Residues of anilinopyrimidine fungicides and suspected metabolites in wine samples

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Abstract

The coexistence of the anilinopyrimidine fungicides pyrimethanil (PYR) and cyprodinil (CYP), and suspected metabolites in wine samples was investigated by liquid chromatography (LC) with tandem mass spectrometry (MS/MS), based on triple quadrupole (QqQ) and quadrupole time-of-flight (QTOF) MS instruments. For the first time, quantitative data obtained after solid-phase extraction (SPE) of wine samples have demonstrated the systematic presence of 4-hydroxyanilino derivatives of PYR and CYP in wines containing residues of parent fungicides, at concentrations from 0.2 to 58 ng mL^{-1}. Higher concentration ratios (hydroxylated derivative/active fungicide) were measured in red than in white wines, particularly in case of PYR. On average, the concentrations of PYR-4OH were twice those measured for PYR in red wines. A targeted search of hydroxyl derivatives in wine extracts by LC-QTOF-MS showed the existence of additional hydroxylation positions in the pyrimidine ring and/or in the alkyl substituents bond to this cycle in the structure of both anti-botrytis fungicides. Moreover, free and glycosylated forms of the hydroxylated metabolites for both fungicides coexist in wine samples. In case of CYP, it is proved that hydroxylated and glycosylated metabolites are already present in grapes before vinification.

Keywords: wine; pyrimethanil; cyprodinil; metabolites; liquid chromatography-mass spectrometry

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1. Introduction

Fungicides are used under conventional agriculture practices to guarantee the productivity and the organoleptic quality of vinification grapes, particularly in those regions with atmospheric conditions promoting the development of fungi. However, the misuse of these compounds and the presence of fungicide residues in elaborated wines are matters of concern for environmentalists, authorities and wine consumers. Thus, the maximum residue limits (MRLs) of approved fungicides in vinification grapes have been regulated [1], studies on transfer factors from grapes to wine are available for most of the marketed fungicides [2–5] and new vine varieties resistant to relevant fungal infections, such as *mildew* and *oidium*, are under evaluation in different field trials [6,7].

The presence of fungicide residues in commercial wines has been investigated by several authors [8–10]; however, little is known about the presence of potential transformation products (TPs), either generated by the vines metabolism, or through reactions catalysed by enzymes existing in fermentation yeasts, in wine. As example, hydroxylation of the fungicide fenhexamide, as well as the conjugation of the parent compound and the hydroxylated derivative with saccarides, has been reported by Polgar and co-workers [11]. However, the extent of compound hydroxylation was less important in vinification grapes in comparison with other fruits and vegetables[¡Error! Marcador no definido.] [11]. In case of the insecticide imidacloprid (IMI), authorized for open applications until 2018, an oxidized derivative (imidacloprid olefin) was found in wine samples at higher concentrations than the active ingredient [12].

Anilinopyrimidines are a group of fungicides employed to control and/or to prevent diseases caused by *Botrytis* fungi in several crops, including vines. Pyrimethanil (PYR) and cyprodinil (CYP) are the most often used congeners of the family. In EU, their current MRLs for vinification grapes are 6000 and 3000 ng g^{-1} for PYR and CYP, respectively. PYR is transferred from grapes to wine with a yield above 90 % [13]. In case of CYP,

literature transfer factors vary in the range from 1 to 20 % [13,14]. Available information regarding the metabolization of PYR by animals, soil microorganisms and plant cultures suggests hydroxylation in both aromatic rings (benzene and pyrimidine cycles) and in the alkyl substituents bonded to the pyrimidine ring, as relevant transformation paths [15]. In fact, aryl hydroxylation is recognized as a significant route in the metabolism phase I of many aromatic pesticides (particularly herbicides) in the vegetable kingdom [16]. Hydroxylation and glycosylation processes have been also reported during *in-vitro* incubation of CYP with wheat cells and microbial cultures [17,18]. EU regulations dictate that residues of PYR in farm animals must be calculated as the sum of the parent fungicide and the 4-hydroxyanilino (PYR-4OH) derivative. For CYP, the sum of compounds (CYP and 4-hydroxyanilino metabolite, CYP-4OH) must be considered when analysing milk and honey [1]. However, for vegetable food commodities, MRLs are just defined for the free form of both fungicides and, as far as we could trace, the existence of hydroxylated forms of anilinopyrimidine fungicides in wine has not been previously reported. Thus, so far, residues of PYR and CYP in this food matrix have been estimated from concentrations measured for the commercial parent fungicides and no analytical methodology has been proposed for the simultaneous determination of their possible hydroxylated metabolites. Even more, the occurrence of these latter compounds has not been previously reported in vegetable origin food commodities.

The aim of this research is to investigate and quantify the potential co-existence of PYR, CYP, PYR-4OH and CYP-4OH in commercial wines. To this end, an analytical approach previously developed for the parent compounds was extended and validated to the above transformation products, using ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry (MS/MS) as determination technique. In addition, a hybrid quadrupole time-of-flight (QTOF) MS instrument was used for retrospective search of additional transformation products of both fungicides, including phase II metabolites generated through glycosylation reactions. Differences between

concentration ratios of both fungicides and their phase-I metabolites in red and white wine are discussed.

2. Experimental

2.1. Solvents, standards and sorbents

Methanol (MeOH) and acetonitrile (ACN), both HPLC grade, as well as, formic acid (FA), ethanol and tartaric acid were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, MA, USA). OASIS HLB solid-phase extraction (SPE) cartridges, containing 200 mg of sorbent, were purchased from Waters (Mildford, MA, USA) and employed for extraction and concentration of wine samples.

Standards of PYR, CYP and their deuterated analogues (PYR-d_5 and CYP-d_5, deuterium atoms are attached to the phenyl ring) were acquired from Sigma-Aldrich (St. Louis, MO, USA). PYR-4OH was supplied by Toronto Research Chemicals (North York, Canada) and CYP-4OH by HPC Standards GmbH (Borsdorf, Germany). Individual standard solutions of each compound were prepared in MeOH and stored at -20 ºC. Mixtures of PYR, CYP, PYR-4OH and CYP-4OH in MeOH were employed to prepare spiked wine samples used during method optimization and validation. Calibration standards were prepared in ACN:MeOH (80:20). The mixture of deuterated fungicides, employed as internal surrogates (ISs) through the analytical procedure, was also made in MeOH. Diluted calibration standards were stored at 4 ºC and used for a maximum of 2 weeks after preparation.

2.2. Samples and sample preparation

Samples employed in the current study correspond to commercial wines. Most of them were acquired from retail markets and the rest kindly provided by local associations of wine producers. Wine bottles were stored in the dark, at a temperature of 16 ºC, for a maximum of one month before opening. The extraction and concentration of fungicides

was performed immediately after opening each bottle of wine. SPE extracts were stored at -20 ºC. Sample collection and analysis, was performed during a period of two years. Only those wines containing concentrations of PYR and CYP above the LOQs of the method were considered in this research.

SPE conditions were adapted from a previous multianalyte study dealing with the determination of pesticide residues in wine[9]. That methodology was re-validated for the two commercially available hydroxylated derivatives of PYR and CYP. Under final working conditions, 10 mL of wine were diluted with ultrapure water and concentrated using a SPE cartridge previously conditioned with 5 mL of MeOH and the same volume of an ethanol:water (12:88) solution. After sample concentration, the cartridges were rinsed with 5 mL of ultrapure water and dried under a gentle stream of nitrogen.Compounds were finally recovered with 2 mL of the ACN:MeOH (80:20) mixture. The extract was homogenized, filtered (0.22 µm) and injected in the LC-MS system without any additional treatment.

Procedural blanks were carried out using aliquots of synthetic wine (ethanol:water, 12:88, containing 4 g L^{-1} of tartaric acid) spiked only with the mixture of ISs. SPE recoveries, independent of matrix effects, were calculated as the ratio between responses obtained for spiked aliquots of wine samples and SPE extracts, obtained from same wines, fortified at the end of the extraction step. Matrix effects during electrospray ionization (ESI) were estimated as the ratio between the slopes obtained from calibration curves prepared using spiked SPE extracts (from red and white wines), and those corresponding to solvent-based standards, multiplied by 100. The range of concentrations varied from 2 to 400 ng mL^{-1}, with 9 different concentration levels.

