Fungicide multiresidue monitoring in international wines by immunoassays

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Abstract

Azoxystrobin, boscalid, cyprodinil, fenhexamid, and pyrimethanil are new generation fungicides extensively employed in order to combat diseases affecting vineyards worldwide. Owing to their physico-chemical characteristics, residues of these compounds on grapes are transferred to must and wine. In this study, a survey of the occurrence of these fungicides in international wines was carried out by using rapid antibody-based assays. Results are discussed as a function of wine type and sample geographical origin. 44.4% of the samples contained at least one of the targets (>10 µg L^{-1}). Fungicide residue occurrences were 22.4%, 19.2%, 18.8%, 6.8%, and 1.2% for pyrimethanil, boscalid, fenhexamid, cyprodinil, and azoxystrobin, respectively, while residue contents higher than 100 µg L^{-1} were found in 8.4% of the samples. This study evidences that contamination of commercial wines with pesticides is an issue of worldwide relevance with potential implications for consumer health and international trade.

Keywords

Fungicide, Wine, Residues, ELISA, Food quality, Food safety, hapten; rapid method

Chemical compounds studied in this article:

Azoxystrobin (PubChem CID: 3034285); Boscalid (PubChem CID: 213013); Cyprodinil (PubChem CID: 86367); Fenhexamid (PubChem CID: 213031); Pyrimethanil (PubChem CID: 91650).

1. Introduction

With a total surface of approximately 7.5 million hectares (http://www.oiv.int), viticulture is a hugely important economic activity in geographical areas with warm climates worldwide. Grapevines are especially vulnerable to attacks by fungal pathogens, namely, *Botrytis cinerea* (gray mold), *Plasmopara viticola* (downy mildew), *Uncinula necator* (powdery mildew), *Elsinoë ampelina* (anthracnose), and *Guignardia bidwellii* (black rot) (Carisse, Bacon, Lasnier, & McFadden-Smith, 2006; Edder, Ortelli, Viret, Cognard, De Montmollin, & Zali, 2009; Pozo-Bayón, Monagas, Bartolomé, & Moreno-Arribas, 2012). Plant diseases caused by fungus may have devastating effects on grape productivity and quality, so vine growers endeavour to efficiently control these parasites, most commonly with chemical fungicides (Cabras & Conte, 2001; Vaquero-Fernández et al., 2008). If good agricultural practices are not met, such as correct fungicide dose and/or pre-harvest safety interval, grapes might be contaminated with unacceptable levels of fungicide residues. Consequently, the quality and safety of the wine can also be compromised, because fungicide residues remaining on grapes at harvest can be transferred to the must, withstand fermentation and the winemaking process, and eventually reach the wine (Cabras & Angioni, 2000; González-Rodríguez, Noguerol-Pato, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2011; Pazzirota, Martin, Mezcua, Ferrer, & Fernández-Alba, 2013). The fungicide transfer rate from grape to wine depends on a number of factors, including the particular oenological technology followed at wineries and the physico-chemical properties of the pesticide, such as solubility, volatility, hydrolytic rate constants, water-octanol partition coefficients, thermal degradation, and adsorption to solid wastes (Keikotlhaile, Spanoghe, & Steurbaut, 2010; Regueiro, López-Fernández, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2015).

Maximum residue limits (MRLs) for the presence of pesticides in processed food products are not commonly regulated, so tolerances established for raw commodities like grapes generally apply to wine (Carpinteiro, Ramil, Rodríguez, & Cela, 2010). In the last few years, several studies have been published wherein the incidence of residues from selected pesticides in commercial wines from particular geographical areas or countries has been reported, including Spain (Fontana, Rodríguez, Ramil, Altamirano, & Cela, 2011b), Republic of Moldova (Duca, Sturza, & Siretanu, 2012), and Slovenia (Cus, Bach, Barnavon, & Pongrac, 2013; Cus, Cesnik, Bolta, & Gregorcic, 2010). Recently, a wider monitoring study was carried out in France in which 100% of 92 bottled French wines were positive for pesticide residues, 33 different plant protection products were identified, and most samples contained multiple residues, including a bottle with 14 different compounds (Humbert & Bonneff, 2013). Pesticide contents higher than 100 ng mL^{-1} were detected in 36 wine samples (39.1%), and as much as 1682 µg kg^{-1} total residue was found in one sample. Edder and Ortelli studied the presence of pesticide residues in wines coming from conventionally cultured grapes, mostly from the European Union and Switzerland (Edder & Ortelli, 2005). They found residues in 95% of the wines, mainly fungicides like carbendazim, fenhexamid, azoxystrobin, cyprodinil, pyrimethanil, and tebuconazol, with most samples containing between 3 and 6 different pesticide residues, and total pesticide contents higher than 100 µg kg^{-1} was detected in 48 samples (27.3%). In 2008, a more limited study on pesticide residues in wines from worldwide countries (40 samples from 8 countries) was undertaken by PAN Europe. Nearly identical results concerning positive samples and mean number of residues were found, with fungicides pyrimethanil, cyprodinil, dimethomorph, and fenhexamid accounting for nearly 50% of the total residues detected (www.pan-europe.info). Despite surveys of this kind are highly appreciated by consumers, regulatory bodies, and even wineries, to the best of our knowledge no studies dealing with the presence of pesticide residues in international wines have been published ever since.

