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Moiety and linker site heterologies for highly sensitive immunoanalysis of cyprodinil in fermented alcoholic drinks

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ABSTRACT

Cyprodinil is a new-generation anilinopyrimidine fungicide widely used in crop protection and frequently found in fruits. In this study, novel derivatives of cyprodinil with linker site heterologies were synthesized and employed in order to produce antibodies with enhanced affinity. Moreover, moiety-heterologous haptens were designed and prepared for assay sensitivity improvement. Two competitive enzyme-linked immunosorbent assays for the analysis of this active substance were developed using direct and indirect formats, achieving IC_{50} values around 0.15 μg/L. Analytical figures of merit and usability of the optimized assays were evaluated with wine and cider as model food processed matrices. The obtained recoveries were from 90% to 120%, and the limit of quantification was in the 1–5 μg/L range. Finally, a monitoring study ($n = 150$) was performed to estimate the occurrence and the concentration of cyprodinil in commercial wine and cider products from different origins. We found that 28% of the analysed wine samples contained cyprodinil residues at levels higher than 5 μg/L.

1. Introduction

Anilinopyrimidines (cyprodinil, pyrimethanil, and mepanipyrim, Table 1) are new-generation and highly efficient compounds active against a broad-spectrum of fungal pests. They show a particular mode of action consisting in the inhibition of methionine biosynthesis, so they are frequently combined with other fungicides possessing a different target site. Cyprodinil was the first anilinopyrimidine active ingredient to be introduced in all European countries (Pesticide Properties DataBase). Nowadays, cyprodinil-based formulations are widely employed for plant and crop protection. According to different monitoring programs, cyprodinil is one of the most commonly found residues (EFSA, 2013; USDA, 2012). In order to regulate the presence of toxic compounds in food commodities, the European Food Safety Authority (EFSA) has established maximum residue levels (MRLs) as the upper legal concentration of a chemical to guarantee the lowest possible consumer exposure whilst reaching adequate phytosanitary efficiency (EU Pesticide Database). Those MRLs generally refer to raw or baby food products, but the legal pesticide contents for most processed foodstuff are not regulated yet in the EU. Nevertheless, different studies have confirmed the persistence of pesticides after industrial processing, for example during fermentation of grape must, with common concentrations in wine at micrograms per litre level (Cabras & Angioni, 2000; Cabras et al., 1997; Edder et al., 2009; González-Rodríguez, Cancho-Grande, & Simal-

Abbreviations: BSA, bovine serum albumin; CR, cross-reactivity; CB, coating buffer; cELISA, competitive enzyme-linked immunosorbent assay; DMF, *N,N*-dimethylformamide; GAR, goat anti-rabbit immunoglobulin; HRP, horseradish peroxidase; LOQ, limit of quantification; MR, molar ratio; OVA, ovalbumin; PB, phosphate buffer; PBS, phosphate buffered saline; PBST, PBS containing Tween 20.

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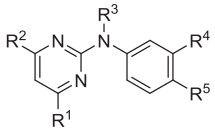
Table 1
Structures of anilinopyrimidine fungicides and synthetic haptens.



	R^1	R^2	R^3	R^4	R^5
Anilinopyrimidine					
Cyprodinil	$CHCH_2CH_2$	CH_3	H	H	H
Pyrimethanil	CH_3	CH_3	H	H	H
Mepanipyrim	$CCCH_3$	CH_3	H	H	H
Immunizing haptens					
CD*p*	$CHCH_2CH_2$	CH_3	H	H	$(CH_2)_5COOH$
CD*m*	$CHCH_2CH_2$	CH_3	H	$(CH_2)_5COOH$	H
CD*n*	$CHCH_2CH_2$	CH_3	$(CH_2)_4COOH$	H	H
CD*b*	$CHCH_2CH_2$	$(CH_2)_4COOH$	H	H	H
Moiety-heterologous haptens					
Cl*p*	Cl	CH_3	H	H	$(CH_2)_5COOH$
Cl*m*	Cl	CH_3	H	$(CH_2)_5COOH$	H

Gándara, 2011). In addition, the presence of more than one residue has been repeatedly found in such high added value goods (Carpinteiro, Ramil, Rodriguez, & Cela, 2010; Pesticide Action Network, 2008; Trosken, Bittner, & Volkel, 2005).