Global recoveries of the method were calculated as the normalized ratio between concentrations measured for spiked and non-spiked aliquots of different wines and added values. Concentrations in sample extracts were determined using solvent-based calibration standards, after correcting the responses measured for each compound with

those of ISs (PYR-d_5 for PYR and PYR-4OH, and CYP-d_5 for CYP and CYP-4OH, respectively). When assessing the recoveries at low addition levels, ecological production wines were selected in order to minimise the content of native compounds in non-spiked samples.

Grapes (*Mencía* variety) were provided by a local farmer who declared a single treatment with a commercial mixture of CYP and Fludioxonil (*Switch*, Syngenta), at the maximum recommended dose, 33 days before harvest. Grapes (around 5 kg of bunches from 4 different vines) were de-stemmed, homogenized and extracted using the same mixture of solvents employed during elution of SPE cartridges. Around 2 g of the homogenized slurry was spiked with the ISs and shaken with 10 mL of solvent for 10 min. An aliquot of the upper phase was filtered (0.22 µm pore size filters) and injected in the LC-MS systems employed in the study. Grape extracts were considered only for semi-quantitative purposes; thus, the extraction yield of the above approach was not evaluated.

2.3. Determination conditions

Two different LC-MS systems were employed in this study. Target determination of anilinopyrimidine fungicides and their 4-hydroxyanilino derivatives was performed using a LC-MS/MS XEVO TQD, triple quadrupole mass spectrometer, from Waters (Milford, MA, USA). Search of additional metabolites of PYR and CYP in wine extracts was carried out using a hybrid QTOF-MS (6550 model) acquired from Agilent (Wilmington, DE, USA). Both MS instruments were furnished with an ESI ionization source working in positive mode and coupled to UPLC systems from same supplier as the MS instrument (Acquity from Waters and Agilent 1290 models). LC separations were carried out under identical conditions in both instruments. To this end, an UPLC Zorbax Eclipse Plus C_{18}, Rapid Resolution column (50 mm x 2.1 mm, 1.8 µm particle size), connected to a C_{18} 2.1 mm i.d. guard cartridge from Phenomenex (Torrance, CA, USA) was employed. The column was maintained at 40 ºC, using ACN (B) and ultrapure water (A), both 0.1 % in FA, as

mobile phases at 0.4 mL min^{-1}. The injected volume was 1 µL and the composition of the mobile phase was programmed as follows: 2 % B (0 min), 30 % B (5 min), 50 % B (7-8 min), 100 % B (9-10.5 min), 2 % B (11-14 min).

Using the LC-QqQ-MS instrument, two transitions were selected per compound and ISs. These transitions were considered for quantification (Q1) and qualification (Q2) purposes attending to their relative intensities. Identification of target species in wine extracts is based on retention time and Q2/Q1 ratio match with solvent-based standards. Maximum differences were set at 0.1 min and ± 30 % of the average Q2/Q1 ratio in calibration standards.

The LC-QTOF-MS system operated in the 2 GHz acquisition mode, offering a mass resolution of 17000 (FWHM) for ions with *m/z* ratios in the range from 200 to 300. The *m/z* axis was continuously recalibrated using reference ions at *m/z* of 121.05087 and 922.00979. MS spectra were acquired in the range of *m/z* values from 50 to 1700, at a frequency of 1 Hz. Product ion scan spectra were recorded in the range from 40 to 700, at a frequency of 4 Hz, considering different collision energies. This system was employed to search additional hydroxylated derivatives of CYP and PYR, as well as their glycosylated forms, produced during phase II of metabolism. Sample (wines or grapes) extracts were first injected in the MS mode to identify the retention time of the potential derivatives of parent fungicides. Selective chromatograms for their pseudo-molecular ($[M+H]^+$) ions were extracted with a mass window of 10 ppm. Thereafter, the product ion scan spectra of candidate peaks were acquired in a 2nd injection. Fragmentation patterns observed in these spectra were compared to those recorded for known compounds in order to propose the chemical structure of the additional metabolites.

3. Results and discussion

3.1. Optimization of determination conditions

Table 1 summarizes retention times, ionization and quantification parameters corresponding to parent fungicides, their isotopically labelled analogues and the commercially available hydroxylated derivatives. Compounds were separated in less than 8 min, with a total LC analysis time of 14 min. The relative intensity of the 2nd transition for each compound stayed above 0.39, and linear responses were attained for standards in the range of concentrations from 1 to 400 ng mL^{-1}. The instrumental limits of quantification (LOQs) of the UPLC-ESI-MS/MS (QqQ) system varied between 0.4 to 0.9 ng mL^{-1}. These values were calculated as the lowest concentration providing a peak with a signal to noise (S/N) ratio of 10 for the qualifying (Q2) transition.

SPE was selected as sample preparation technique to extract and concentrate compounds from wine. ACN and ACN:MeOH (80:20) mixtures were investigated for compounds elution. The first solvent permitted the effective elution of the two parent fungicides, leading to free-pigment extracts even from red wines; however, PYR-4OH and CYP-4OH required up to 10 mL of ACN for their complete desorption. Addition of a 20 % of MeOH to the elution solvent permitted to recover all the compounds from the SPE sorbent using just 2 mL. Breakthrough studies, considering 10 mL of wine diluted with the same volume of ultrapure water, showed that all compounds were quantitative retained in the 200 mg cartridge (data not shown). Recoveries of the SPE process, without considering MEs, were estimated for samples spiked at 20 ng mL^{-1} (equivalent to 100 ng mL^{-1} in the SPE extract). Spiked and non-spiked fractions of each wine were processed in triplicate. SPE recoveries were in the range of 92-101 %, with relative standard deviations (RSDs) between 2 and 3 %, Table 2. The MEs, calculated as described in section 2.2, varied between 88 and 112% (Table 2), which points out to

minor changes in the efficiency of compounds ionization between wine extracts and solvent standards.

The accuracy of the procedure was investigated with spiked samples of different wines considering three different addition levels: 4, 10 and 20 ng mL^{-1} for analytes involved in the quantitative study. ISs were also added to samples at a concentration of 20 ng mL^{-1}. Responses obtained for each compound were corrected with ISs and compared to those obtained for solvent-based standards. Recoveries stayed between 70 and 107 % depending on the considered compound and the addition level, Table 3. In summary, after ISs correction, solvent-based standards are suitable for the accurate quantification of wine samples. Procedural blanks were prepared using 10 mL of synthetic wine with addition of ISs. Contamination problems were not detected in the SPE extracts. The calculated LOQs of the method were in the range of 0.1 and 0.2 ng mL^{-1} depending on the compound. The linear response range of the procedure, referred to wine samples, extends up to 80 ng mL^{-1}.

3.2. Levels of fungicides and 4-hydroxyanilino derivatives in wine samples

The validated methodology was applied to the analysis of selected compounds in commercial wine samples. Most of them correspond to wines elaborated in Galicia (Spain). Table S1 shows the selection of samples containing concentrations of, at least, one of the parent fungicides above the LOQs of the method. The list of analysed samples corresponds to wines produced between years 2014 and 2018, including 28 whites and 32 red wines, and the measured concentrations are presented as supplementary information (Table S1). A summary of the obtained values is shown in Table 4. The highest concentrations of PYR and CYP in the set of processed wines stayed below 100 and 32 ng mL^{-1}, which represents less than 2 % of MRLs set by the EU in vinification grapes (6000 and 3000 ng g^{-1} for PYR and CYP respectively). The 4-hydroxyanilino

derivatives (PYR-4OH and CYP-4OH) were found nearly in all the wines containing residues of the commercial fungicides. To the best of our knowledge, data summarized in Table 4 represent the first measurements of PYR-4OH and CYP-4OH in the vegetable kingdom.