Gas-chromatography and liquid-chromatography coupled to different detection systems and sample extraction procedures are the commonly chosen analytical methods for the determination of multiple pesticide residues in wines, including those studies mentioned above (Fontana, Rodríguez, Ramil, Altamirano, & Cela, 2011a; Martins, Esteves, Simoes, Correia, & Delerue-Matos, 2011; Wang, Cheng, Zhou, Li, & Cheng, 2013; Wang & Telepchak, 2013; Wong, Webster, Halverson, Hengel, Ngim, & Ebeler, 2003; You, Wang, Liu, & Shi, 2013). These separative methods are endowed with high sensitivity and selectivity, even though laborious sample manipulation steps are usually required, which increases analysis time and cost. Enzyme-linked immunosorbent assays (ELISAs) have also been occasionally employed for the analysis of specific pesticide residues in wine, like metalaxyl (Bushway & Thome, 1998), benalaxyl (Rosso, Giraudi, Gamberini, Baggiani, & Vanni, 2000), tebufenozide (Irwin, Tolhurst, Jackson, & Gale, 2003), fenhexamid (Mercader & Abad-Fuentes, 2009), atrazine, bromopropylate, 2,4,6−trichlorophenol (Argárate et al., 2010), and cyprodinil (Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2015a). The main advantages of immunoassays over instrumental methods are simplicity, high sample throughput, and portability, making them an attractive alternative to chromatographic methods for extensive monitoring programmes focused on particular analytical targets.

In the present study, a high number of bottled wines were analysed using rapid immunoanalytical methods for the occurrence of chemical residues from a set of relevant fungicides. Commercial wines (n=280) of different geographical areas and characteristics, including wine type, country of origin, protected designation of origin (PDO), grape variety, alcoholic grade, and price, were included in the survey. Several innovative fungicides belonging to new chemical families, like anilinopyrimidines, strobilurins, and succinate dehydrogenase inhibitors, and widely used for the treatment of diseases affecting wine grapes, were selected for this investigation (Gabriolotto, Monchiero, Negre, Spadaro, & Gullino, 2009). These target compounds – azoxystrobin, boscalid, cyprodinil, fenhexamid, and pyrimethanil – are regularly used for final sprayings early before harvest, and they show a remarkable transfer rate from grapes to wine due to their physico-chemical characteristics (Table S1), so their residues have been repeatedly reported in recent studies as often encountered in wines (Cus, Bach, Barnavon, & Pongrac, 2013). The main aim of this work was testing the usefulness of immunoassays for the rapid and simple monitoring of fungicides in wines, and figuring out the incidence of those selected fungicides in commercial wines from around the world.

2. Experimental

2.1. Reagents and instrumentation

Fungicide analytical standards were purchased from Sigma/Aldrich (Madrid, Spain). Stock solutions were prepared at 10 g L^{-1} in acetonitrile and kept at −20 ºC in amber glass vials. RAM–HRP was from Dako (Glostrup, Denmark). Costar flat-bottom high-binding polystyrene

ELISA plates were from Corning (Corning, NY, USA). ELISA absorbances were read in dual wavelength mode with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments. Acetonitrile for LC–MS was obtained from Scharlau (Barcelona, Spain). *o*-Phenylenediamine was purchased from Sigma/Aldrich (Madrid, Spain).

2.2. ELISA procedure

Ninety-six-well polystyrene ELISA plates were coated with 100 µL of coating antigen solution in 50 mM carbonate−bicarbonate buffer (pH 9.6) by overnight incubation at room temperature. Coated plates were washed four times with 150 mM NaCl solution containing 0.05% (v/v) Tween 20, and received, afterwards, 50 µL per well of standard/sample in Milli-Q water plus 50 µL per well of the specific antibody solution in 20 mM sodium phosphate buffer (pH 7.4) with 280 mM NaCl and 0.05% (v/v) Tween 20. Plates were run simultaneously, and each plate analysed the samples for a particular compound. The immunochemical reaction took place during 1 h at room temperature, and plates were washed again as described. Next, 100 µL per well of HRP-labelled secondary antibody (1/2000) in 10 mM sodium phosphate buffer (pH 7.4) with 140 mM NaCl and 0.05% (v/v) Tween 20 was added, and plates were incubated an additional hour. After washing the plates, signal was generated by addition of 100 µL per well of freshly prepared 2 mg mL^{-1} *o*-phenylenediamine and 0.012% (v/v) hydrogen peroxide 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 1 M sulphuric acid, and absorbances were immediately read at 492 nm with a reference wavelength at 650 nm. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). Assay sensitivity was estimated as the concentration of the fungicide at the inflection point of the sigmoidal curve, typically corresponding to a 50% inhibition (IC_{50}) of the maximum absorbance reached at the zero dose of analyte (A_{max}).

