences; and (iv) to avoid a potential damage to the immobilized antibody. Different strategies have been described in the literature for the elution of analytes from immunosorbents. Displacer molecules or chaotropic agents were discarded because they are not usually adequate for small molecules such as the fungicide PY. Acidic or basic aqueous buffers are less aggressive for the antibody but, as many previous studies have demonstrated, these mild conditions do not always provide a complete elution of low molecular weight analytes [22,30]. Therefore, mixtures of water and an organic solvent such as methanol or acetonitrile at several concentrations (from 0 to 75%, v/v, in water) were evaluated for release of PY from the IAC columns.

Columns were loaded with 1 μg of PY and then eluted with five aliquots of the elution mixtures under study in independent experiments. Methanol or acetonitrile at or below 10% were unable to efficiently elute PY (data not shown). The analyte began to be detected in the eluted fractions at organic solvent concentrations at or above 25%. Results obtained with acetonitrile as organic solvent are shown in Fig. 2. Methanol performed similarly (data not shown) but acetonitrile was preferred because it provided a superior capacity to disrupt the antibody–antigen interaction, resulting in complete elution of PY in a lower volume.

Different elution patterns were observed for each immobilized antibody. On the one hand, immunosorbents produced with mAb PYs5#21 could be eluted with 5 mL of 25% acetonitrile, and 50% acetonitrile was sufficient for a complete elution in 2–3 mL. On the other hand, columns made with mAbs PYs5#14 and PYs5#33 required 75% acetonitrile to quantitatively recover the analyte in a small volume (2 mL), which suggested a tighter capture of the analyte by these two immunoaffinity supports. As mentioned above, all three mAbs displayed affinities to PY in the low nanomolar range. Nonetheless, mAb PYs5#14, the one displaying the highest affinity to the target, required elution conditions similar to those necessary for mAb PYs5#33, an antibody with a 30-fold lower affinity. These results pointed out that not a direct and clear relationship existed between IC_{50} values in competitive ELISA experiments and the harshness of the elution conditions required to break the interaction between the receptor and the ligand. Finally, 75% acetonitrile was selected as the optimum solvent for elution because it provided satisfactory recoveries (from 88 to 96%) for all of the evaluated IAC columns in just a 2 mL fraction.

No relevant analyte binding was detected in control columns without antibody or with an immobilized mAb directed to an unrelated target [35]. More precisely, a small quantity of PY (less than 10% of the percolated amount) was unspecifically retained by the control columns, but it was rapidly eluted in the first volume of eluent. For this reason, a washing step with 5 mL of 10% (v/v) acetonitrile in water was included in the proposed SPE methodology in order to elute any compound unspecifically retained by the column.

3.3. Immunosorbent binding capacity

The amount of analyte selectively retained by IAC columns was calculated with cartridges containing 0.15 g of Sepharose beads. Columns were loaded with 10 μg of PY, washed, and eluted with 75% acetonitrile in water. The amount of analyte in the eluted frac-