Concentration plots of PYR-4OH and CYP-4OH versus those corresponding to parent fungicides are shown in Fig. 1A and Fig. 1B for white and red wines, respectively. In the first case, the levels of hydroxylated transformation products represented on average 20 and 12 % of those measured for PYR and CYP, respectively. However, for the pair CYP-4OH/CYP shows a relatively poor correlation in the white wine matrix (R^2 0.365). For red wines, higher concentration ratios were noticed. The levels of CYP-4OH in the processed wines represented around 50% of those obtained for CYP, whereas, on average the concentrations of PYR-4OH were twice those of PYR.

3.3. LC-QTOF-MS investigation of additional derivatives

SPE extracts from wine samples, and the available standards (PYR, CYP, PYR-4OH and CYP-4OH) were injected in the LC-QTOF-MS system in order to investigate the presence of additional hydroxylated species and/or their glycosylated forms. As previously commented, the formation of these species has been already proved with *in-vitro* experiments using microbial cultures[¡Error! Marcador no definido.], but never evaluated in the metabolism of vines. Injections were made operating the ESI source in positive and negative modes (in different runs); however, only relevant signals (chromatographic peaks) were obtained under positive polarity. Table 5 shows the chromatographic and spectral data of detected compounds. The latter include the mass errors for their pseudo-molecular ($[M+H]^+$) ions, and a normalized score (0-100) corresponding to the match between the cluster of ions for the protonated species in experimental and theoretical spectra. Above information is also given for PYR-4OH and CYP-4OH. Despite using the same column and gradient than in the LC-QqQ-MS instrument, the retention times of these two compounds were shifted to slightly lower values than those obtained with the

LC-QqQ-MS system (Table 1). Such difference is probably related to the different design of each UPLC instrument.

Data compiled in Table 5 point out to the existence of two additional hydroxylated derivatives of PYR (apart from PYR-4OH) and three analogues of CYP-4OH. In both cases, the new hydroxylated compounds displayed longer retention times than those obtained for 4-hydroxyanilino species. Mass errors associated to the $[M+H]^+$ ion in their spectra stayed below 8 ppm, the normalized score (accounting for mass accuracy and isotopic profile) varied from 75 to 99. Mass accuracy and normalized scores in the same range of values were noticed for potential glycosylated forms (phase II metabolism) of hydroxylated derivatives: PYR-TP378 and CYP-TP404A to CYP-TP404D.

Further information regarding the structures of species in Table 5 was inferred from their product ion scan spectra, comparing fragment ions with those observed for species with known structures: PYR, CYP, PYR-4OH and CYP-4OH. The spectrum of PYR-TP378 shows a single fragment corresponding to PYR-OH ($[M+H]^+$ ion), with removal of the sugar moiety ($C_6H_{10}O_5$), Fig. S1A. Same transition was noticed in the spectra of CYP-TP404A to CYP-TP404D, Fig S1B. Attending to their retention times, we deem that PYR-TP378 and CYP-TP404A are the glycosylated analogues of PYR-4OH and CYP-4OH, respectively; however, confirming such statement requires recording their MS^3 spectra (for comparison with the MS^2 ones of PYR-4OH and CYP-4OH), which is beyond the possibilities of LC-MS instruments employed in this study.

Fig. 2A shows the spectrum for PYR-TP216B, with the structure proposed for this compound and some of its relevant fragments. PYR-TP216B follows parallel fragmentation routes to those identified in the spectra of PYR and PYR-4OH, Fig. S2. One of these routes involves cleavage of the carbon nitrogen bond between aniline and pyrimidine rings to render fragments at *m/z* 107.0604 (pyrimidine cycle) and *m/z* 93.0573 (aniline, as radical cation), Fig. S2A. The 2nd competitive route consists of re-arrangement of both rings with removal of ammonia (NH_3) and methyl (CH_3) moieties

(Fig. S2A). So, for PYR-4OH, the first route renders an ion at *m/z* 109.0522, instead of that at 93.0573 appearing in the spectrum of PYR (Fig. S2B). The spectrum in Fig. 2A contains ions at *m/z* 93.0575 and 123.0543. These two fragment ions, together with the absence of that at *m/z* 107.0604, mean that the hydroxyl group is attached to the pyrimidine ring. Fragments at higher *m/z* values showed consecutive losses of NH_3 and CH_2O, instead of CH_3, as observed for PYR and PYR-4OH (Fig. S2). Thus, PYR-TP216B is hydroxylated in one of the methyl substituents of the pyrimidine cycle.

PYR-TP216A displayed a different product ion spectrum (Fig. 2B) than the other two hydroxylated forms of PYR. Fragmentation of the $[M+H]^+$ ion of this compound needed less energy than the rest of PYR derivatives rendering a loss of H_2O (216.1122>198.1022), as the first and the most intense transition. Neither cleavage of the bond between aniline and pyrimidine cycles, nor rearrangement of the protonated molecular ion were noticed in the spectrum of this compound, Fig. 2B. Instead, after removal of H_2O, the positive charge of the product ion is stabilized in the pyrimidine ring. Further losses involve removal of CH_3 (198.1022>183.0785) and HCN (183.0785>156.0681), or HCN (198.1022>171.0907) followed by CH_3 (171.0907>156.0681). On the basis of such fragmentation pattern, we propose that PYR-TP218C is hydroxylated in the non-substituted carbon of the pyrimidine ring.

Hydroxylation positions of CYP are expected to be equivalent to those reported for PYR. However, the product ion scan spectra of CYP derivatives are more complex than those of PYR, with many product ions of low intensity. Thus, the tentative identification of the exact positions of hydroxyl groups, based on fragment ions, was possible just in case of CYP-TP242B. Comparison between the spectrum of this compound (Fig. 3B) with that obtained for a commercial standard of CYP-4OH (Fig. 3A) showed some relevant differences. As example, the spectrum of CYP-TP242B did not contain the characteristic fragment of the hydroxylated aniline ring (*m/z* 109.0516), so hydroxylation is expected to take place in the pyrimidine ring. The removal of H_2O (242.1287>222.1025) followed by

replacement of the methyl group by hydrogen in the spectrum of CYP-TP242B are compatible with the introduction of an additional bond in the cyclopropane cycle. Another specific fragment in the spectrum of this compound is that at *m/z* 131.0599, again compatible with the pyrimidine cycle after water removal. Thus, CYP-TP242B is assumed to be hydroxylated in one of the carbons forming the cyclopropane cycle in the molecule of CYP.

The spectra of CYP-TP242C is given as supplementary information, Fig. S3. The structure tentatively proposed for this compound corresponds to hydroxylation in the aromatic carbon of the pyrimidine ring. Finally, the response obtained for CYP-TP242D was too weak to record its product ion scan spectra.

3.4. Origen of anilinopyrimidine metabolites

Free and glycosylated forms of the hydroxyl derivatives of PYR and CYP might be produced at vines and/or they might be generated through enzymatic reactions occurring during must fermentation. Considering that for red wines, the concentration of PYR-4OH was, in general, higher than that of PYR (see Fig. 1) and that the parent fungicide has a transfer factor from grapes to wine around 0.9 [**¡Error! Marcador no definido.**], it is evident that formation of PYR-4OH already starts at vines, and both compounds are necessarily present in grapes before fermentation.

In case of CYP, extraction of grapes treated with a commercial formulation including this fungicide was performed as described in the experimental section. Average concentrations of CYP and CYP-4OH in the grapes sample were 1898 ± 93 and 21.5 ± 2.3 ng g^{-1}, respectively. Thus, although the accuracy of compounds extraction was not evaluated, it is obvious that hydroxylation reactions start in vines. Fig. 4 shows the extracted ion chromatograms (EICs) corresponding to $[M+H]^+$ ions of hydroxylated (A) and glycosylated (B) forms of CYP in the extracts from these grapes. In the 1st case, Fig.

4A, the most intense peak corresponded to CYP-4OH. Apparent signals for the four conjugated forms compiled in Table 4 are also evident, with intensities higher than those observed for free hydroxylated species.

Even assuming that formation of hydroxyl and glycosylated metabolites starts at vines, and that the transfer factors from grapes to wine are different to those of active fungicides, there is not a clear explanation why the ratios between 4-hydroxyanilino derivatives and parent fungicides varied significantly between red and white wines. A possible explanation is that free and glycosylated forms of the hydroxyl derivatives of CYP and PYR are exchangeable species, as reported in case of terpenoid compounds [19]. So, the balance between both forms might vary depending on wine elaboration conditions. These balances might affect also the ratio between 4-hydroxyaniline species and free fungicides.