2.3. Wine sample processing and analysis

Wine bottles from worldwide brands were purchased at retail outlets, specialized shops, and wine importers. Upon reception and bottle opening, wine samples were transferred to glass vials and stored at 4 ºC and −20 ºC for short and long-term storage, respectively. For residue determination by ELISA, samples were 50-fold diluted with Milli-Q water and analysed twice in consecutive days in duplicated wells. When two independent measurements differed more than 20%, a third analysis was carried out. Every 96-well plate contained an eight-point standard curve for a particular fungicide, 38 wine samples, and 2 quality control (QC) samples. QC samples were prepared using a white wine and a red wine spiked with the full set of fungicides at 50 and 100 µg L^{-1}, respectively. Absence of fungicides in QC wines was previously checked by ELISA and confirmed by UPLC–MS/MS.

3. Results and discussion

3.1. Sampling study

A total of 250 wine samples from conventionally grown grapes were screened for the analysis of fungicide residues (134 red, 96 white, and 20 rosé wines), 33 of them being sparkling wines, mostly from white musts. Wines came from different geographical areas of Spain (n=80), France (n=33), Italy (n=29), Argentina (n=27), Chile (n=21), Germany (n=14), Portugal (n=12), USA (n=9), Australia (n=7), South Africa (n=4), Austria (n=4), and other countries (n=10). Particularly, 64.8% of the samples were monovarietal wines, while 14.0% employed a mixture of two grape varieties, 15.6% used three grape types in the winemaking process, and 5.9% employed more than three cultivars. Due to the own scope of the study, wines were made from both international and local grape varieties, being cabernet sauvignon (15.2%), tempranillo (14.8%), chardonnay (12.4%), merlot (10.4%), garnacha tinta (5.6%), and syrah (3.2%) the most common cultivars. Most wine bottles (86.8%) were from grapes harvested in the period 2008–2013. Alcoholic grade ranged between 5.5 and 20.0%, with an average value of 12.7%. Wine price was also heterogeneous, with an average price of 8.4 € per bottle, covering a range from 1.5 to 36.6 €. A detailed description of wine samples included in this survey (origin, year, grape varieties, price, wine type, and alcoholic grade) can be found in the supplementary data file (Table S2). Commercial names of wines and wineries have been intentionally hidden.

3.2. Immunoassay performance

Antibodies and coating conjugates for each fungicide were produced in-house. With the only exception of boscalid, the particular immunoassays employed in this study have been previously reported (Esteve-Turrillas, Abad-Fuentes, & Mercader, 2011; Esteve-Turrillas, Abad-Somovilla, Quinones-Reyes, Agulló, Mercader, & Abad-Fuentes, 2015; Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2015b; Parra, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2012). Immunoreagent concentrations, main analytical parameters, and calibration standard curves are reported in the supplementary data file (Table S3 and Fig. S1). Wine samples were diluted at least 50 times in order to avoid matrix interferences, and 10 µg L^{-1} was selected as the threshold concentration to designate positive samples.

For immunoassay quality control, repeatability of ELISA inhibition curves was evaluated. RSD values lower than 20% for IC_{50} and A_{max} parameters were found. Trueness and precision were evaluated using the QC samples that were run in parallel with the real wine samples as described. Recoveries calculated at 50 and 100 µg L^{-1} ranged from 95 to 111% (Table S4). Intra-day and inter-day precision was satisfactory, with RSD values from 7 to 11% and from 8 to 15%, respectively.

3.3. Occurrence of fungicide residues

A total of 111 wine samples (44.4%) were found to contain at least one of the five studied fungicides, at a residue level higher than 10 µg L^{-1}. In 26.0% of the samples, one of those fungicides was detected, and 18.4% of the samples contained multiple residues (Fig. 1). No sample containing simultaneously the five fungicides was encountered. The most frequently found fungicide was pyrimethanil (22.4%), followed by boscalid (19.2%), and fenhexamid (18.8%); cyprodinil was detected in 6.8% of the samples and azoxystrobin occurrence was rare in this study (1.2%). The distribution of the residue concentrations for each fungicide in positives samples is depicted in Fig. S2. The highest residue concentration found for each fungicide was 920 μg L^{-1} for pyrimethanil, 267 μg L^{-1} for fenhexamid, 136 μg L^{-1} for boscalid, 88 μg L^{-1} for cyprodinil, and 54 μg L^{-1} for azoxystrobin. The fungicide average concentration ranged from 67 μg L^{-1} for pyrimethanil to 25 μg L^{-1} for cyprodinil (Table 1). Twenty-one wines (8.4%) had total fungicide residue contents higher than 100 μg L^{-1}, most of them from France (9 out of 33 analysed samples).

