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Quantitation of 13 azole fungicides in wine samples by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed for the direct quantitation of residues of 13 azole fungicides in wine and has been successfully applied to 103 wine samples. The method utilizing 25 transitions is highly sensitive and specific with centrifugation as the only sample work-up step. Precision was better than 14% and accuracy ranged between 80 and 120%. Quantitation limits (LOQs) ranged between 0.25 (penconazole) and 7.5 ng/mL (triadimefon). Since the LOQs achieved are at least four times lower than the maximum residue levels for azole-fungicides in wine prescribed, the method presented here can be conveniently used as a screening assay for azole-residues in wine samples.

Keywords: LC–MS/MS; Electrospray (ESI); Azole; Endocrine disruption; Wine

1. Introduction

Azoles (imidazoles and triazoles) are widely used as fungicides in agriculture. The antifungal activity is based on the inhibition of fungal CYP51 (lanosterol-14α-demethylase) and thus inhibition of sterol biosyntheses [1]. Azoles are also discussed as a new group of endocrine active agents in humans disturbing the biosyntheses of steroids by inhibition of human cytochrome P450 enzymes like aromatase (CYP19) [2] and human CYP51 [3,4].

In many countries wine is a very prestigious alcoholic beverage and therefore grapes are an important cultivated plant. Grapes are especially vulnerable to moulds, which leads to a widespread use of antifungal agents. The physicochemical and biological processes of wine-making do not break down fungicides, leading to observable amounts in wine samples [5]. The determination of residue levels is necessary for food safety monitoring and regulatory purposes.

Up to now azole levels in wine samples were mainly analyzed by gas chromatography/electron ionization mass spectrometry (GC/EI-MS) [6,7]. This technique involves many preceding sample work-up steps like extraction, clean up or interference removal, and derivatization. Sample preparation could be simplified by automated solid phase micro-extraction techniques as described for triadimefon, propiconazole, myclobutanil and penconazole [8]. Methods based on liquid chromatography/tandem mass spectrometry (LC–MS/MS) are described for the quantitation of several azoles used in human medicine in body fluids of humans and rats [9,10]. A single analytical procedure is desirable to screen beverage samples for all azoles applied as fungicides. In complex matrix this is only achievable with single MS or more conveniently with MS–MS based methods and has been described previously for eight and more analytes within a single LC-run [11,12]. The method presented here allows the quantitation of 13 azoles in wine samples without any sample preparation other than centrifugation, within one LC-run using a tandem mass spectrometer as detector. Analytes can be distinctly detected by GC–MS [5,7] and LC–MS [13,14] with similar sensitivity to the method described but tandem mass spectrometry has the advantage of unequivocal analyte identification due to the ion selection of two quadrupole mass analysers.

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2. Materials and methods

2.1. Chemicals and reagents

Epoxiconazole was a kind gift from the Swiss Federal Research Station, Wädenswil, Switzerland. All other azole compounds were from Dr. Ehrenstorfer (Augsburg, Germany). LC grade water was from Roth (Karlsruhe, Germany). All other chemicals were from Sigma/Fluka (Taufkirchen, Germany). All solvents used were LC grade or better. Wine samples were chosen randomly out of private donations from members of the department.

2.2. Sample preparation

Wine samples were centrifuged at 15,000 × *g* for 5 min to remove particles. The supernatant was analyzed by LC–MS/MS without further sample work-up.

2.3. Optimization of mass spectrometry methods

The LC–ESI-MS/MS method was optimized with respect to sensitivity, analyte identification, and quantitative measurement. Infusion experiments were carried out to examine ionization and fragmentation patterns of the analytes. A syringe pump (Single Syringe Pump 11, Harvard Apparatus Inc., Holliston, USA) was used to provide a constant analyte infusion (300 μL/min) into the LC eluent via a T-connection. Analyte concentrations were chosen in a range of 5–100 ng/μL to obtain a constant signal in the Q1 scan mode. Basic source and MS parameters such as declustering potential (DP), focussing potential (FP), collision energy (CE) and exit potential (CXP) were optimized using the "quantitative optimization" function of "Analyst 1.3.1" (Applied Biosystems, Darmstadt, Germany).

