A class-selective immunoassay for simultaneous analysis of anilinopyrimidine fungicides using a rationally designed hapten

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The development of multianalyte immunoassays constitutes a main research issue in the field of bioanalytical techniques. In the present study, class-specific antibodies against the three members of the anilinopyrimidine family of fungicides (pyrimethanil, cyprodinil, and mepanipyrim) were raised by using a bioconjugate of a rationally designed hapten [5-(6-methyl-2-(phenylamino)pyrimidin-4-yl)pentanoic acid]. Highly sensitive immunoassays were developed for the generic determination of these compounds, using the competitive enzyme-linked immunosorbent assay (ELISA). Particularly, a direct antibody-coated competitive ELISA afforded identical sensitivity for the three anilinopyrimidines, with IC₅₀ values of 0.26, 0.27, and 0.25 µg L⁻¹ for pyrimethanil, cyprodinil, and mepanipyrim, respectively. This immunoassay was fully characterized and applied to the multianalyte determination of anilinopyrimidine fungicides in white and red wines, with a limit of quantification of 1 µg L⁻¹, average recoveries from 93.1% to 114.4%, and relative standard deviations lower than 20%. Commercial wine samples were analyzed and those containing detectable anilinopyrimidine residues were verified by a reference chromatographic technique.

Introduction

Pyrimethanil, cyprodinil, and mepanipyrim are the three members of the anilinopyrimidine group of fungicides, which are highly active against a broad range of pathogenic fungi due to an innovative mode of action based on the inhibition of both methionine biosynthesis and secretion of hydrolytic enzymes.¹ The application of anilinopyrimidine fungicides in vineyards is a common practice to fight pests like mildew and botrytis; consequently, residues of these compounds may remain on the grapes. Several studies have appointed the actual transference of fungicide residues from grapes to wine,^{2–4} being cyprodinil and pyrimethanil among the most common pesticides found in this valuable commodity.^{5–10} Fungicide residues in wine are usually analyzed by chromatographic multianalyte methods, particularly high-performance liquid chromatography with mass spectrometry detection (HPLC–MS), supported by sample extraction procedures like QuEChERS (acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe).^{11–13} Moreover, alternative extraction methodologies have also been developed recently for the analysis of anilinopyrimidine residues in wine using

liquid–liquid extraction,¹⁴ solid-phase extraction,^{15,8,16} solid-phase microextraction,¹⁷ dispersive liquid–liquid microextraction,⁷ bar adsorptive microextraction,¹⁸ and ultrasound-assisted microextraction.^{19,20}

Adoption of bioanalytical techniques for the determination of chemical residues and contaminants has been increasing during the last decades, being the competitive enzyme-linked immunosorbent assay (ELISA) one of the most extended immunochemical methods because of a series of great advantages like simplicity, high sensitivity, portability, affordable cost, and high sample throughput.²¹ Competitive ELISAs have been developed for the determination of a number of single pesticides in wine, such as benalaxyl,²² tebufenozide,²³ fenitrothion,²⁴ fenhexamid,²⁵ bromopropylate,²⁶ and cyprodinil.²⁷ However, the development of multianalyte immunoassays constitutes currently a major challenge.²⁸ Complementary strategies exist in order to develop multianalyte competitive ELISAs, which can be based on specific²⁹ or broad-specificity antibodies,³⁰ or a combination of both.³¹ Raising generic receptors that recognize simultaneously several compounds requires a rational design of haptens to be used for the preparation of immunogens.^{32–35} Class-specific antibodies have been previously obtained and multiresidue immunoassays have been developed for the analysis of families of compounds, such as chlorinated cyclodiene insecticides,³⁶ triazine herbicides,³⁷ and organophosphorus insecticides.³⁸

In preceding studies, a collection of specific high-affinity antibodies to pyrimethanil,³⁹ cyprodinil,⁴⁰ and mepanipyrim⁴¹ fungicides was generated. Now, a novel hapten has been rationally designed and bioconjugates have been prepared

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aiming at the generation of antibodies that could recognize with equivalent affinity the three anilinoypyrimidine fungicides. Pyrimethanil, cyprodinil, and mepanipyrim have a very similar molecular structure, with different substituent groups at the C-3 position of the pyrimidine ring. Following the Landsteiner's principle,⁴² hapten AP has been conceived by introducing a functional carboxylate group at such position (Fig. 1) in order to promote a higher recognition of the distal moieties of the molecule, that is, the aniline ring which is common to the three compounds. A linear five-carbon spacer has been proposed to link the carboxylate group to the pyrimidine moiety, so to preserve as much as possible the conformation and electronic properties of the original anilinoypyrimidine compounds. The final objective was the development of a multiresidue immunoassay for the simultaneous analysis of pyrimethanil, cyprodinil, and mepanipyrim in wine samples, and the validation of this new methodology by comparison with a reference chromatographic technique.

Fig. 1 Molecular structures of pyrimethanil, cyprodinil, mepanipyrim, and hapten AP.