In order to further confirm the results attained by competitive immunochemical methods, 10 random wine samples with no measurable levels of the selected fungicides and 20 wine samples selected among those with a higher number of different residues and/or higher total fungicide contents, were analysed by UPLC–MS/MS. No false negative results were found, and the chromatographic method confirmed that the fungicides detected by ELISA were present in the positive samples at equivalent concentrations. For specific details concerning the UPLC–MS/MS method and a selection of chromatograms for positive wine samples, the reader is referred to the supplementary data file.

The aforementioned results are in accordance to previous monitoring studies carried out to assess pesticide levels in wines, even though a lowest global incidence was found in this study, in part because of the limited number of residues that were sought, and also because samples with residues at trace levels (<10 ng L^{-1}) were prudently not scored as positives.

In the next sections, the analytical information that was gathered by ELISA in this study was evaluated as a function of the wine type and the geographical origin of the sample. Parameters like vintage, grape variety, alcohol content, and price showed no correlation with the fungicide content.

3.3.1. Wine type

Fungicide residue concentrations on positive samples were examined by type of wine. For the studied compounds, a similar incidence of samples with residues was noticed among red wines (42.5%) and white wines (52.1%), while in rosé wines the frequency of positives was lower (20.0%). In sparkling wines, the rate of positives was 36.4% (Table 1). However, clear differences were observed when data were filtered for each particular fungicide (Fig. 2). Thus, pyrimethanil occurred more frequently in white and sparkling wines, cyprodinil and azoxystrobin were found almost exclusively in red wines, and boscalid and fenhexamid were more evenly distributed among the different wine types. This differential distribution of specific fungicide residues among wine types may be related to the effect of winemaking processes, like maceration and clarification, on the transfer rate of each substance from grape to wine.

3.3.2. Geographical origin

The presence and concentration of the five studied fungicides was assessed as a function of the country of origin (Fig. 3). Wines produced in the USA (88.9%), Germany (78.6%), France (75.8%), and Austria (75.0%) contained fungicide residues in most of the analysed bottles, while samples from Italy, Chile, Portugal, and Spain showed occurrence values close to 30−40%. The lowest fungicide contents were in samples from Australia (14.3%), Argentina (7.4%), and South Africa (0%). As treatments with other agrochemicals are feasible and their occurrence was not monitored, a lower amount of positive samples in a country or area does not necessarily mean a lower use of fungicides or better agricultural practices.

Regarding the use of particular fungicides, azoxystrobin was only found in French and Spanish wines, and at a low extent; boscalid was preferentially encountered in wines from USA, Germany, and Austria; cyprodinil residues were only present in samples from USA, Spain, Chile, France, and New Zealand; the highest occurrence of fenhexamid was in wines produced in Germany, USA, and France; and residues of pyrimethanil were mainly found in French, Austrian, and German wines (Fig. 3). The average concentration of fungicides in positive samples for each country is depicted in Fig. 4. It is worthy to note that Portugal and Spain, with intermediate figures in the percentage of positive samples, ranked high in this aspect.

A more detailed inspection of the data was carried out with wines from PDOs for which at least 4 samples were analysed (Fig. 5). PDOs with the highest number of representatives were Luján de Cuyo (Argentina, n=19), Rioja (Spain, n=17), Valle de Curicó (Chile, n=14), Bordeaux (France, n=12), California (USA, n=9), Cava (Spain, n=9), and Champagne (France, n=8). All analysed samples from Bierzo (Spain) and Pfalz (Germany) were positives, and wines from California (USA), Champagne (France), Bordeaux (France), Mosel-Saar-Ruwer (Germany), and

Piamonte (Italy) contained fungicide residues in at least 80% of the analysed samples. On the contrary, wines from the POD Cava (Spain), Luján de Cuyo (Argentina), Emilia-Romagna (Italy), La Mancha (Spain), and Utiel-Requena (Spain) contained residues in less than 20% of the samples. Wines with the highest average residue levels were from the PDO Champagne (France), Bordeaux (France), Valencia (Spain), Sicilia (Italy), and Rheinhessen (Germany).

Unfavourable weather conditions most commonly occurring in geographical areas with cold wet climates favour the development of fungal diseases in the vineyards, so more intense chemical treatments are generally required in order to safeguard grape quality, which eventually may result in greater fungicide residues in wines. Nonetheless, some exceptions to this reasoning were actually found, probably because adverse weather conditions promoting fungal diseases may occasionally take place also in warm climates.