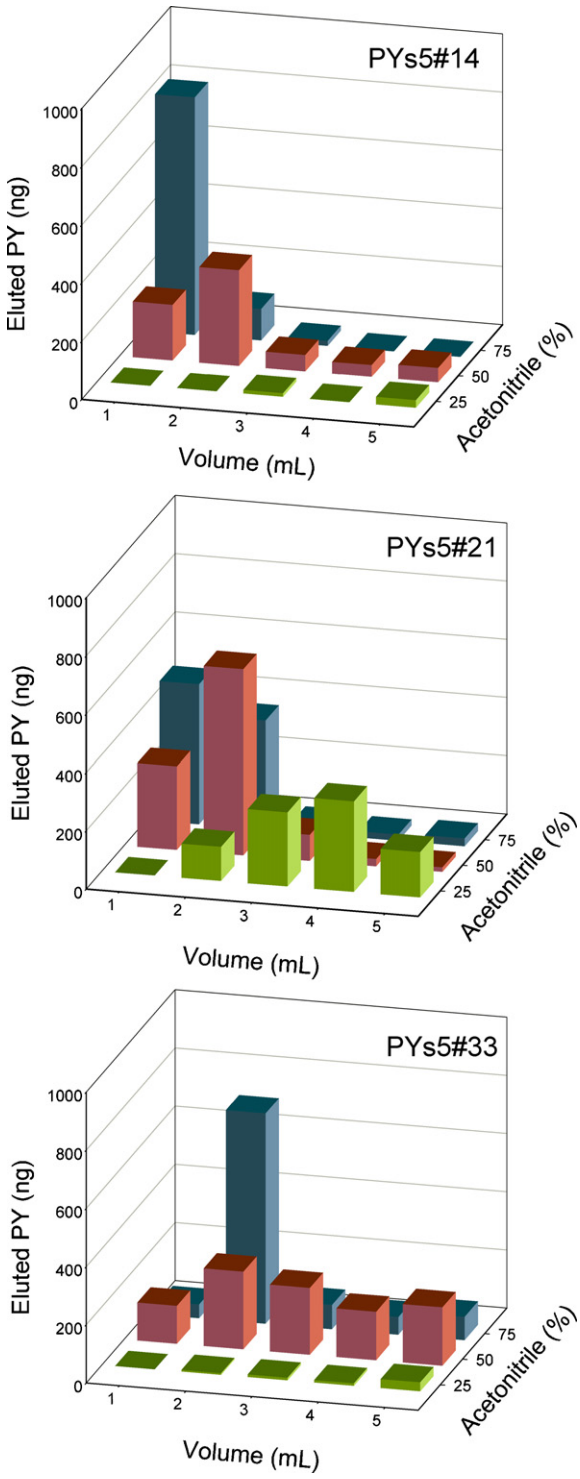

Fig. 2. Elution profile of pyraclostrobin. Amount of pyraclostrobin in the elution fractions (1 mL each) of PYs5#14-, PYs5#21- and PYs5#33-based immunoaffinity columns with 25, 50 and 75% (v/v) acetonitrile. One microgram of pyraclostrobin was loaded into every column. This experiment was carried out twice with equivalent results, so for improved clarity only the data from one replicate are depicted in the graphs.

tions was determined by competitive ELISA. Not surprisingly, the higher the mass of antibody immobilized to the gel, the higher the amount of analyte that was bound to the column. Thus, columns prepared with mAbs PYs5#14, #21, and #33 at 4 mg g^{-1} were able to retain 14.1, 10.0, and 7.8 μg of PY, respectively, whereas columns with these antibodies at 25 mg g^{-1} entrapped as much as 52.9, 46.1,

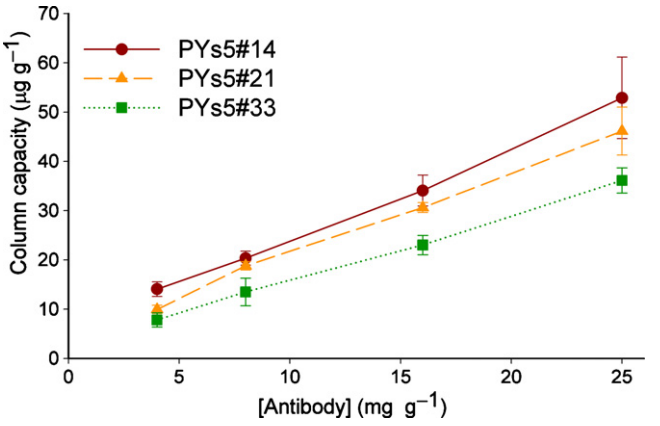

Fig. 3. Evolution of the immunosorbent binding capacity with the amount of antibody employed for the preparation of the immunoaffinity columns. Values are the mean of two replicates.

and 36.1 μg of analyte (Table 2). As a matter of fact, a linear relationship was observed between the immunosorbent binding capacity and the amount of immobilized antibody (Fig. 3). When the ELISA IC_{50} values for each mAb (see Table 2) were compared with the capacity of the respective immunosorbent, it was observed that the highest binding capacity corresponded to columns prepared with mAb PYs5#14, the antibody with the highest affinity to PY. Nevertheless, the other two mAbs, and in particular PYs5#21, behaved optimally, despite their lower affinity exhibited in competitive ELISA. Shelver et al. [31] previously reported a poor correlation between IC_{50} values in competitive experiments and column performance, yet a minimum affinity to the target was obviously required for efficient binding. They rightly recognized that the determinations of IC_{50} values are commonly conducted under equilibrium conditions, whereas the kinetics of the binding process dominates IAC column operation. In addition, immunosorbents are produced with as much antibody as possible, and the target analyte does not have to compete with a bioconjugate for the active sites of the antibody.