The Box-Whisker plots for the response ratio between the peak assigned to the glycosylated form of CYP-4OH (CYP-TP404A) and the free compound, as well those for the pair PYR-TP378/PYR-4OH, are shown in Fig. 5. In case of red wines, the ratio CYP-TP404/CYP-4OH is only slightly higher than the observed for PYR-TP378/PYR-OH (Fig. 5A). For white wines, the difference is evident. So, the lowest response ratios for CYP-4OH/CYP versus PYR-4OH/PYR (see Fig. 1) might suggest that the equilibrium between the glycosylated and free forms of CYP-4OH is shifted to the first species, whilst the opposite behaviour occurs for the pair PYR-TP378/PYR-4OH. Obviously, data in Fig. 5 have just a qualitative significance, since they correspond to peak areas, measured for the most intense MS/MS transition, and they do not represent concentration values.

4. Conclusions

Herein, for the first time, a sensitive procedure for the simultaneous quantification of the 4-hydroxyanilino metabolites of PYR and CYP, and the parent fungicides, in wine

samples is developed and validated. Moreover, the co-occurrence of both kinds of compounds (free and 4-hydroxyanilino forms) is reported through analysis of a relevant number of commercial wines, produced in different years. Ratios between 4-hydroxyanilino species and parent compounds were higher in red than in white wines. Moreover, PYR-4OH showed higher concentration levels than PYR in red wines. Thus, the sum of concentrations for parent and 4-hydroxyanilino species is recommended to estimate their total residues. Qualitative data obtained in this study demonstrate, again for the first time, that hydroxylation takes place also in different positions of the pyrimidine ring of parent fungicides, and also that these compounds are already present in grapes treated with parent fungicides (proved for CYP). In addition, the hydroxyl derivatives of anilinopyrimidine fungicides coexist in wine with their glycosylated forms. Further research is required to understand those parameters controlling the extent of hydroxylation and glycosylation reactions at vines and during wine elaboration, as well as the relative toxicity of the metabolites described in this research versus that of parent fungicides sprayed in vineyards.

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Table 1. LC-ESI-MS/MS determination parameters, including quantification (Q1) and qualification (Q2) ions, linearity assessment and instrumental LOQs values corresponding to the UPLC-QqQ-MS instrument.

Compound	Retention time (min)	$[M+H]^+$ ion	Cone Voltage (V)	Q1 (CE)	Q2(CE)	Q2/Q1 ratio	Linearity (1-400 ng mL^{-1}, R^2)	LOQs (ng mL^{-1})
PYR	5.67	200.1	51	107.1 (24)	82.1 (24)	0.76	0.996	0.5
CYP	7.08	226.1	56	93.1 (33)	108.1 (25)	0.72	0.996	0.4
PYR-4OH	3.01	216.1	55	107.1 (25)	82.1 (28)	0.75	0.995	0.8
CYP-4OH	4.24	242.1	60	93.1 (34)	109.1 (30)	0.39	0.998	0.9
PYR-d_5	5.64	205.1	51	107.1 (24)	82.1 (24)	0.82	-	-
CYP-d_5	7.03	231.2	56	93.1 (33)	108.1 (25)	0.53	-	-

CE, collision energy.

Table 2. Summary of SPE recoveries and matrix effects (MEs, %) of the analytical procedure.

Compound	Recoveries (%, SDs)		MEs (%, SDs)	
	Red wine	White wine	Red wine	White wine
PYR	101 (3)	98 (3)	106 (4)	109 (4)
CYP	99 (3)	99 (2)	112 (6)	108 (4)
PYR-4OH	98 (3)	99 (3)	95 (6)	95 (6)
CYP-4OH	92 (2)	98 (2)	88 (2)	94 (6)

Table 3. Global recoveries (%) of the method, with standard deviations, for wine samples spiked at 3 different concentration levels. Values obtained using solvent-based calibration standards, after ISs correction.

Compound	Recoveries (%, SD)						LOQs (ng mL^{-1})
	4 ng mL^{-1}		10 ng mL^{-1}		20 ng mL^{-1}		
	Red wine	White wine	Red wine	White wine	Red wine	White wine	
PYR	70 (1)	86 (8)	103 (1)	107 (2)	73 (1)	81 (1)	0.1
CYP	87 (2)	88 (9)	101 (1)	107 (1)	87 (2)	83 (3)	0.1
PYR-4OH	78 (2)	80 (9)	97 (3)	85 (2)	72 (2)	83 (4)	0.2
CYP-4OH	87 (2)	90 (9)	101 (1)	82 (2)	83 (1)	87 (1)	0.2

Table 4. Summary of concentrations in commercial wine samples. Data obtained for a set of 60 wines processed in duplicate. Values in ng mL^{-1}.

Compound	Average	Median	Maximum	Positive samples
PYR	18.7	8.0	99.6	54
CYP	7.9	7.6	31.9	50
PYR-4OH	11.4	4.6	58.0	54
CYP-4OH	2.7	1.7	17.3	45

Table 5. Summary of potential transformation products of PYR and CYP identified by LC-QTOF-MS.

Parent fungicide	TP	Formula	$[M+H]^+$ Calculated mass (Da)	Retention time (min)	$[M+H]^+$ Experimental Mass (Da)	Mass error (ppm)	Normalized score (0-100)
PYR	PYR-4OH	$C_{12}H_{13}N_3O$	216.1131	2.55	216.1128	-1.4	95
	PYR-TP216A	$C_{12}H_{13}N_3O$	216.1131	4.50	216.1122	-4.2	96
	PYR-TP216B	$C_{12}H_{13}N_3O$	216.1131	4.71	216.1117	-2.8	97
	PYR-TP378	$C_{18}H_{23}N_3O_6$	378.1660	2.17	378.1654	-1.6	94
CYP	CYP-4OH	$C_{14}H_{15}N_3O$	242.1288	3.96	242.1287	-0.4	94
	CYP-TP242A	$C_{14}H_{15}N_3O$	242.1288	6.30	242.1292	1.7	99
	CYP-TP242B	$C_{14}H_{15}N_3O$	242.1288	6.60	242.1270	-7.4	97
	CYP-TP242C	$C_{14}H_{15}N_3O$	242.1288	6.81	242.1290	0.8	75
	CYP-TP404A	$C_{20}H_{25}N_3O_6$	404.1816	2.97	404.1814	-0.5	76
	CYP-TP404B	$C_{20}H_{25}N_3O_6$	404.1816	4.94	404.1815	-0.2	94
	CYP-TP404C	$C_{20}H_{25}N_3O_6$	404.1816	5.03	404.1820	1.0	88
	CYP-TP404D	$C_{20}H_{25}N_3O_6$	404.1816	5.28	404.1808	-2.0	87