Results and discussion

Synthesis of hapten and protein–hapten conjugates

Hapten AP was designed in order to obtain generic antibodies capable of recognizing with high affinity the three anilinoypyrimidine fungicides (pyrimethanil, cyprodinil, and mepanipyrim). In this hapten, the spacer arm, which joined the anilinoypyrimidine moiety to the carrier protein of immunogenic and assay conjugates, was a carboxylated five-membered linear chain replacing the structural element that differentiates the three target anilinoypyrimidine fungicides (Fig. 1). This substitution did not imply a drastic change in the electronic distribution of the anilinoypyrimidine system with respect to the fungicides themselves, nor had a significant influence on the conformational distribution that they reach (Fig. 2). The lowest energy conformers of pyrimethanil, cyprodinil, mepanipyrim, and hapten AP showed nearly planar structures that perfectly overlap. Only slight differences in the angular position of some atoms, such as the anilinic nitrogen atom, were observed; though the position of the bonded hydrogen atom displaying a partial positive charge (Fig. 2) – and thus highly available for hydrogen bonding – was practically the same for the four compounds. Therefore, exposure of the common 6-methyl-2-(phenylamino)pyrimidin-

Fig. 2 A) Overlap of lowest energy conformers of pyrimethanil, cyprodinil, mepanipyrim, and hapten AP. The elements are represented in the following manner: carbon, dark grey; oxygen, red; nitrogen, blue; hydrogen, light grey. B) Calculated partial atomic charges of pyrimethanil ($R = \text{CH}_3$), cyprodinil ($R = \text{cyclopropyl}$), mepanipyrim ($R = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), and hapten AP ($R = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$). Only those atoms that are common to the four structures are depicted. Hydrogen atoms are not included with the exception of the amine hydrogen (H-15). The numbering in the figure does not reflect the systematic IUPAC numbering. Calculations were performed using the SCIGRESS program (version 3.0.0, Fujitsu Limited, FQS, Poland). First, global minimum search was performed using MM3 parameters (all rotatable bonds were rotated by 24 degree steps) and the geometry of the generated conformers was refined performing an optimized geometry calculation in MOPAC using the PM3 method with COSMO water solvation parameters.

yl moiety was allowed and mimicking of the conformation and electronic distribution of the target molecules was accomplished in hapten AP; consequently, the generation of class-specific antibodies should be advantaged.

As shown in Fig. 3, the synthesis of hapten AP was based on the initial preparation of a 1,3-dicarbonyl compound, *i.e.* diketone **4**, which incorporated the carboxylated hydrocarbon chain that constituted the spacer arm of the target hapten and a β -diketone group appropriate for the elaboration of the pyrimidine moiety. This compound was prepared from β -keto ester **1** via C-acylation reaction of the active methylene group by treatment with acyl chloride **2**, to give tricarbonyl derivative **3**, followed by subsequent removal of the allyl ester group through a Pd(0)-catalyzed desallylation-decarboxylation reaction. With the β -diketone **4** at hands, the preparation of the pyrimidine ring was completed through a condensation reaction in basic medium of the two ketonic carbonyl groups with the amino groups of phenylguanidine, generated in situ from phenylguanidinium nitrate (**5**). This condensation reaction afforded anilinoypyrimidine **6**, thus completing the skeleton of hapten AP, whose synthesis ended with the basic hydrolysis of the methyl ester moiety under standard conditions. The synthesis of the hapten AP through this

Fig. 3 Synthesis of hapten AP and its active NHS-ester.

synthetic route was simple and efficient; it took place with an overall yield of nearly 55% for the four involved steps.

In order to control more effectively the conjugation of hapten AP to the carrier proteins (BSA, bovine serum albumin; OVA, ovalbumin; HRP, horseradish peroxidase), we proceed first to the activation of the carboxylate group through its transformation into the corresponding *N*-hydroxysuccinimide ester. This transformation was accomplished in high yield using *N,N'*-disuccinimidyl carbonate (DSC) and Et_3N . The so-obtained activated ester, AP-NHS ester (Fig. 3), was in a high degree of purity after its purification by column chromatography, allowing the preparation of the required hapten–protein immunizing (BSA–AP) and assay (OVA–AP and HRP–AP) conjugates with precise hapten-to-protein ratios. The obtained hapten-to-protein molar ratio (MR) for BSA, OVA, and HRP conjugates were 11, 4, and 3, respectively.

Generic immunoassay selection

Two antibodies (AP#1 and AP#2) were generated using the BSA–AP conjugate as immunogen. Checkerboard competitive ELISAs using the direct and indirect formats were run for immunoreagent selection. For direct assays, plates were coated with different dilutions of the antiserum (from 3×10^3 to 3×10^4), and the competitive step was carried out with the HRP–AP tracer at concentrations from 1 to $300 \mu\text{g L}^{-1}$. For indirect assays, plates were coated with the OVA–AP conjugate at 10, 100, and 1000 ng mL^{-1} , and a range of antibody dilutions (from 3×10^3 to 1×10^6) was assayed. Thus, one inhibition curve was obtained for every immunoreagent combination in both competitive ELISA formats using pyrimethanil, cyprodinil, and mepanipyrim standard curves. Table 1 lists a summary of the selected assay conditions that afforded A_{max} values around 1.0 with negligible background signal, and the respective IC_{50}

values for each anilino-pyrimidine fungicide. The selected assays recognized the three anilino-pyrimidine fungicides with high sensitivity, mainly showing IC_{50} values lower than $1 \mu\text{g L}^{-1}$.