2.4. Liquid chromatography–tandem mass spectrometry

To determine the 13 different azole compounds qualitatively or quantitatively in wine samples, 10 μL of supernatant were injected on a Synergi Hydro RP LC column (80 A, 4 μm, 150 mm × 2 mm Phenomenex, Aschaffenburg, Germany) with a C18 ODS guard column (4 mm × 3 mm, Phenomenex, Aschaffenburg, Germany) using an Agilent 1100 autosampler and an Agilent 1100 LC-pump (Agilent Waldbronn, Germany). The samples were separated by gra-

Table 1
MS/MS-transitions, declustering potential and collision energy used

Compound		Transition (*m*/*z*)	Ret. time (min)	DP (V)	CE (V)	CXP (V)
Penconazole	Quantifier	284.0 → 158.9	8.76	56	45	4
	Qualifier	284.0 → 70.0		56	27	4
Myclobutanil	Quantifier	289 → 70.1	7.31	76	31	4
	Qualifier	289 → 125.1		76	41	4
Cyproconazole	Quantifier	292.0 → 70.1	6.65	66	31	4
	Qualifier	292.0 → 125.1		66	49	4
Triadimefon	Quantifier	294.0 → 57.1	7.67	71	45	2
	Qualifier	294.0 → 69.1		71	31	4
Triadimenol	Quantifier	296.0 → 70.1	5.83, 6.24[a]	41	17	2
Imazalil	Quantifier	297.1 → 159	2.25	86	27	6
	Qualifier	297.1 → 200.9		86	21	4
Tebuconazole	Quantifier	308.1 → 70.0	7.88	76	53	2
	Qualifier	308.1 → 124.8		76	51	4
Hexaconazole	Quantifier	314.0 → 70.1	8.40	71	33	4
	Qualifier	314.0 → 159.1		71	41	6
Flusilazole	Quantifier	316.1 → 165.3	8.06	66	39	4
	Qualifier	316.1 → 109		66	79	4
Epoxiconazole	Quantifier	330.0 → 121.2	7.57	46	29	4
	Qualifier	330.0 → 129.1		46	61	4
Bitertanol	Quantifier	338.1 → 70.0	8.13	41	23	2
	Qualifier	338.1 → 99.0		41	21	4
Propiconazole	Quantifier	342 → 159.0	8.97	71	47	4
	Qualifier	342 → 69.2		71	33	4
Prochloraz	Quantifier	377.9 → 309.7	6.99	41	19	14
	Qualifier	377.9 → 70		41	33	4

[a] Two isomers are separated on the column. For quantitation purposes the second peak was used.

dient elution with water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) using the following conditions: 50% A isocratic for 1 min, linear to 0% A within 5 min, and isocratic for 5 min at 0% A with a flow rate of 250 μL/min. The LC system was coupled directly to a triple stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with a turbo ion spray source. Analytes were detected in the positive ion mode at a vaporizer temperature of 400 °C.

An ion spray voltage of 2000 V was applied. Spectral data were recorded with N_2 as collision gas (CAD = 4) in the multiple reaction monitoring (MRM) mode with a dwell time of 50 ms for each transition monitoring the following MS/MS ion-transitions (Table 1).

Quantitation of azoles was based on calibration curves obtained after addition of known amounts of azoles (0.1–50 ng/mL) to two red wine and one white wine sample containing no azoles. Calibration curves were calculated from 11 duplicate data points using "Analyst 1.3.1". Limits of detection (LODs) and limits of quantitation (LOQs) were measured in three independently spiked and analyzed samples. Signal to noise (s/n) ratios where determined with Analyst 1.3.1 software applying a standard deviation of three. A peak with s/n ≥ 3 was judged as above the LOD and a peak with s/n ≥ 9 as above the LOQ. Precision values at the 1 ng/mL and at the 10 ng/mL level where each calculated from 20 consecutive injections of the same sample.

3. Results and discussion

3.1. Sample work-up

Centrifugation was the only sample preparation step used to eliminate particles, such as crystals of tartar and yeast of very young wines. The same is true for beer samples (data not shown).