3.3.3. Organic farming wines

In order to complete the study, 30 additional wine bottles produced from grapes grown following organic farming practices, and labelled accordingly by the corresponding national and international authorities, were analysed. In this case, most wines were from Spain (24), four wines were from France, and 2 samples were from Chile. As it should be expected, no residues of the studied fungicides were found with our cut-off limits (10 µg L^{-1}), although in other studies pesticide residues at trace levels were detected in some organic wines (Edder & Ortelli, 2005)

4. Conclusions

By taking advantage of the remarkable features of immunochemical methods for the cost-effective and rapid screening of particular analytes in foodstuff, a multiresidue survey on the presence of rationally selected fungicides in bottled wines from all over the world was carried out. Our results are in line with those from other authors, and evidence that contamination of conventional wines with pesticide residues is an issue of worldwide significance, even though the incidence seems to differ among geographical areas. Although the contamination levels that are commonly found should not raise serious toxicological concerns among consumers, farmers and wine-makers should make additional efforts to implement quality control procedures and innovative technological processes aimed at keeping to a minimum the presence of pesticide residues in wines. Since long ago, some authors, consumer organizations, and professional associations are stressing the need to legislate on specific and more restrictive MRLs for pesticide residues in wine, so a 10-fold reduction in MRLs for wine with respect to those established for grapes has been suggested. Likewise, limits to the total pesticide residue contents in wines might be legislated, mimicking European guidelines for pesticide residues in drinking water. Consumers perceive wines as a high value-added commodity that should meet the uppermost quality standards, and preventive measures and reliable analytical methods, such as immunoassays and chromatographic techniques, may certainly complement each other in order to contribute to achieve this goal.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at

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Figure legends

Fig. 1. Occurrence of fungicide residues in wine samples. Blue, negative samples; red, positive samples; yellow, samples with 1 residue; green, samples with 2 residues; purple, samples with 3 residues; black, samples with 4 residues.

Fig. 2. Radar plot of fungicide occurrence as a function of wine type.

Fig. 3. Fungicide occurrence classified by country of origin (A, total amount; B, azoxystrobin; C, boscalid; D, cyprodinil; E, fenhexamid; and F, pyrimethanil).

Fig. 4. Fungicide average concentration classified by country of origin.

Fig. 5. Fungicide occurrence (A) and average concentration (B) as a function of the Protected Designation of Origin. ISO country codes and number of analysed samples are indicated in parenthesis.

Table 1
Residue concentrations in positive samples and occurrence of fungicides in analysed wines.

Fungicide	Concentration ($\mu g\ L^{-1}$)			Occurrence (%)				
	Maximum	Average	Median	Total (n=250)	Red (n=134)	White (n=96)	Rose (n=20)	Sparkling (n=33)
Azoxystrobin	54	30	20	1.2	2.2	-[a]	-[a]	-[a]
Boscalid	136	33	24	19.2	13.4	28.1	15.0	18.2
Cyprodinil	88	28	19	6.8	11.2	2.1	-[a]	-[a]
Fenhexamid	267	49	31	18.8	17.2	22.9	10.0	18.2
Pyrimethanil	920	70	25	22.4	17.2	31.3	15.0	30.3
Total	967	75	37	44.4	42.5	52.1	20.0	36.4

[a] No positive samples were found (concentration lower than 10 $\mu g\ L^{-1}$).