Cross-reactivity (CR) was calculated as the percentage of the quotient between the IC_{50} value for pyrimethanil and the IC_{50} value for cyprodinil or mepanipyrim (Table 1). Antibody AP#1 showed the most generic pattern, with CR values for cyprodinil and mepanipyrim close to 100%, which means that the affinity to the three analytes was almost nearly identical. On the other hand, antibody AP#2 recognized pyrimethanil and cyprodinil with comparable affinities, but it showed a lower recognition of mepanipyrim, with CR values of 69% and 56% for the direct and the indirect format, respectively. Considering the higher affinity (IC_{50} values from 0.25 to $0.27 \mu\text{g L}^{-1}$) and the more generic character of antibody AP#1 (CR from 97% to 104%), as well as the short analysis time of the direct immunoassay format (1 h in a single incubation step), further assay development and validation were carried out with antibody AP#1 by direct competitive ELISA.

Table 2 Recovery values obtained by the direct AP#1-based immunoassay from water spiked with single analytes and mixtures of analytes ($n = 3$)

Spiked ($\mu\text{g L}^{-1}$)	Analytes ^a	Found ($\mu\text{g L}^{-1} \pm s$)	Average recovery (%)	RSD (%)
0.1	PM	0.093 ± 0.001	95.8	12.5
	CD	0.097 ± 0.001		
	MP	0.112 ± 0.018		
	PM + CD	0.096 ± 0.007		
	PM + MP	0.091 ± 0.016		
	CD + MP	0.094 ± 0.003		
	PM + CD + MP	0.092 ± 0.019		
1.0	PM	1.12 ± 0.11	105.6	13.1
	CD	1.17 ± 0.13		
	MP	1.15 ± 0.17		
	PM + CD	0.94 ± 0.08		
	PM + MP	0.97 ± 0.10		
	CD + MP	1.13 ± 0.09		
	PM + CD + MP	0.93 ± 0.08		
10.0	PM	10.5 ± 1.4	101.1	10.5
	CD	10.6 ± 1.4		
	MP	10.1 ± 0.3		
	PM + CD	9.4 ± 1.0		
	PM + MP	9.4 ± 0.7		
	CD + MP	10.8 ± 0.3		
	PM + CD + MP	9.7 ± 1.5		

^a PM, pyrimethanil; CD, cyprodinil; MP, mepanipyrim.

Table 1 Assay parameters and cross-reactivity values from checkerboard assays ($n = 3$)

Ab ^a	Assay format	Ab dilution ($\times 10^3$)	$[\text{C}]^b$ ($\mu\text{g L}^{-1}$)	A_{max}	IC_{50} ($\mu\text{g L}^{-1}$)			$\text{CR}^{\text{PM}} (\%)^c$	
					PM ^d	CD ^e	MP ^f	CD	MP
AP#1	Direct	10	5	0.91	0.26	0.27	0.25	97	104
	Indirect	100	10	1.13	0.43	0.36	0.42	118	103
AP#2	Direct	3	10	1.12	0.25	0.22	0.36	112	69
	Indirect	100	10	0.92	0.60	0.56	1.07	107	56

^a Antibody. ^b Conjugates were HRP–AP and OVA–AP for direct and indirect assay, respectively. ^c Cross-reactivity to pyrimethanil. ^d Pyrimethanil. ^e Cyprodinil. ^f Mepanipyrim.

The class-selective character of the AP#1-based direct assay was confirmed by two additional tests. Firstly, solutions of the three anilinoypyrimidine fungicides were prepared at 0.1, 1, and 10 $\mu\text{g L}^{-1}$ in deionized water, which were analyzed using three different situations: i) each fungicide alone, ii) an equimolar mixture of two of them, and iii) an equimolar mixture of the three compounds. As shown in Table 2, all of the analyte combinations provided quantitative results with average recoveries ranging from 95.8% to 105.6%, and relative standard deviations (RSD) of less than 15%. The second test consisted in the analysis and comparison of two standard curves, one prepared only with pyrimethanil, and the second with an equimolar mixture of pyrimethanil, cyprodinil, and mepanipyrim. As seen in Fig. 4, the obtained inhibition curves were absolutely equivalent, having the same IC_{50} and slope values; thus, showing the truly generic character of the proposed AP#1-based direct immunoassay for the analysis of the three members of the anilinoypyrimidine family of fungicides.