The antibody efficacy can be defined as the percentage of active sites in the support capable of efficiently binding the analyte, and it can be calculated by comparison of the maximum amount of ligand that theoretically can be retained by the column with the experimental maximum value. Considering the amount of antibody coupled to the gel, the maximum theoretical capacity of the immunosorbents can be established considering that IgGs are molecules with bivalent binding properties and with a molecular weight of 150,000. Accordingly, the maximum immunosorbent capacities were 20.7, 41.4, 82.7, and 129.3 μg of PY per gram of gel for columns produced with antibodies at 4, 8, 16 and 25 mg g^{-1}, respectively. Maximum antibody efficacy was reached at low antibody loadings, and it decreased with higher amounts of coupled IgG (Table 3). That decrease in binding efficacy has commonly been ascribed to random orientation of the attached antibodies, partial denaturalization of the immobilized molecules, and steric hindrance in overloaded columns, all factors contributing to limiting the number of effective active sites [27,30].

Taking into account a high immunosorbent capacity and moderate mAb consumption, an antibody amount of 16 mg g^{-1} (2.4 mg of antibody per column) was selected as the most appropriate for further work.

3.3.1. *Reusability*

The cost of IAC columns for sample extraction and clean-up is typically higher than conventional SPE cartridges (such as C_{18} or activated carbon) due to the high value of antibodies. Although

Table 3
Immunosorbent capacity, relative standard deviation (RSD) and antibody efficacy of the evaluated immunoaffinity columns.

Antibody	[Antibody] ($mg\,g^{-1}$)	Immunosorbent capacity ($\mu g\,g^{-1}$)[a]	RSD (%)	Efficacy (%)[b]
PYs5#14	4	14.1	10.5	68
	8	20.4	6.9	50
	16	34.1	9.2	41
	25	52.9	15.7	41
PYs5#21	4	10.0	8.4	48
	8	18.8	4.4	46
	16	30.6	3.3	39
	25	46.1	10.5	40
PYs5#33	4	7.8	18.5	38
	8	13.5	20.7	33
	16	23.0	8.6	28
	25	36.1	7.1	28

[a] Maximum amount of analyte retained by one gram of immunosorbent (two independent determinations).
[b] Percentage of immobilized antibody able to retain analyte.

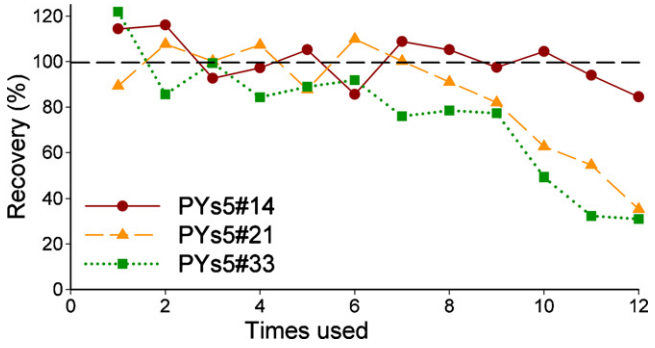

Fig. 4. Performance of the developed IAC columns in recovery studies after repeated uses in buffer. Cartridges containing $16\,mg\,g^{-1}$ of mAb were loaded with 0.2 μg of pyraclostrobin and recoveries were determined by competitive ELISA.

modern in vitro technologies enable the production of large amounts of mAbs at a reasonable price, an alternative approach to partially overcome the economical disadvantage of IAC columns consists in the repeated use of the same support for a number of samples. Even though the use of organic solvents in the elution step is a very efficient way to quantitatively recover the analyte from the column, this desorption procedure may damage the coupled antibody and consequently it may considerably decrease the immunosorbent capacity of the support [22].