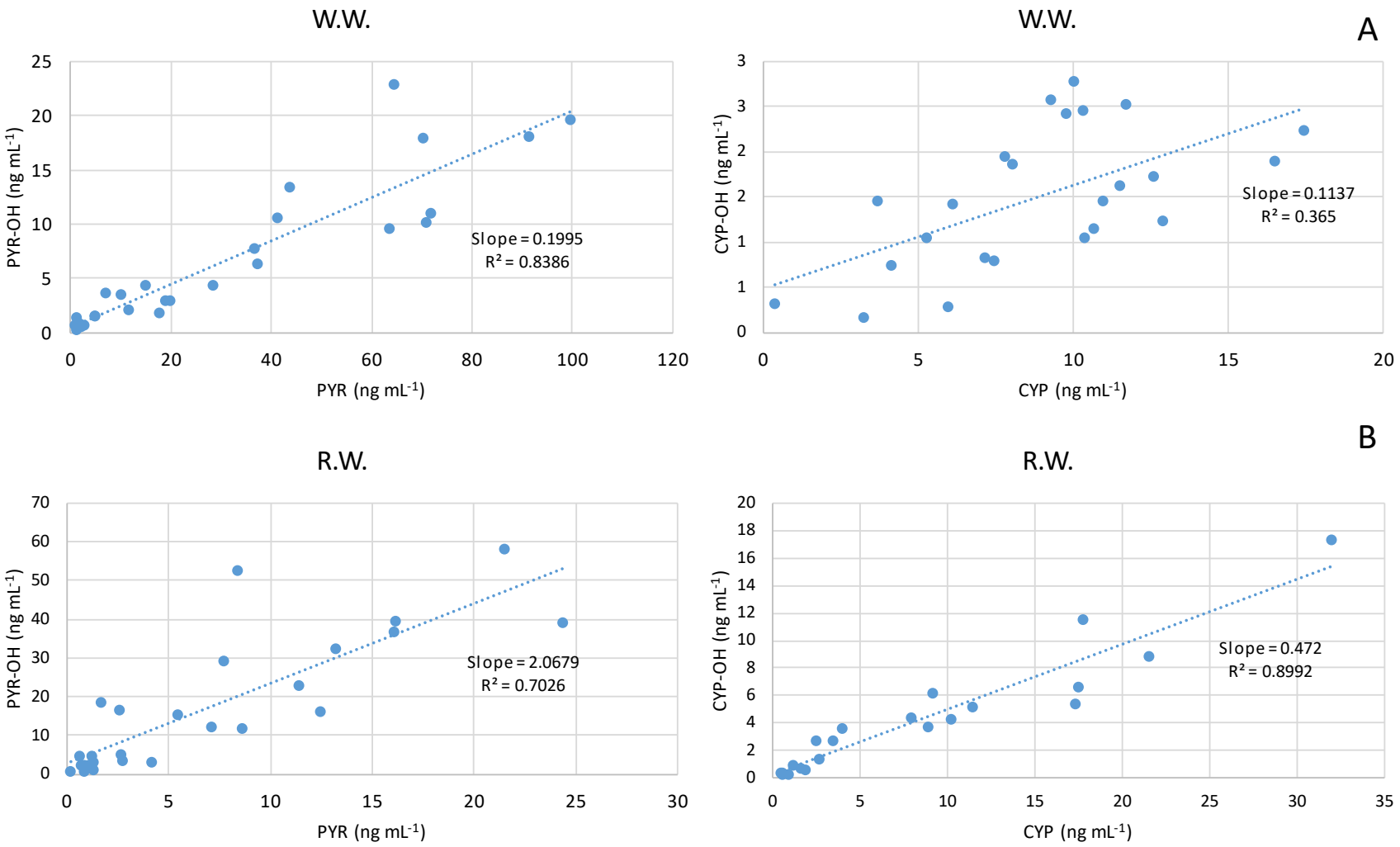

Fig. 1. Plots of concentrations for PYR-4OH/PYR and CYP-4OH/CYP in white wine (W.W.) (A) and red wines (R.W.) (B).

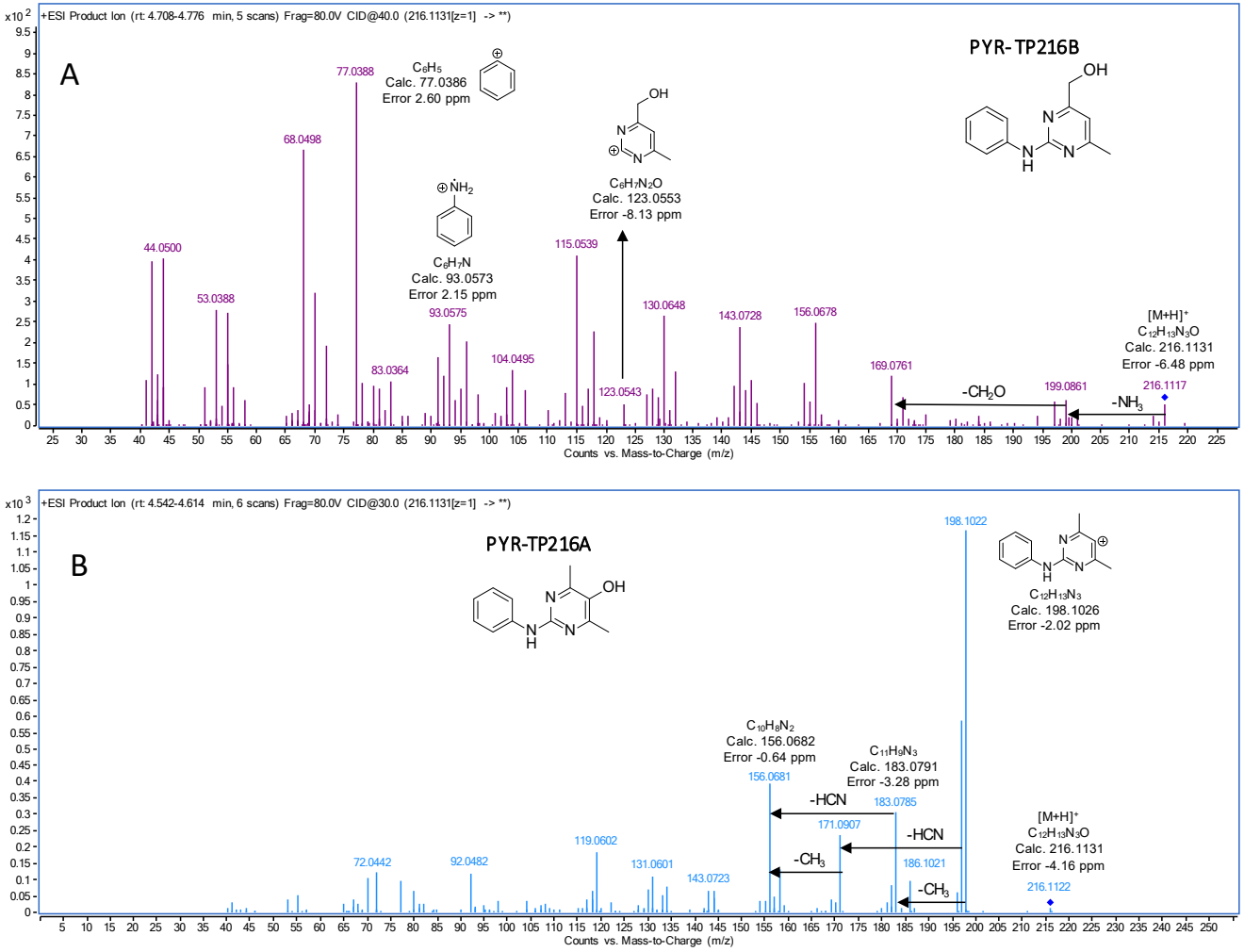

Fig. 2. Product ion scan spectra of PYR-TP216B (A) and PYR-TP216A (B).