Immunoassay characterization

Assay robustness against changes in pH and ionic strength during the competitive immunochemical reaction was also evaluated, taking PBS (phosphate buffered saline) as the reference buffer (10 mM sodium phosphate at pH 7.4 and 140 mM NaCl). Assays were carried out in 10 mM phosphate buffers with varied pH values and with fixed ionic strength for pH studies, and with different NaCl concentration with fixed pH 7.4 for ionic strength studies. Pyrimethanil standard curves were prepared in water and analyzed by the direct AP#1-based assay using the aforementioned buffers. Particularly, the maximum A_{max} variation was found at the highest assayed ionic strength and pH values as well as at pH 6.0 (Fig. S1 in the Supplementary Information). On the other hand, the IC_{50} was

Fig. 4 Inhibition curves obtained for pyrimethanil (PM) and a mixture of the three anilinoypyrimidine fungicides (PM+CD+MP) using the developed competitive ELISA.

Fig. 5 Tolerance to ethanol and acetonitrile of the AP#1-based direct immunoassay.

mainly changed also at the most far-out assayed conditions (pH 6.0 and 250 mM NaCl concentration). In general, it was observed that the A_{max} and IC_{50} values of these inhibition curves were similar to those obtained with the reference buffer, showing variations lower than 21% and 32% for each parameter, respectively.

The presence of solvents in the sample extract may have an effect on the standard curve parameters – particularly over the A_{max} and the IC_{50} values – of the competitive ELISA. In this study, two solvents were selected in order to evaluate the tolerance of the proposed immunoassay, *i.e.*, ethanol, because of the high concentration found in fermented drinks, and acetonitrile, due to its extensive usage as extraction solvent in multiresidue pesticide analysis. Thus, pyrimethanil standard curves were prepared in water containing different concentrations of ethanol and acetonitrile, and analyzed by the selected immunoassay. Fig. 5 shows the variation of the A_{max} and IC_{50} values of the inhibition curve as a function of the solvent concentration. It was found that ethanol was quite well tolerated up to 10% (v/v) contents, displaying a gradual and smooth increment of the IC_{50} value and an alike descent of the A_{max} value by increasing the solvent concentration. On the contrary, acetonitrile had a little effect over the A_{max} value, but high solvent contents sharply incremented the IC_{50} value of the assay, thus limiting the tolerable (variations lower than 50%) acetonitrile concentration to 2% (v/v).

Additional CR studies were performed with the selected immunoassay using twenty-four fungicides frequently found in grapes and wine, such as azoxystrobin, pyraclostrobin, picoxystrobin, kresoxim-methyl, trifloxystrobin, fluoxastrobin, fenhexamid, boscalid, fludioxonil, proquinazid, imidacloprid, procimidone, cyazofamid, tolylfluanid, spirotetramat, fluopyram, epoxiconazole, tebuconazole, fluxapyroxad, benzanilide, chlorothalonil, fenamidone, vinclozolin, and captan. In this case no inhibition was observed with any of the assayed fungicides (CR lower than 0.1 %), so no interferences from these fungicides are expected to be found using the proposed immunoassay.

Multiresidue analysis in wine

Wine constituents may considerably influence the immunoassay response, such as the ethanol content and the presence of polyphenols. This interference can be easily avoided by dilution of wine samples in water. Organic-farming

Table 3 Recovery values obtained from spiked wines using direct AP#1-based immunoassay (n = 3)

Spiked ($\mu\text{g L}^{-1}$)	Analytes ^a	Sample dilution	White wine			Red wine		
			Found ($\mu\text{g L}^{-1} \pm s$)	Average recovery (%)	RSD (%)	Found ($\mu\text{g L}^{-1} \pm s$)	Average recovery (%)	RSD (%)
1	PM	1/15	1.04 $\pm$ 0.09	114.4	5.6	0.95 $\pm$ 0.10	105.9	15.6
	CD		1.19 $\pm$ 0.12			1.13 $\pm$ 0.20		
	MP		1.13 $\pm$ 0.10			1.26 $\pm$ 0.17		
	PM + CD + MP		1.10 $\pm$ 0.01			0.91 $\pm$ 0.17		
5	PM	1/15	5.0 $\pm$ 0.4	99.7	1.2	4.9 $\pm$ 0.6	97.9	2.9
	CD		4.9 $\pm$ 0.3			4.8 $\pm$ 0.7		
	MP		5.0 $\pm$ 0.5			5.1 $\pm$ 0.4		
	PM + CD + MP		5.0 $\pm$ 0.5			4.8 $\pm$ 0.6		
10	PM	1/15	10.3 $\pm$ 0.5	106.9	9.2	8.9 $\pm$ 0.1	93.8	5.7
	CD		9.5 $\pm$ 0.3			9.1 $\pm$ 1.1		
	MP		11.3 $\pm$ 1.0			9.5 $\pm$ 0.8		
	PM + CD + MP		11.7 $\pm$ 0.9			10.1 $\pm$ 0.4		
50	PM	1/15	48.7 $\pm$ 3.4	93.1	8.5	48.4 $\pm$ 2.1	94.5	7.2
	CD		44.6 $\pm$ 6.2			43.3 $\pm$ 4.3		
	MP		42.0 $\pm$ 2.0			46.0 $\pm$ 2.9		
	PM + CD + MP		50.9 $\pm$ 1.8			51.3 $\pm$ 2.4		
100	PM	1/150	110 $\pm$ 10	102.7	8.4	105 $\pm$ 2	101.2	4.3
	CD		90 $\pm$ 10			100 $\pm$ 6		
	MP		99 $\pm$ 8			104 $\pm$ 5		
	PM + CD + MP		107 $\pm$ 9			95 $\pm$ 1		
500	PM	1/150	473 $\pm$ 6	94.8	5.4	539 $\pm$ 10	106.2	8.0
	CD		490 $\pm$ 40			486 $\pm$ 40		
	MP		440 $\pm$ 70			513 $\pm$ 30		
	PM + CD + MP		500 $\pm$ 30			586 $\pm$ 50		