To evaluate column performance after repetitive uses under our experimental conditions, the same immunosorbent was loaded with 0.2 μg of PY, washed, eluted, and conditioned for a new use. This cycle was repeated twelve times, and the amount of analyte in the eluted fractions after each cycle, as determined by competitive ELISA, was used to calculate recoveries (Fig. 4). Excellent performance was obtained with the three columns for at least nine repetitive uses. In the case of the support made with the mAb PYs5#14, it perfectly withstood the complete procedure, with almost quantitative recoveries even at cycle number twelve.

At this point, we wondered whether column reusability may depend on the amount of analyte loaded on the gel. With this aim, a new column containing immobilized mAb PYs5#14 was challenged with a much higher amount of PY (10 μg). In fact, the procedure was essentially the same as the one previously employed for the determination of the column binding capacity, but repeated twelve times. Under these demanding conditions, we observed a continuous reduction in column performance, with binding capacity values decreasing from 34 μg of PY in the first use to 12 μg of PY after twelve repetitive usages (Fig. 5). This result emphasized the dependence of column reusability studies on analyte loading amounts. Similar results concerning a different behaviour of immunosorbents when loaded with the analyte at under-saturating or overloading concentrations has been previously described for acetochlor immunoaffinity columns [34].

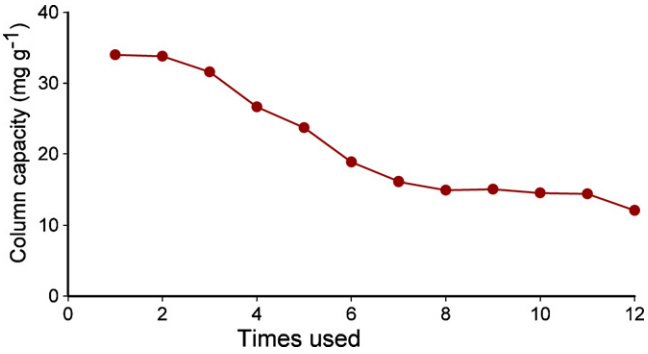

Fig. 5. Variation of the column binding capacity with usage. A Sepharose immunosorbent with immobilized mAb PYs5#14 was overloaded with pyraclostrobin and the amount eluted after washing was determined by competitive ELISA.

3.4. Determination of pyraclostrobin in fruit juices

Taking into account immunosorbent binding capacity, reusability, and global performance, IAC columns with immobilized mAb PYs5#14 at $16\,mg\,g^{-1}$ were selected as the best choice to develop a SPE procedure for the analysis of PY residues.

The selectivity of an IAC application depends on the specificity of the employed antibodies. As mentioned above, mAb PYs5#14 exhibited an exquisite specificity to its target analyte in competitive ELISA experiments. However, conclusions from cross-reactivity studies carried out with competitive methods should not be assumed as entirely valid for IAC [31]. To evaluate column selectivity, fortified samples instead of water were used. Red grape must and apple juice were both spiked with PY and with two additional agrochemicals as model interferents: boscalid, a fungicide usually mixed with PY in many commercial formulations, and azoxystrobin, a member, like PY, of the strobilurin family of fungicides, and one of the most frequently found pesticides in fruits and vegetables [4].

Samples were spiked with a $1\,mg\,L^{-1}$ mixture of PY, azoxystrobin, and boscalid, and 2 mL were extracted with the PYs5#14-based IAC column as shown in the experimental section. Fig. 6 shows the HPLC–UV chromatogram of a spiked red grape must sample measured directly and after the IAC clean-up step (chromatograms A and D, respectively). Interfering compounds from the grape must were efficiently removed by the immunosorbent and, unlike directly injected samples, a well-defined peak corresponding to PY was observed. Accordingly, percolation of the sample through the IAC column allowed an accurate quantification of the analyte, while increasing the HPLC column half-life by protecting it from damages caused by matrix components. In addition, the other spiked fungicides, azoxystrobin and boscalid, were not detected in the elution fractions (see inset of Fig. 6, line D), which evidenced the selectivity of the developed IAC column.