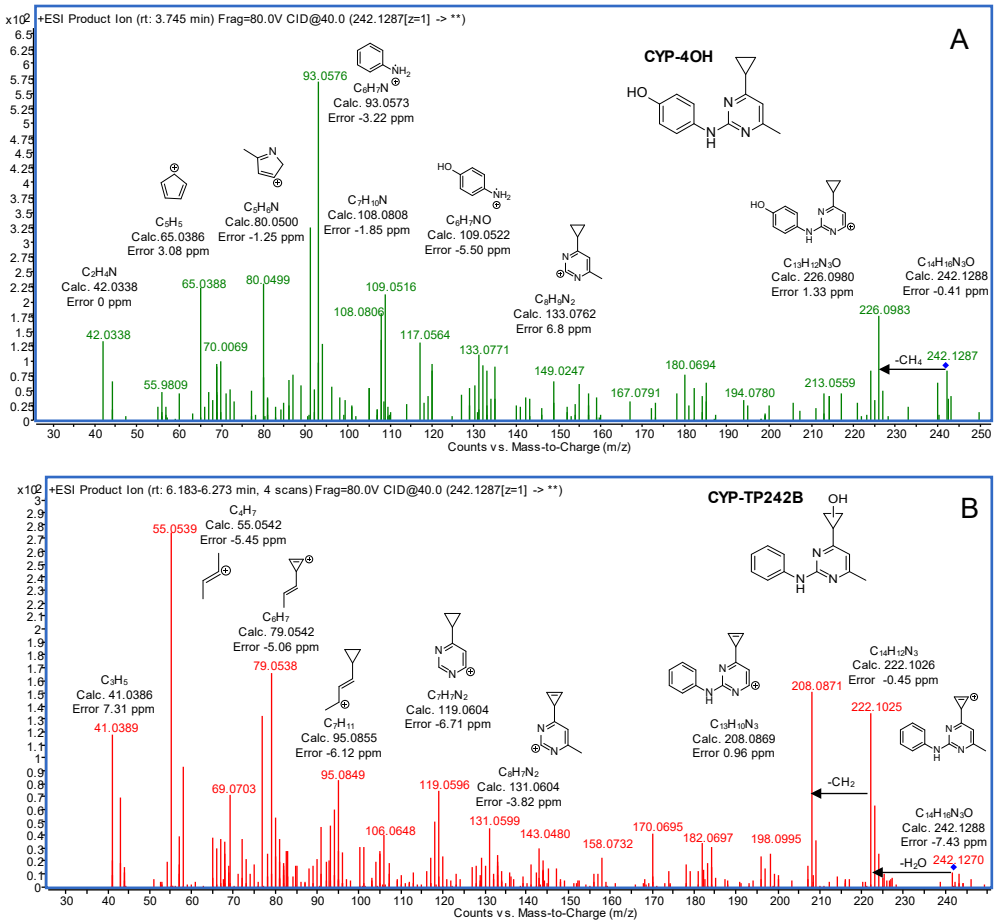

Fig. 3. Product ion scan spectra of CYP-4OH (A) and CYP-TP242B (B).

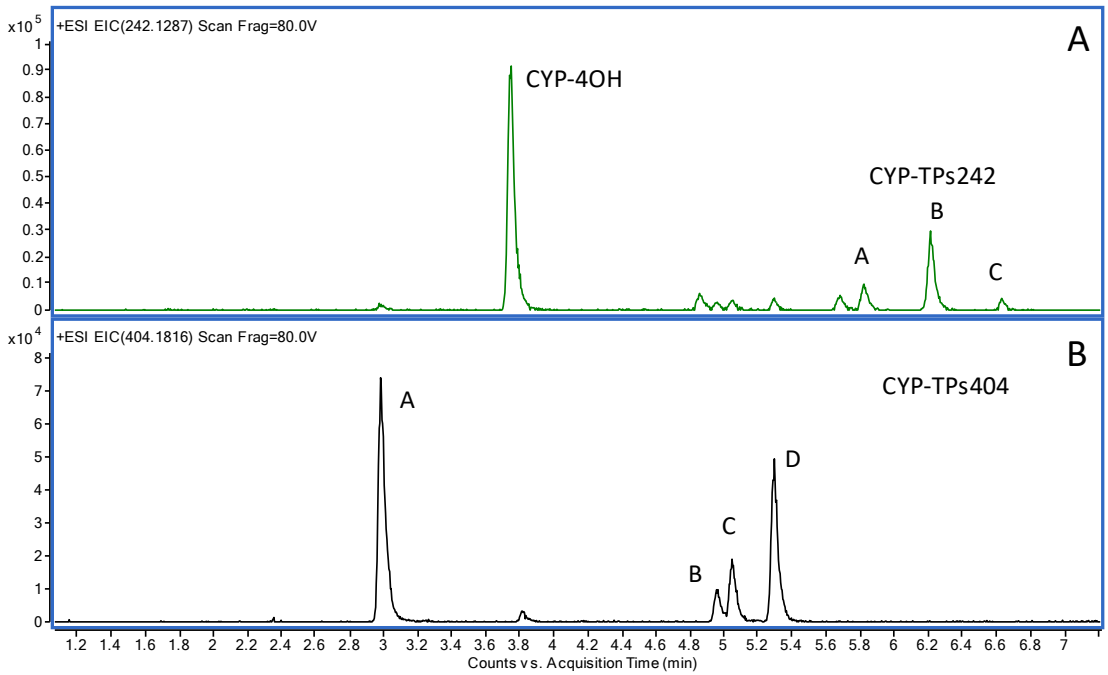

Fig. 4. Extracted ion chromatograms (extraction window 10 ppm) for the $[M+H]^+$ ion of hydroxylated (A) and glycosylated (B) derivatives of CYP in the extract from red grapes.

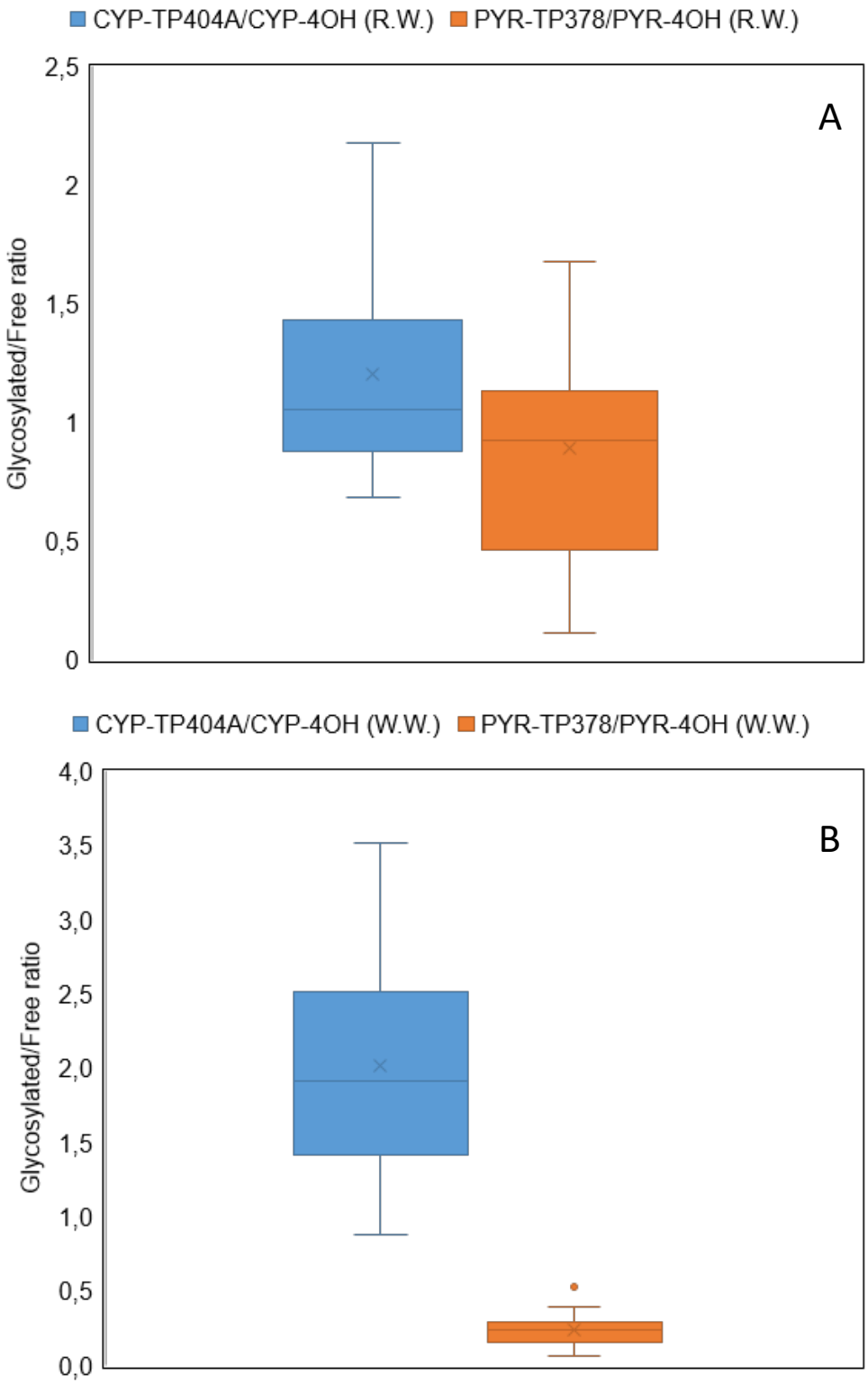

Fig. 5. Box-Whisker plots for response ratios of CYP-TP404A/CYP-4OH and PYR-TP378/PYR-4OH in red (A) and white (B) wines.