3.2. High performance liquid chromatography–mass spectrometry

3.2.1. Ionization and fragmentation

3.2.1.1. Ionization. Table 1 summarises precursor ions of all azoles of which analytical standards were available. In the positive ion mode, protonation was the major ionization mechanism for all azoles. Triadimefon was the only compound that could be sufficiently ionized in the negative ion mode to produce detectable product ions in the third quadrupole. Due to adequate sensitivity in the electrospray mode, other ionization techniques like atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) were not tested. For different azoles literature describes APCI, in positive and negative ion mode [13,15], and APPI [16] as ionization techniques. However, references are not consistent in the evaluation of the different ionization techniques: for imazalil APCI and ESI gave similar

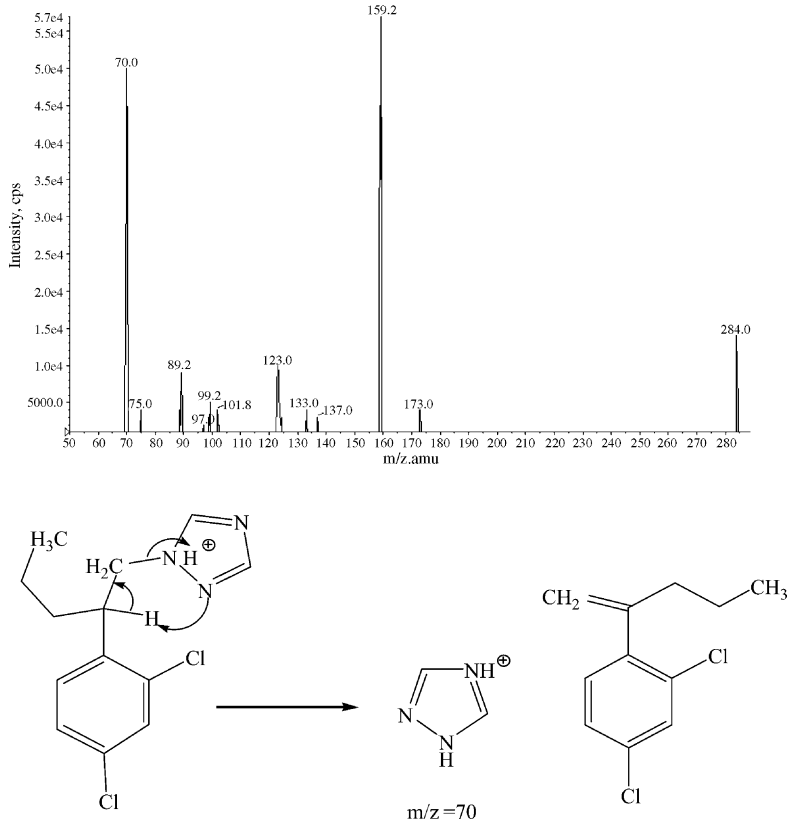

Fig. 1. Product ion spectrum of penconazole and proposed McLafferty rearrangement.