To further prove the selectivity of the IAC columns, two well-known extraction protocols, a SPE with a standard C_{18} column, and a QuEChERS procedure, were also carried out with samples spiked with $1\,mg\,L^{-1}$ of PY, azoxystrobin, and boscalid. Fig. 6 shows the respective HPLC–UV chromatograms (see lines B and C). As expected, matrix components were also efficiently removed with both classical clean-up procedures, but the two other fortified pesticides (azoxystrobin and boscalid) remained in the measured extract, which demonstrated the selectivity of the IAC column, a

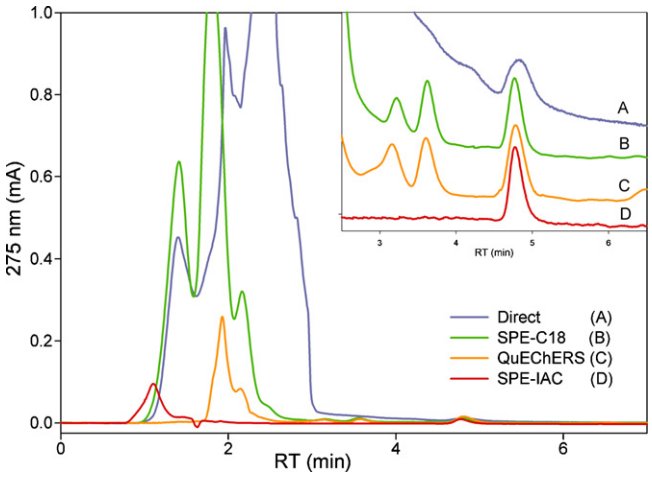

Fig. 6. HPLC–UV chromatograms of a red grape must spiked with a 1 mg L^{-1} mixture of pyraclostrobin, boscalid and azoxystrobin. Samples were measured directly (A) and after a clean-up based on SPE with C_{18} (B), QuEChERS (C) and SPE with the developed PYs5#14-based IAC column (D). Retention times were 3.1 min for azoxystrobin, 3.5 min for boscalid, and 4.8 min for pyraclostrobin.

feature of crucial importance when detectors of limited selectivity, such as UV–DAD, are employed.

An additional relevant aspect of column performance is the ability of the immunosorbent to efficiently preconcentrate samples, because it would allow for an improvement in method sensitivity. With this aim, several volumes (1, 10, 50 and 100 mL) of grape must and apple juice samples were spiked with 1 μg of PY. After washing and eluting as usual, extracts were injected into the chromatographic system. Fig. 7 shows the HPLC–UV chromatograms of the apple juice extracts. Again, interfering compounds remained low and they eluted well before PY, so even the highest loaded volume did not negatively affect analyte quantification. Importantly, recoveries were identical for all four samples irrespective of the processed volume, as evidenced by the overlapping PY peaks shown in the inset of Fig. 7. Therefore, no breakthrough occurred when up to 100-mL samples were percolated through the cartridge. The limit of detection (LOD) of the HPLC–UV equipment, calculated as the analyte concentration that provided a peak with a signal-to-noise ratio of three, was 250 μg L^{-1} for PY. Consequently, the feasibility of concentrating samples 50 times with the IAC column

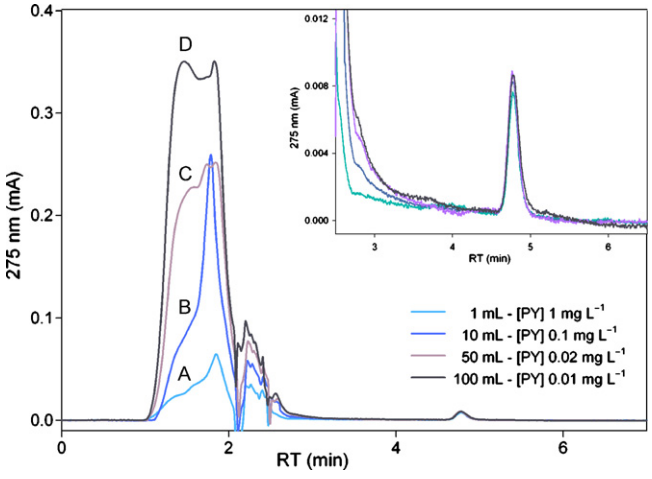

Fig. 7. HPLC–UV chromatograms of apple juice samples spiked with 1 μg of pyraclostrobin after extraction with PYs5#14-based IAC column. Sample volumes percolated through the column were 1 mL (A), 10 mL (B), 50 mL (C), and 100 mL (D).