Supplementary information to manuscript:

Residues of anilinopyrimidine fungicides and suspected metabolites in wine samples

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Table S1. Summary of concentrations measured in wine samples. Average values for duplicate extractions. W, white wines; R, red wines.

Sample code	Production year	Origin	Concentration (ng mL^{-1})			
			PYR	CYP	PYR-4OH	CYP-4OH
W1	2016	SPAIN	99.6	9.2	19.6	2.6
W2	2016	SPAIN	6.8	0.4	3.7	0.3
W3	2016	SPAIN	71.6	10.3	11.0	2.5
W4	2016	SPAIN	64.4	11.7	22.9	2.5
W5	2016	SPAIN	37.1	10.9	6.3	1.5
W6	2016	FRANCE	1.2	n.d.	1.3	n.d.
W7	2017	SPAIN	1.0	5.2	0.5	1.0
W8	2017	SPAIN	0.7	0.4	0.7	n.d.
W9	2017	SPAIN	1.9	6.1	0.5	1.4
W10	2017	SPAIN	11.6	9.7	2.0	2.4
W11	2018	SPAIN	19.6	17.4	3.0	2.2
W12	2018	SPAIN	36.7	10.3	7.7	1.1
W13	2018	SPAIN	91.4	7.8	18.0	2.0
W14	2018	SPAIN	4.9	11.5	1.5	1.6
W15	2018	SPAIN	4.7	16.5	1.5	1.9
W16	2018	SPAIN	18.8	3.2	2.9	0.2
W17	2018	SPAIN	70.3	12.6	18.0	1.7
W18	2018	SPAIN	17.5	8.0	1.8	1.9
W19	2018	SPAIN	1.1	10.0	0.3	2.8
W20	2017	SPAIN	9.9	0.4	3.5	n.d.
W21	2017	SPAIN	70.8	12.9	10.2	1.2
W22	2017	SPAIN	63.5	10.6	9.5	1.1
W23	2017	SPAIN	1.6	n.d.	0.8	n.d.
W24	2017	SPAIN	15.0	3.7	4.4	1.4
W25	2017	SPAIN	2.5	4.1	0.7	0.7
W26	2017	SPAIN	43.7	7.1	13.4	0.8
W27	2017	SPAIN	41.1	7.4	10.6	0.8
W28	2017	SPAIN	28.2	5.9	4.4	0.3

Table S1. Cont.

Sample code	Production year	Origin	Concentration (ng mL^{-1})			
			PYR	CYP	PYR-4OH	CYP-4OH
R1	2016	FRANCE	0.2	0.4	0.6	0.3
R2	2016	SPAIN	11.4	31.9	22.7	17.3
R3	2019	SOUTH AFRICA	n.d.	0.5	n.d.	0.2
R4	2016	FRANCE	1.3	2.4	4.6	2.7
R5	2015	CHILE	1.3	0.8	1.0	0.3
R6	2016	SPAIN	13.2	17.3	32.4	5.3
R7	2016	SPAIN	2.6	4.0	4.9	3.6
R8	2016	SPAIN	0.8	n.d.	2.4	n.d.
R9	2016	SPAIN	21.5	7.9	58.0	4.4
R10	2016	SPAIN	12.4	21.5	16.1	8.8
R11	2016	SPAIN	7.1	11.4	12.1	5.1
R12	2014	SPAIN	2.7	n.d.	3.6	n.d.
R13	2015	SPAIN	n.d.	9.1	n.d.	6.2
R14	2016	SPAIN	n.d.	3.4	n.d.	2.7
R15	2017	SPAIN	16.0	n.d.	36.8	n.d.
R16	2017	SPAIN	n.d.	n.d.	n.d.	n.d.
R17	2017	SPAIN	5.5	n.d.	15.2	n.d.
R18	2017	SPAIN	4.2	1.0	3.0	n.d.
R19	2017	SPAIN	n.d.	2.7	n.d.	1.4
R20	2017	SPAIN	16.1	5.3	39.6	n.d.
R21	2015	SPAIN	24.4	1.6	39.2	0.6
R22	2016	SPAIN	n.d.	17.5	n.d.	6.6
R23	2016	SPAIN	8.6	1.1	11.6	0.9
R24	2018	SPAIN	1.1	17.7	2.2	11.5
R25	2017	SPAIN	0.7	0.5	2.2	0.3
R26	2017	SPAIN	0.8	n.d.	0.7	n.d.
R27	2017	SPAIN	0.6	n.d.	4.7	n.d.
R28	2017	SPAIN	8.4	10.2	52.7	4.2
R29	2017	CHILE	1.3	0.3	3.2	n.d.
R30	2017	SPAIN	2.6	1.9	16.6	0.6
R31	2017	SPAIN	1.7	n.d.	18.4	n.d.
R32	2017	SPAIN	7.7	8.9	29.3	3.7

n.d., not detected

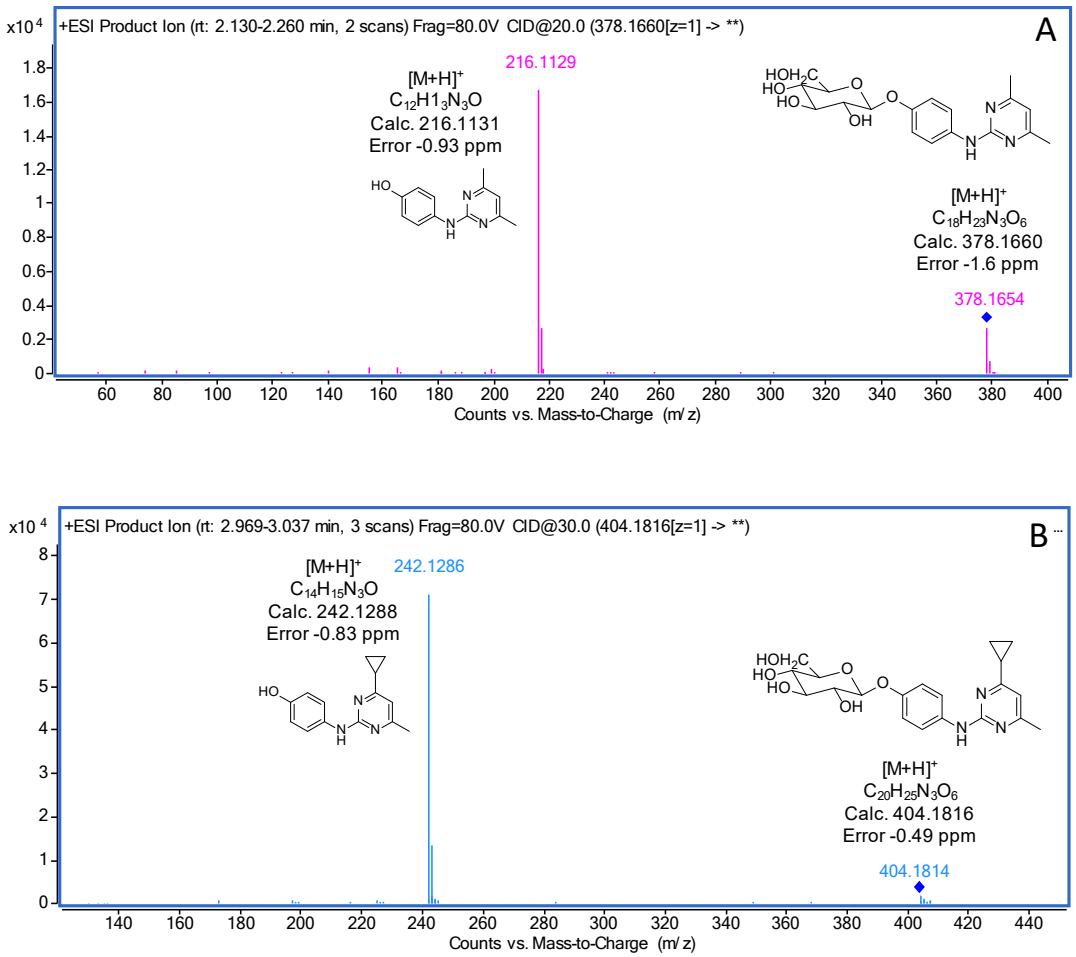

Fig. S1. Product ion scan spectra of PYR-TP378 and CYP-TP404A.