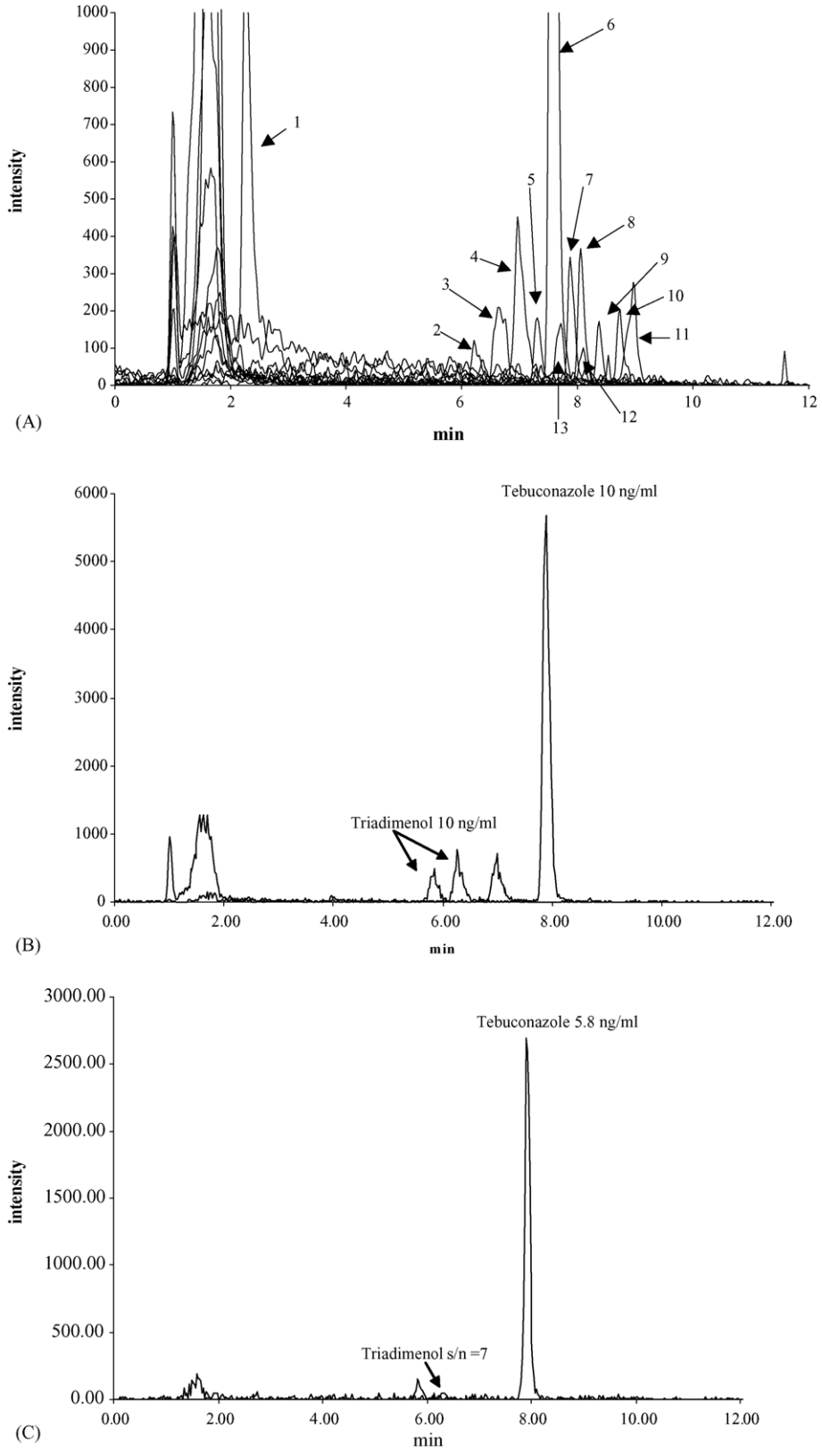

Fig. 2. Chromatograms showing. (A) Transitions of all 13 azoles of a standard in wine matrix spiked at LOQ level: imazalil (1), triadimenol (2), cyproconazole (3), prochloraz (4), myclobutanil (5), epoxiconazole (6), tebuconazole (7), flusilazole (8), hexaconazole (9), penconazole (10), propiconazole (11), bitertanol (12), triadimefon (13); (B) transitions of tebuconazole and triadimenol from a 10 ng/mL standard; (C) transitions of tebuconazole (5.8 ng/mL) and triadimenol (<LOQ) of a real sample.

results in terms of sensitivity [14]; for bitertanol, tebuconazole and triadimefon APCI in positive mode was considered superior to ESI in positive and negative mode and to APCI in negative mode [17]. The declustering potential was optimized for each compound in the positive ion mode to achieve highest sensitivity.