Table 4
Recovery and relative standard deviation (RSD) in the pyraclostrobin determination of spiked fruit juices by the proposed IAC–HPLC–UV procedure.[a]

Spiked PY (mg L^{-1})	Volume loaded (mL)	Apple juice		Red grape must	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0.01	100	104.0	3.1	101.6	1.7
0.10	10	92.5	2.9	93.1	1.3
1.00	1	98.6	0.9	98.5	4.2

[a] Values correspond to three independent determinations.

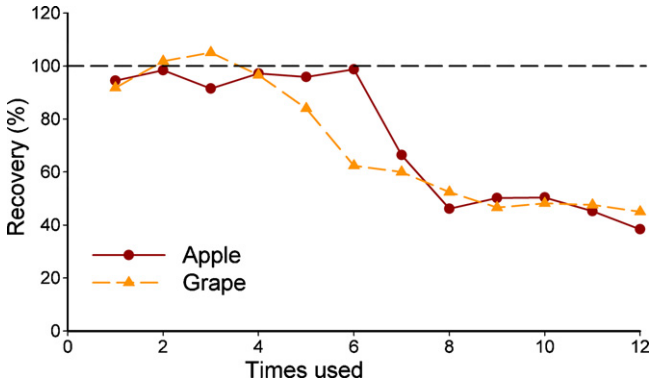

Fig. 8. Performance of the developed IAC columns in recovery studies after repeated uses with apple and grape samples spiked with pyraclostrobin at 1 mg L^{-1}.

made it possible to bring the LOD for the whole analytical procedure (IAC–HPLC–UV) to 5 μg L^{-1}, a value in line with the expected sensitivity for a residue analytical method. Juice samples fortified at this level were unequivocally positive, but recovery values were not consistent. In the case of samples with a high PY concentration, the percolated sample volume must be adjusted to avoid overloading the column.

Finally, the analytical effectiveness of the developed IAC columns for residue analysis was evaluated. Grape must and apple juice samples were spiked at different concentrations (0.01, 0.1 and 1 mg L^{-1}) and they were determined by the proposed methodology (Table 4). Different sample volumes were loaded on the column depending on the spiked level. Three independent determinations were conducted, and recoveries from 93 to 104% for apple juice and from 93 to 102% for grape must were obtained, with relative standard deviations lower than 5% (Table 4). Taking into account the observed accuracy and precision, a limit of quantification (LOQ) of 10 μg L^{-1} was defined for the determination of PY residues in fruit juice samples. Remarkably, when juice samples were fortified with PY at 1 mg L^{-1}, IAC columns could be reused 4–6 times, depending on the commodity (Fig. 8).

4. Conclusions

Herein, we describe the development of the first immunoaffinity supports for the solid phase extraction of pyraclostrobin residues from foodstuffs. Three anti-PY mAbs have been evaluated for the production of IAC columns using Sepharose gels for covalent antibody immobilization. After immunosorbent characterization in terms of binding capacity, elution conditions, and reusability, the antibody with the highest affinity to the target analyte (mAb PYs5#14) was shown to display superior binding features. Nevertheless, affinity does not seem to be the sole criterion for antibody selection in IAC development. Otherwise, testing available antibodies for IAC application in order to find the most suitable candidate for the production of immunosorbents is highly advisable. In our study, the high specificity of the selected antibody allowed a very

selective retention of PY from fruit juice samples, while other agrochemicals and most matrix components were washed out. In addition, sample preconcentration provided a substantial improvement in method sensitivity, reaching a LOQ of 10 $\mu g\,L^{-1}$. Finally, due to its high sample-throughput, robustness, and simplicity, ELISA has been confirmed as an excellent complementary tool for the analysis of IAC fractions during characterization of affinity columns.

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Limited amounts of the immunoreagents described in this work are available upon request for evaluation purposes.

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