^a PM, pyrimethanil; CD, cyprodinil; MP, mepanipyrim.

white and red wines were early analyzed by HPLC–MS/MS in order to check for the absence of anilinopyrimidine fungicides, and then diluted (1/5, 1/15, 1/50, 1/150, and 1/500) with MilliQ water. Pyrimethanil standard curves were prepared in the aforementioned wine dilutions and analyzed by the proposed AP#1-based direct assay. Fig. S2 shows the obtained inhibition curves for white and red wines. Matrix effects were higher in red than in white wines probably due to the higher tannin contents, although they could be completely avoided using a 1/15 dilution in water for both matrices.

Organic wines were spiked with each individual anilinopyrimidine fungicide and using a mixture of the three compounds, at equimolar concentrations, with total fungicide concentrations from 1 to 500 $\mu\text{g L}^{-1}$. Then, spiked wines were homogenized by vortex shaking and analyzed by the developed competitive ELISA using a pyrimethanil standard curve. Mean recoveries were from 94.8% to 114.4% for white wine, and from 93.8% to 106.2% for red wine, with RSD values mostly lower than 10% (Table 3). Thus, the limit of quantification (LOQ) of the developed immunoassay for the analysis of anilinopyrimidine fungicide residues in wines was established at 1 $\mu\text{g L}^{-1}$.

Validation study

Residues of anilinopyrimidine fungicides were measured in 75 commercially available wine samples using the developed immunoassay. Six positive samples were detected by the

developed immunoassay, which were also measured by UPLC–MS/MS. As listed in Table 4, equivalent results were obtained from both methodologies. Thus, the generic pattern of the developed immunoassay was confirmed again with the correct identification and quantification of residues of pyrimethanil and cyprodinil in wines. No detectable residues of mepanipyrim were found, as also observed in other studies with wine samples.^{43,44} The maximum residue concentration that was found was in a white sparkling wine with an anilinopyrimidine fungicide content of 204 $\mu\text{g L}^{-1}$. The obtained results are in accordance to literature data where the maximum anilinopyrimidine concentrations were from 15 to 234 $\mu\text{g L}^{-1}$.^{5–10} The European Union does not establish maximum residue limits (MRL) for processed food beverages, and application of MRL values for the raw commodity – wine grapes in this case – is not probably adequate to establish quality and safety parameters for wine.

Table 4 Wine samples with measurable anilinopyrimidine contents

Wine type	UPLC–MS/MS ($\mu\text{g L}^{-1} \pm s$, n=2)			ELISA ($\mu\text{g L}^{-1} \pm s$, n=3)
	Pyrimethanil	Cyprodinil	Mepanipyrim	
Sparkling	88.4 $\pm$ 6.5	– ^a	–	94.8 $\pm$ 1.7
Sparkling	222.6 $\pm$ 19.0	–	–	204.1 $\pm$ 9.4
White	81.2 $\pm$ 5.8	–	–	100.4 $\pm$ 7.1
Red	11.6 $\pm$ 0.3	–	–	23.3 $\pm$ 2.9
Red	8.4 $\pm$ 0.1	12.0 $\pm$ 0.1	–	31.6 $\pm$ 2.3
Red	35.9 $\pm$ 1.6	6.7 $\pm$ 0.1	–	39.5 $\pm$ 4.1

^a Below the UPLC–MS/MS limit of quantification (1 $\mu\text{g L}^{-1}$).

Experimental

Reagents and instrumentation

Anhydrous solvents were freshly distilled under nitrogen from Na/benzophenone for THF or calcium hydride for CH₂Cl₂ and CH₃CN, or dried over KOH for Et₃N. Other solvents and reagents were obtained from commercial sources and used without purification. Anilinopyrimidine fungicides and other pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). HRP, OVA, *o*-phenylenediamine, fetal bovine serum (FBS), and Freund's adjuvants were purchased from Sigma/Aldrich (Madrid, Spain). BSA was purchased from Roche Applied Science (Mannheim, Germany). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification. Polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR-HRP) was from BioRad (Hercules, CA, USA). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Triphenyl phosphate (TPP) and Supelco C₁₈ SPE cartridges were purchased from Sigma/Aldrich (Madrid, Spain). Hydrogen peroxide (30%), sulfuric acid (95%), high purity organic solvents for chromatography, and buffer constituents were purchased from Prolabo-VWR International Eurolab S.L (Barcelona, Spain) or Scharlau (Barcelona, Spain).