Figure 1

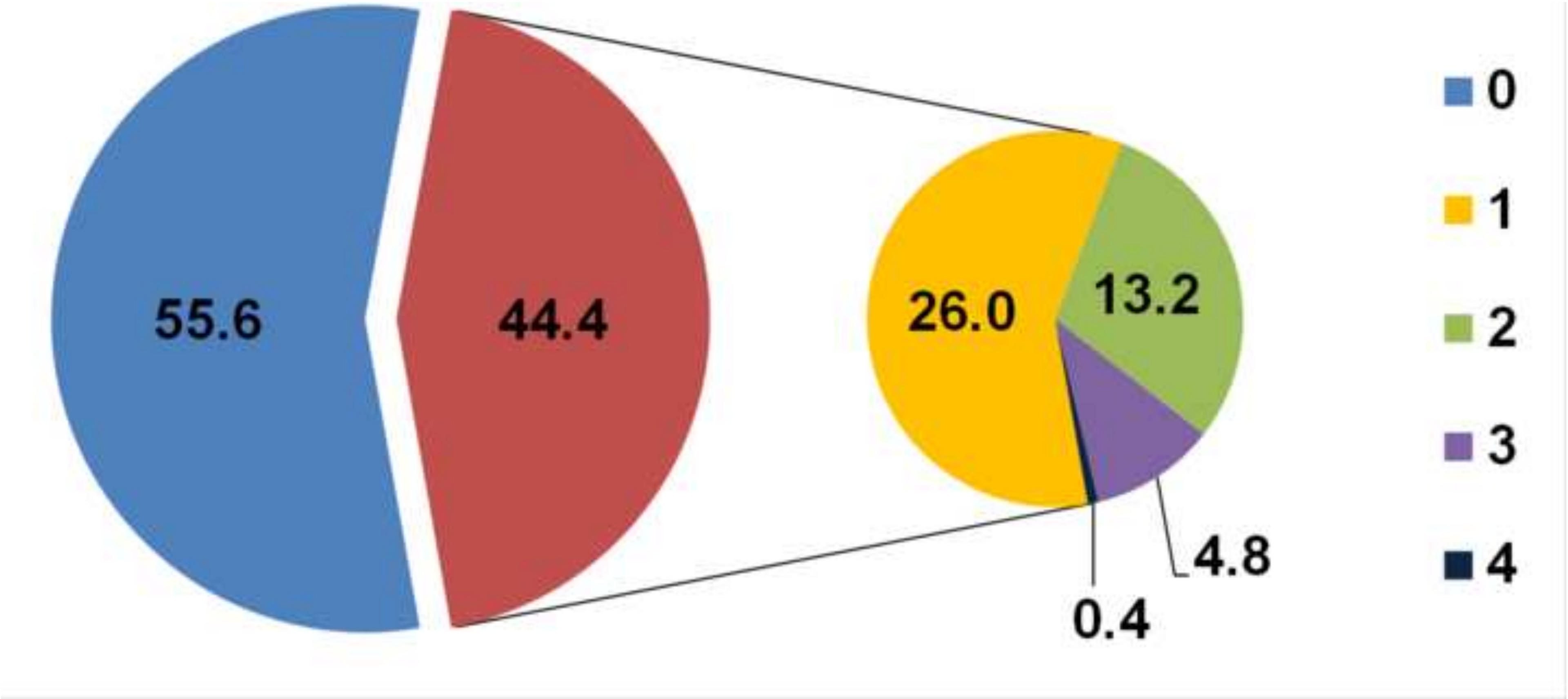

Figure 2

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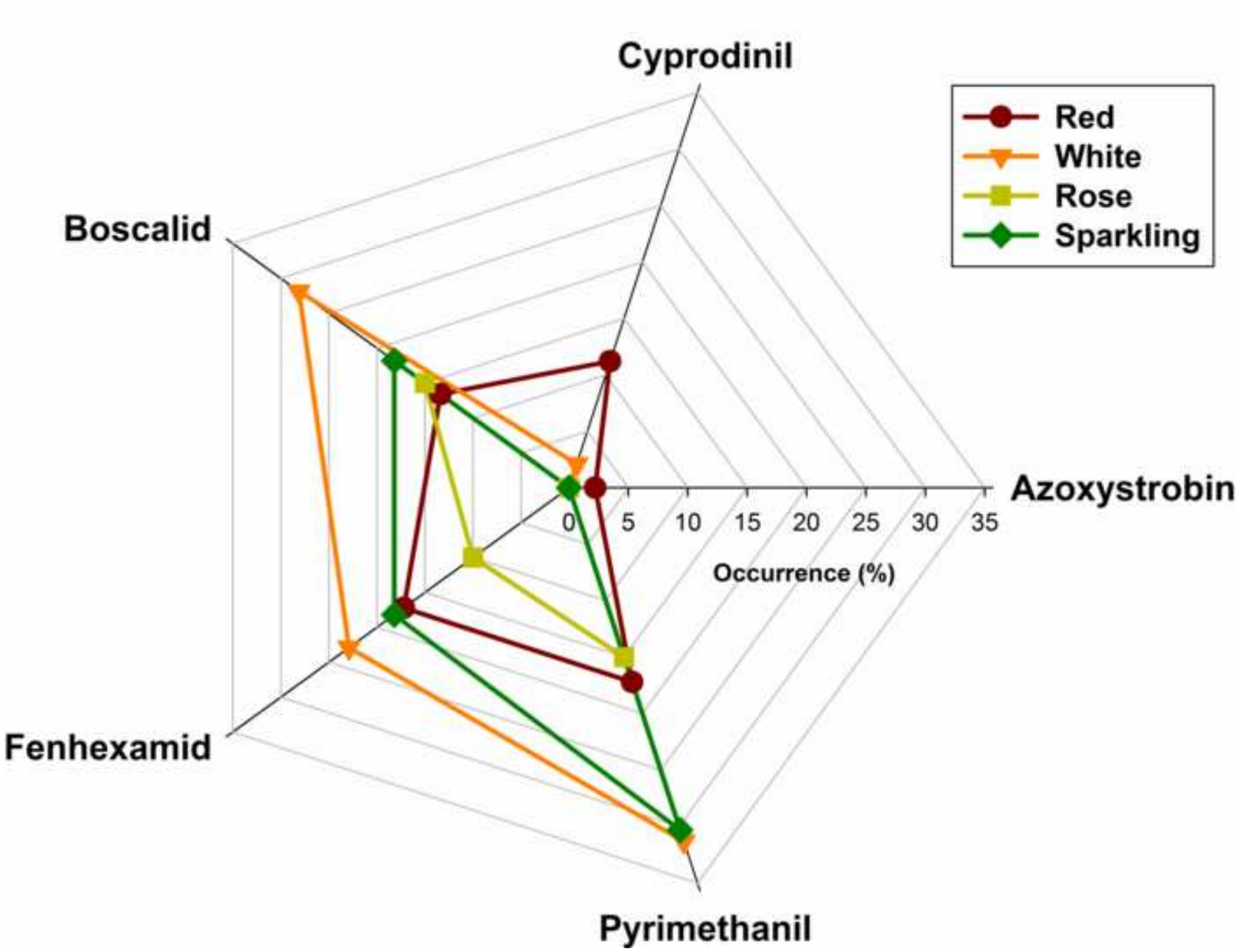