Several methods have been described for the detection of cyprodinil traces in wines, based on gas or liquid chromatography with mass spectrometry detection. A previous extraction step is usually employed to avoid matrix effects, such as solid-phase microextraction with divinylbenzene–carboxen–polydimethylsiloxane fibres (Rial-Otero, Yagüe-Ruiz, Cancho-Grande, & Simal-Gándara, 2002), liquid–liquid extraction (Vaquero-Fernández et al., 2008), solid-phase extraction with Oasis HLB cartridges (Fontana, Rodríguez, Ramil, Altamirano, & Cela, 2011), dispersive liquid–liquid microextraction (Rodríguez-Cabo, Rodríguez, Ramil, & Cela, 2011), or the QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure (Moeder, Bauer, Popp, van Pinxteren, & Reemtsma, 2012; Walorczyk, Drozdzynski, & Gnusowski, 2011). Also, immunoassays have been developed and validated in wines for the analysis of pesticides like benalaxyl (Rosso, Giraudi, Gamberini, Baggiani, & Vanni, 2000), tebufenozide (Irwin, Tolhurst, Jackson, & Gale, 2003), fenhexamid (Mercader & Abad-Fuentes, 2009), or bromopropylate (Ramon-Azcón, Sánchez-Baeza, Sanvicens, & Marco, 2009), showing excellent performance.

Immunoassays can be nowadays considered a mature technology for the analysis of chemical residues and contaminants in food and the environment, being the competitive enzyme-linked immunosorbent assay (cELISA) one of the most employed setups. This immunochemical method is rapid, simple, sensitive, and specific; nevertheless, when cELISAs are compared with standard chromatographic methods, the main advantages of the bioanalytical method are the high sample throughput and the minimum sample treatment. Immunoassays are strongly recommended for the analysis of a high number of samples with reduced costs (Meulenberg, Mulder, & Stoks, 1995). In a previous study, polyclonal antibodies against cyprodinil were generated for the first time by our group (Esteve-Turrillas, Agulló, Abad-Fuentes, Abad-Somovilla, & Mercader, 2012). Now, with the aim of analysing traces of cyprodinil in fermented drinks by immunoassay, we have designed two novel regioisomeric immunizing haptens with linker site heterologies in order to produce higher-affinity antibodies. Moreover, assay conjugates were prepared with new haptens bearing moiety heterologies for assay sensitivity enhancement. With those novel immunoreagents, a direct and an indirect cELISA have been optimized for the analysis of cyprodinil in white, red, and sparkling wines and in cider samples. For further validation, the developed immunoassays were applied to the analysis of cyprodinil in a variety of commercial wines from different origins.

2. Materials and methods

2.1. Reagents and instrumentation

Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidin-2-amine, CAS Registry 121552-61-2, Mw 225.29 g/mol) and other pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Horseradish peroxidase (HRP), ovalbumin (OVA), and *o*-phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain). Bovine serum albumin (BSA) was from Roche Applied Science (Mannheim, Germany). Sephadex G-25 HiTrap Desalting and HiTrap Protein G HP 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). Foetal bovine serum and Freund's adjuvants were from Sigma/Aldrich (Madrid, Spain). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Ultraviolet–visible spectra and ELISA absorbances were read (in dual wavelength mode, 492–650 nm) with a PowerWave HT microplate reader from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments. Most stable conformers were calculated using CONFLEX with MM3 molecular mechanics to systematically search for low-energy conformers, followed by geometry optimization in MOG using AM1 parameters and including aqueous solvation effects simulated by COSMO (CAChe WorkSystem Pro, version 7.5.0.85).

Different buffers were employed in this study. The composition, concentration, and pH of the employed buffers were: i) Phosphate buffer (PB), 100 mM sodium phosphate buffer (pH 7.4); ii) Phosphate buffered saline (PBS), 10 mM sodium phosphate buffer (pH 7.4) with 140 mM NaCl; iii) PBST, PBS containing 0.05% (v/v) Tween 20; iv) Carbonate buffer (CB), 50 mM carbonate–bicarbonate buffer (pH 9.6); v) Washing solution, 15 mM NaCl and 0.05% (v/v) Tween 20; vi) Developing buffer, 25 mM citrate and 62 mM sodium phosphate buffer (pH 5.4); and vii) PBST-2x, 20 mM sodium phosphate buffer (pH 7.4) with 280 mM NaCl and 0.05% (v/v) Tween 20.