Ultraviolet-visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an EL405 microplate washer also from BioTek Instruments. A Waters Acquity ultra-performance liquid chromatography system from Waters (Milford, MA, USA), coupled to a Waters Acquity triple quadrupole mass spectrometry detector equipped with a Z-spray electrospray ionization source, was employed for chromatographic analysis. The system was set up with a binary solvent delivery system, an autosampler, and a BEH C₁₈ (1.7 µm, 2.1 x 50 mm) column.

The composition, concentration, and pH of the employed buffers were as follows: i) PB, 100 mM sodium phosphate buffer (pH 7.4); ii) PBS, 10 mM sodium phosphate buffer (pH 7.4) with 140 mM NaCl; iii) PBST, PBS containing 0.05% (v/v) Tween 20; iv) PBST-FBS, PBST containing 10% (v/v) fetal bovine serum; v) CB, 50 mM carbonate-bicarbonate buffer (pH 9.6); vi) washing solution, 15 mM NaCl and 0.05% (v/v) Tween 20; vii) developing buffer, 25 mM citrate and 62 mM sodium phosphate buffer (pH 5.4); and viii) PBS2xT, 20 mM sodium phosphate buffer (pH 7.4) with 280 mM NaCl and 0.05% (v/v) Tween 20.

Hapten synthesis and activation

A schematic representation of the synthetic procedure followed for the preparation of hapten AP and its *N*-hydroxysuccinimidyl ester (AP-NHS ester) is depicted in Fig. 3. Spectrometric characterization data of all of the intermediates and of the active ester of hapten AP can be found in the Supplementary Information.

Preparation of 1-allyl 8-methyl 2-acetyl-3-oxooctanedioate (3). Allyl 3-oxobutanoate (**1**, 299 mg, 0.288 mL, 2.1 mmol) was

added dropwise over a stirred suspension of anhydrous MgCl₂ (200 mg, 2.1 mmol) in dry CH₂Cl₂ (2.5 mL) under a nitrogen atmosphere at room temperature. The mixture was cooled to 0 °C in an ice bath, and dry pyridine (0.339 mL, 4.2 mmol) was added. Stirring was continued at this temperature for 15 min, and methyl 6-chloro-6-oxohexanoate (**2**, 0.327 mL, 2.1 mmol) was added dropwise, whereupon stirring was maintained at 0 °C for an additional 15 min. The ice bath was then removed and the mixture was allowed to stir at room temperature for 5 h. The mixture was then cooled again in an ice bath and 6 M HCl was added until acid pH was attained (approximately 1.5 mL), then diluted with water and extracted with ethyl ether. The combined organic extracts were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure to provide compound **3** (580 mg, 97%) as a dense colorless oil which did not require purification and whose ¹H NMR spectrum showed that it was exclusively present in the keto-enol tautomeric form.

Preparation of methyl 6,8-dioxononanoate (4). Formic acid (0.064 mL, 1.7 mmol) and Et₃N (0.113 mL, 0.81 mmol) were added consecutively to a solution of Pd(OAc)₂ in dry THF (1.5 mL) under nitrogen at room temperature. After a few minutes of stirring, a solution of compound **3** (200 mg, 0.70 mmol) in dry THF (1.5 mL) was added and the resulting mixture was refluxed for 4 h. The reaction mixture was cooled to room temperature and filtered through a small column of silica using ethyl ether for washing. The obtained ethereal solution was washed with water and brine, dried over anhydrous MgSO₄, filtered, and concentrated to dryness to give compound **4** (127 mg, 90%) as a viscous oil, which was shown by ¹H NMR spectroscopy to be an equilibrium mixture of keto-enol (72%) and dicarbonyl (28%) tautomeric forms.

Preparation of methyl 5-(6-methyl-2-(phenylamino)pyrimidin-4-yl)pentanoate (6). A mixture of diketone **4** (161 mg, 0.8 mmol), *N*-phenylguanidinium nitrate (**5**, 175 mg, 0.88 mmol), Na₂CO₃ (47 mg, 0.44 mmol), and EtOH (3 mL) placed in a dried ampule was degassed using the freeze-thaw method. The mixture was cooled under nitrogen and sealed under vacuum. Then, it was vigorously stirred and heated at 80 °C for 18 h, cooled to room temperature, and concentrated at reduced pressure. The obtained residue was chromatographed, eluting with EtOAc 9:1, to afford pyrimidine **6** (169 mg, 70%) as an oil.