Figure 3

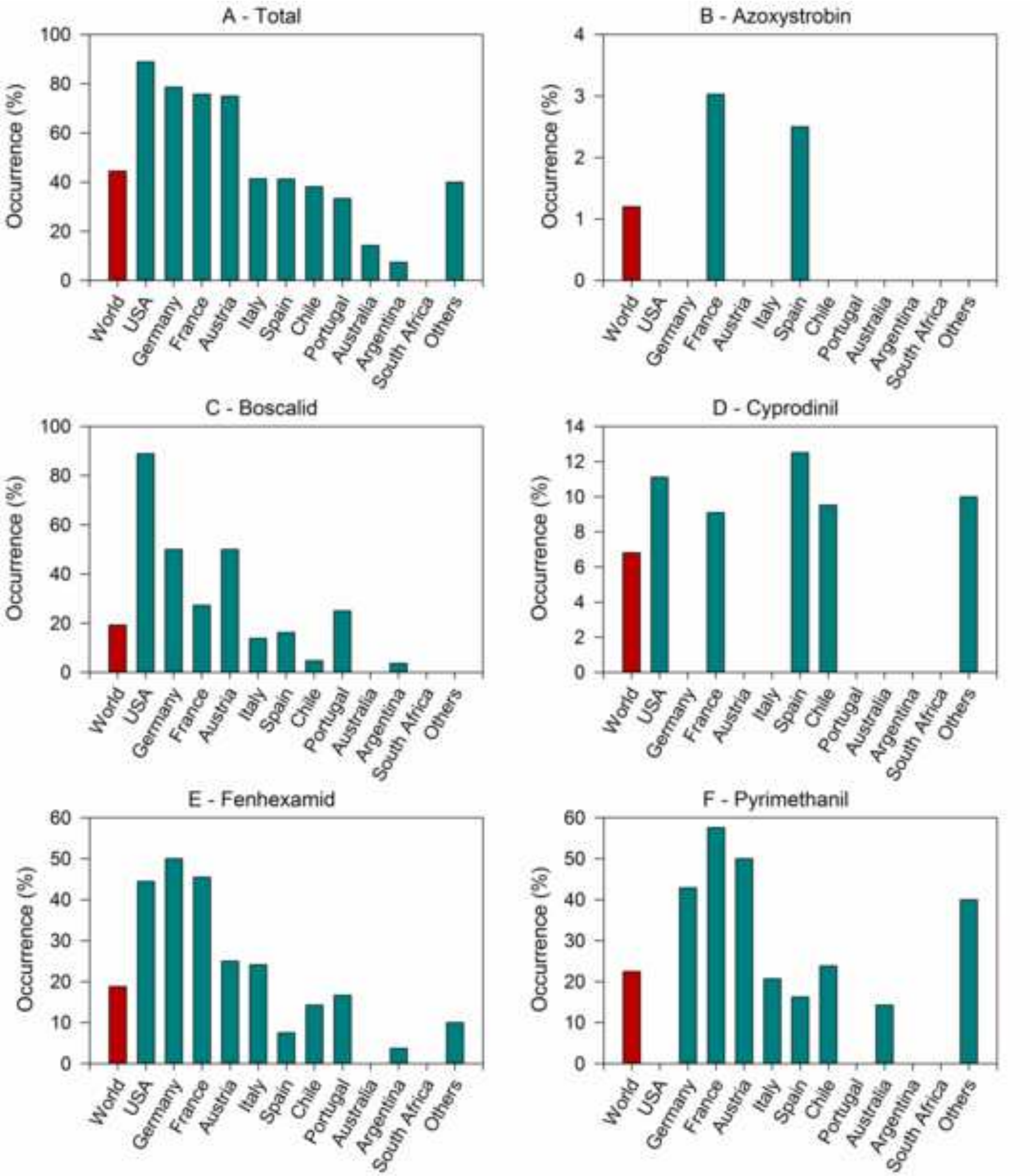
A - Total
B - Azoxystrobin
C - Boscalid
D - Cyprodinil
E - Fenhexamid
F - Pyrimethanil
Occurrence (%)
World
USA
Germany
France
Austria
Italy
Spain
Chile
Portugal
Australia
Argentina
South Africa
Others
0
20
40
60
80
100
1
2
3
4
6
8
10
12
14
30
50