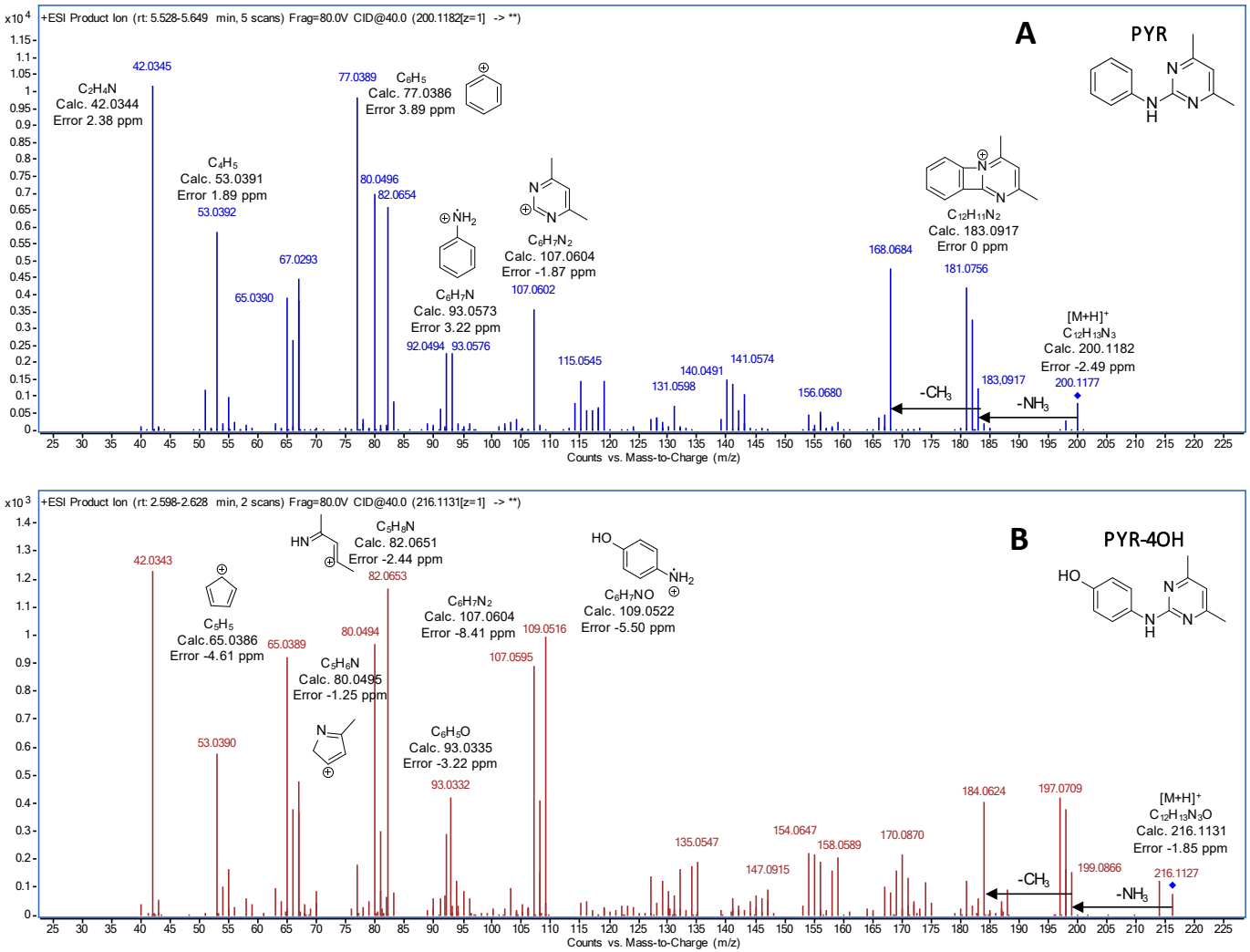

Fig. S2. Product ion scan spectra for PYR (A) and PYR-4OH (B).

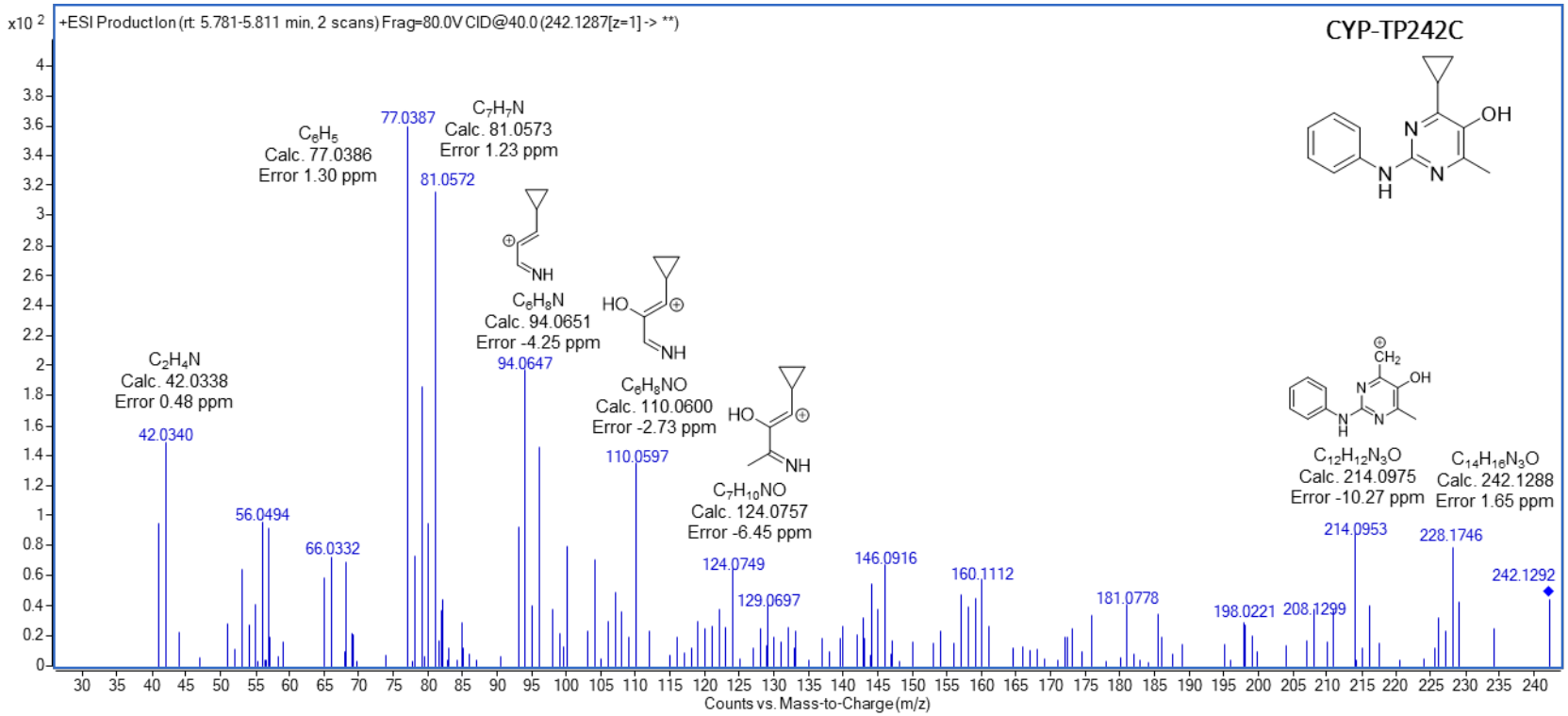

Fig. S3. Product ion scan spectra for CYP-TP242C