Preparation of 5-(6-methyl-2-(phenylamino)pyrimidin-4-yl)pentanoic acid (Hapten AP). A 2 M aqueous solution of NaOH (1 mL) was added to a solution of methyl ester **6** (167 mg, 0.56 mmol) in MeOH (3 mL), and the mixture was refluxed with stirring for 1.5 h. After this time, the solvent was removed on a rotary evaporator and the obtained dried residue was dissolved in the minimum amount of HCO₂H, and then water was added until the total precipitation of the product, which was collected by vacuum filtration, washed with cold water, and dried under vacuum to afford hapten AP (143 mg, 90%) as a white solid. Mp 201–204 °C (crystallized from DMSO/H₂O); ¹H NMR (300 MHz, DMSO-d₆) δ (ppm) 12.06 (1H, br s, COOH), 9.44 (1H, br s, NH), 7.80 (2H, br d, *J* = 7.6 Hz, H-2/H-6 Ph), 7.24 (2H, br t, *J* = 7.9 Hz, H-3/H-5 Ph), 6.89 (1H, tt, *J* = 7.3, 1.2 Hz, H-

4 Ph), 6.62 (2H, t, $J = 7.2$ Hz, H-5 Pyrim), 2.57 (2H, t, $J = 7.4$ Hz, H-5), 2.32 (3H, s, CH₃-Pyrim), 2.25 (2H, t, $J = 7.2$ Hz, H-2), 1.69 (2H, m, H-4), 1.54 (2H, m, H-3); ¹³C NMR (75 MHz, DMSO-d₆) δ (ppm) 174.3 (C-1), 170.3 (C-4 Pyrim), 167.0 (C-2 Pyrim), 159.7 (C-6 Pyrim), 140.9 (C-1 Ph), 128.3 (C-3/C-5 Ph), 120.7 (C-4 Ph), 118.4 (C-2/C-6 Ph), 110.4 (C-5 Pyrim), 36.4 (C-5), 33.4 (C-2), 27.4 (C-4), 24.1 (C-3), 23.5 (CH₃-Pyrim); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3295, 3202, 3082, 2918, 2852, 2503, 1694, 1618, 1585, 1552, 1508, 1372, 842, 727; MS (EI) m/z (%) 285 (M⁺, 10), 284 (2), 226 (7), 212 (5), 199 (40), 178 (7), 73 (100); HRMS, m/z calcd for C₁₆H₁₉N₃O₂ (M⁺) 285.14773, found 285.14819.

Preparation of 2,5-dioxypyrrolidin-1-yl 5-(6-methyl-2-(phenylamino)pyrimidin-4-yl)pentanoate (AP-NHS ester). Hapten AP (30.5 mg, 0.107 mmol) and DSC (35.6 mg, 0.139 mmol, 1.3 equiv.) were dissolved in dry CH₃CN (1 mL) under nitrogen atmosphere. Et₃N (57 μ L, 0.406 mmol, 3.8 equiv.) was added and the reaction mixture was stirred at room temperature for 15 h. The solution was diluted with CHCl₃, washed with an aqueous saturated solution of NaHCO₃ and brine, and then dried over anhydrous Na₂SO₄. Flash column chromatography of the residue that was left after evaporation of the solvent, using CHCl₃ as the eluent, afforded the *N*-succinimidyl ester of hapten AP (28.5 mg, 72%) as an oil.

Synthesis of protein–hapten conjugates

Protein conjugates were prepared by coupling the NHS-activated ester of hapten AP with three different carrier proteins (BSA, HRP, and OVA) which were employed as immunizing, enzyme tracer, and coating conjugate, respectively. The hapten-to-protein MR of the purified conjugates was determined from the absorbance values of the conjugate at 260 and 280 nm by assuming that the molar absorptions of the hapten and the protein were the same for the free and the conjugated forms.

Immunizing BSA–AP conjugate. A 50 mM solution (200 μ L) of AP–NHS in *N,N*-dimethylformamide was added dropwise over 2 mL of a 15 g L^{−1} BSA solution in CB. The conjugation reaction was carried out during 4 h at room temperature with moderate stirring. Next, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using PB as eluent. The conjugate was diluted to 1 g L^{−1} with PB and stored at −20 °C.

Tracer HRP–AP conjugate. A 10 mM solution (100 μ L) of AP–NHS in *N,N*-dimethylformamide was added dropwise over 1 mL of a 2.2 g L^{−1} HRP solution in CB. Reagents were allowed to couple during 4 h at room temperature with gentle stirring. The prepared conjugate was separated from uncoupled hapten by gel chromatography as described before. The purified conjugate was diluted 1:1 (v/v) with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal, and stored at 4 °C in amber vials.

Coating OVA–AP conjugate. A 100 mM solution (50 μ L) of AP–NHS in *N,N*-dimethylformamide was added dropwise over 2 mL of a 15 g L^{−1} OVA solution in CB. The reaction was carried out during 4 h at room temperature with moderate stirring. Then, conjugates were purified by gel chromatography as

described. The conjugate was stored at −20 °C in amber vial at 1 g L^{−1} in PB containing 0.01% (w/v) thimerosal.

Generation of antibodies

Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Food, and Environment, and approved by the Ethics Committee of the University of Valencia. Two female New Zealand white rabbits weighing 1–2 kg were immunized by subcutaneous injection with 0.3 mg of BSA–AP protein conjugate in 1 mL of a 1:1 mixture of PB and complete Freund's adjuvant. Animals were boosted at 21-day intervals with the same immunogen suspended in a mixture of 0.5 mL of PB and 0.5 mL of incomplete Freund's adjuvant. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4 °C. Then, the serum was separated by centrifugation, and an antibody fraction was diluted 1/5 with PBS containing 0.01% thimerosal (w/v) and kept at 4 °C for daily usage. The remainder of each antiserum was precipitated with a solution of saturated ammonium sulfate and long-term stored at 4 °C.