Figure 4

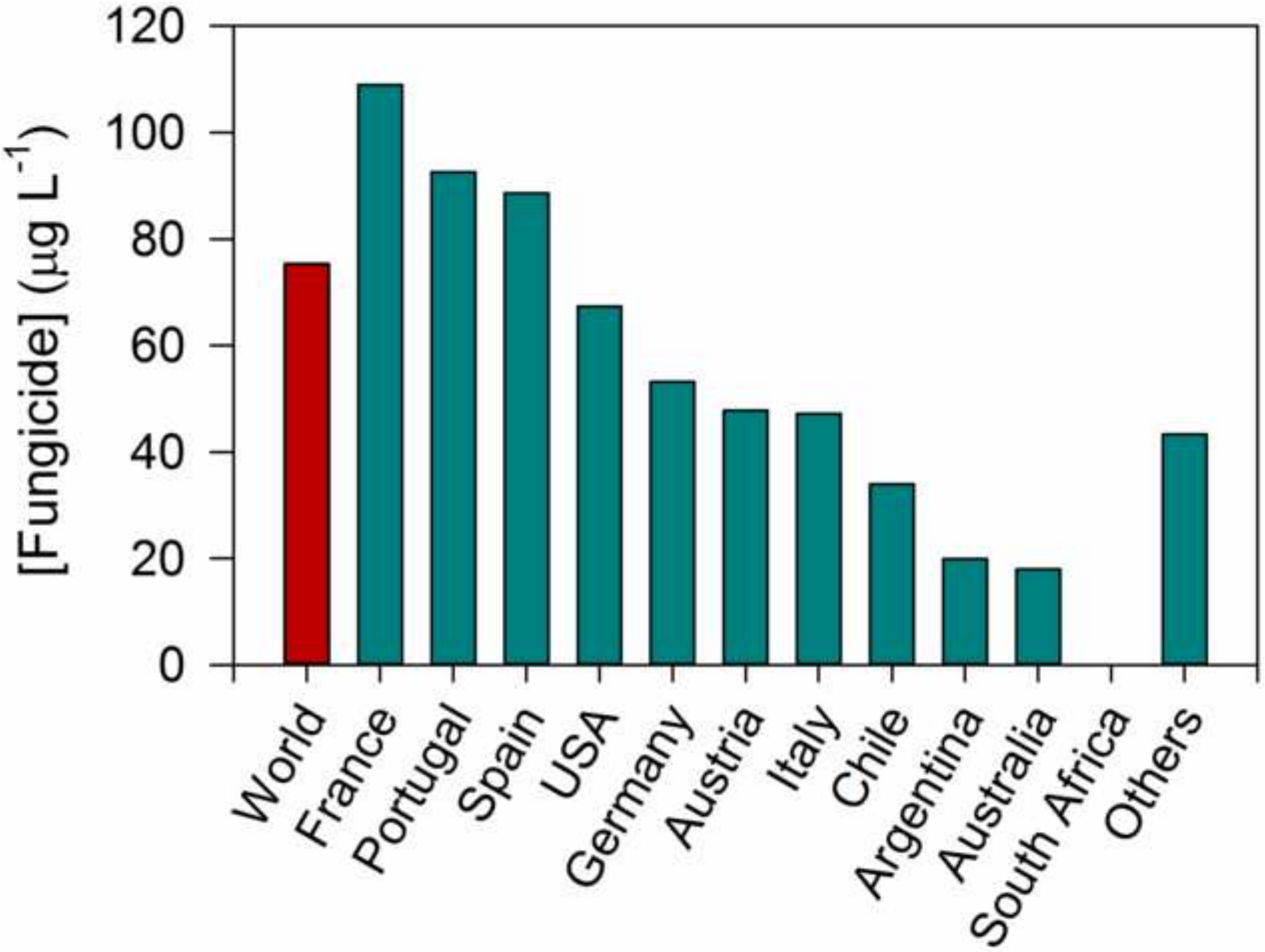
[Fungicide] (µg L^{-1})
120
100
80
60
40
20
0
World
France
Portugal
Spain
USA
Germany
Austria
Italy
Chile
Argentina
Australia
South Africa
Others

Figure 5

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