Antibody-coated direct competitive ELISA

Ninety-six-well polystyrene ELISA plates were coated with 100 μ L antibody solution in CB and incubated overnight at room temperature. Coated plates were washed four times with washing solution and received, afterwards, 50 μ L per well of analyte in PBS plus 50 μ L per well of HRP–AP in PBST. 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 μ L per well of freshly prepared 2 mg mL^{−1} *o*-phenylenediamine and 0.012% (v/v) hydrogen peroxide in developing buffer. The enzymatic reaction was stopped after 10 min at room temperature by the addition of 100 μ L per well of 1 M sulfuric acid and then the absorbance was immediately read at 492 nm with a reference wavelength at 650 nm. Wine samples were conveniently diluted with deionized water and analyzed as aforementioned using HRP–AP tracer in PBS2xT buffer.

Conjugate-coated indirect competitive ELISA

Ninety-six-well polystyrene ELISA plates were coated with 100 μ L per well of OVA–AP in CB by overnight incubation at room temperature. Coated plates were washed four times with washing solution, and then received 50 μ L per well of analyte in PBS plus 50 μ L per well of antibody solution in PBST. The immunological reaction took place during 1 h at room temperature, and plates were washed again as described. Next, 100 μ L per well of a 1/10⁴ dilution of GAR–HRP conjugate in PBST-FBS was added, and plates were incubated 1 h at room temperature. Finally, the signal was generated as aforementioned. Wine samples were diluted with deionized water and analyzed using the corresponding antibody solution in PBS2xT.

ELISA data processing

Eight-point standard curves were prepared from 100 mg L⁻¹ pyrimethanil, cyprodinil, and mepanipyrim stock solutions in acetonitrile. Curves were arranged from 200 µg L⁻¹ (8-fold serial dilution and including a blank point) in PBS for assay characterization, and in deionized water for sample analysis. Individual pyrimethanil, cyprodinil, and mepanipyrim curves were prepared for preliminar assay characterization. Only pyrimethanil curves were employed for multianalyte immunoassay development. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was estimated as the concentration of the fungicide at the inflection point of the sigmoidal curve, typically corresponding to a 50% inhibition (IC₅₀) of the maximum absorbance reached at the zero dose of analyte (A_{max}) if the background signal approaches to zero.

UPLC-MS/MS procedure

A solid-phase extraction method was employed for the analysis of wine samples by UPLC-MS/MS.¹⁶ Briefly, 100 mg C₁₈ cartridge was activated with 3 mL methanol and 2 mL deionized water. Then, 2 mL wine was passed through the cartridge and it was washed with 1 mL water/methanol (9:1 v/v). Elution of fungicides was achieved with 2 mL of ethyl acetate. Finally, the extract was evaporated to dryness and reconstituted in 200 µL acetonitrile. Wine samples were previously spiked with TPP at 500 µg L⁻¹, which was employed as internal standard.

For the chromatographic separation a mobile phase consisting of 0.5% (v/v) formic acid in water (A) and acetonitrile (B) was used. The gradient started at 50% B, which was linearly increased to 95% B in 4 min, and then maintained two more minutes. The injection volume was set at 5 µL and the flow-rate was 400 µL min⁻¹. Tandem mass acquisitions were performed using electrospray ionization in positive mode with 3.5 KV capillary voltage, 120 °C source temperature and 300 °C desolvation temperature. The employed m/z transitions were: 199.9 → 107.4 + 82.1 for pyrimethanil; 226.2 → 108.1 + 93.1 for cyprodinil; 224.4 → 131.1 + 106.1 for mepanipyrim; and 328.3 → 77.0 + 152.4 for TPP.

Conclusions

A novel hapten molecule was designed and employed in the preparation of high-affinity antibodies class-specific to the anilinopyrimidine family of fungicides. High-sensitivity immunoassays were developed that recognized simultaneously pyrimethanil, cyprodinil, and mepanipyrim. The proposed direct competitive ELISA with antibody AP#1 provided an equal recognition of each anilinopyrimidine compound, with CR values close to 100%. Several tests were performed in order to assure the generic pattern of the developed immunoassay towards the three compounds. The proposed assay was fully characterized and applied to the

determination of pyrimethanil, cyprodinil, and mepanipyrim fungicides in wine samples, with excellent sensitivity (LOQ of 1 µg L⁻¹) and precision (RSD lower than 20%). Commercial wines were sampled and analyzed by the proposed immunoassay in order to quantify residues of anilinopyrimidine fungicides, and positive samples were confirmed by a reference chromatographic procedure. Thus, the use of this generic immunoassay is a promising tool for the multiresidue determination of fungicides in this valuable commodity.

Conflict of interest

There are no conflicts to declare.

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