3.2.1.2. Fragmentation. Fragmentation was performed with nitrogen as collision gas and the important potentials like the collision energy were optimized for each azole and transition (Table 1). The most sensitive fragment ion was used to generate a quantifier transition and the second most sensitive one to generate a qualifier transition. Triadimenol did not generate a second utilisable transition, for myclobutanil the qualifier transition was less sensitive than the quantifier transition by a factor of five. For all other compounds the qualifier transition was as sensitive as the quantifier transition. Bitertanol, cyproconazole, epoxiconazole, hexaconazole, myclobutanil,

Table 2
Method validation parameters

Compound	MRL (ng/g)[a]		LOD (ng/mL)	LOQ (ng/mL)	Accuracy (%)
Penconazole	200	Quantifier	0.25	0.25	80–120
		Qualifier	0.25		
Myclobutanil	1000	Quantifier	0.5	0.75	80–116
		Qualifier	2.5		
Cyproconazole	–	Quantifier	0.5	0.75	80–117
		Qualifier	0.5		
Triadimefon	2000	Quantifier	5	7.5	80–118
		Qualifier	0.75		
Triadimenol	2000	Quantifier	0.5	0.75	80–116
Imazalil	20	Quantifier	2.5	5	80–117
		Qualifier	2.5		
Tebuconazole	–	Quantifier	0.25	0.5	80–118
		Qualifier	0.25		
Hexaconazole	100	Quantifier	0.25	0.5	80–116
		Qualifier	0.25		
Flusilazole	–	Quantifier	0.5	0.75	80–115
		Qualifier	0.5		
Epoxiconazole	50	Quantifier	n.d.[b]	n.d.[b]	80–119
		Qualifier	n.d.[b]		
Bitertanol	50	Quantifier	0.75	1	80–116
		Qualifier	0.5		
Propiconazole	500	Quantifier	0.5	0.75	80–117
		Qualifier	0.25		
Prochloraz	50	Quantifier	0.5	0.75	80–120
		Qualifier	0.25		

Compound	Linearity	r^2	Precision (%) (1 ng/mL)	Precision (%) (10 ng/mL)
Penconazole	0.25–50	0.983	13.3	5.8
Myclobutanil	0.75–50	0.995	7.9	2.6
Cyproconazole	0.75–50	0.994	9.0	4.2
Triadimefon	7.5–50	0.990	n.d.	13.7
Triadimenol	0.75–50	0.992	12	5.3
Imazalil	5–50	0.987	n.d.	5.1
Tebuconazole	0.5–50	0.992	9.2	6.9
Hexaconazole	0.5–50	0.994	12.6	6.1
Flusilazole	0.75–50	0.994	9.1	10.7
Epoxiconazole	5–50	0.998	10.1	7
Bitertanol	1–50	0.977	12.1	8.6
Propiconazole	0.75–50	0.994	5.3	4.1
Prochloraz	0.75–50	0.993	8.4	3.5

[a] Appendix II German Regulation for Maximum Residue Levels in Food (RHmV).
[b] Contamination in water and wine with a signal to noise of 8, thus LOD and LOQ could not be determined.

penconazole, prochloraz, tebuconazole and triadimenol all showed $m/z = 70$ as a prominent product ion. With the exception of epoxiconazol the transition of $[M + H]^+ \rightarrow m/z = 70$ was chosen as qualifier or quantifier transition. For bitertanol, penconazole and triadimenol this product ion can be explained by a McLafferty rearrangement since these compounds have one γ-hydrogen available [18]. Fig. 1 shows a product ion spectrum of penconazole with the proposed McLafferty rearrangement. Cyproconazole, epoxiconazole, hexaconazole myclobutanil and tebuconazole do not have a γ-hydrogen. The product ion of $m/z = 70$ could be explained by a three or four-membered transition state with the neighbouring methylene group, followed by a cleavage of the nitrogen–carbon-bond.

However, the product ion $m/z = 70$ of prochloraz cannot be explained by this, since prochloraz does not have a neighboured methylene group.

Both propiconazole and triadimefon show $m/z = 69$ as product ion corresponding to a simple cleavage of the triazole ring plus hydrogen. To a small extent they also show $m/z = 70$ as product ion, which could be explained by the above mentioned three to four membered transition state.

Imazalil has $m/z = 69$ as characteristic product ion which corresponds to a McLafferty rearrangement of an imidazole ring.

Flusilazole shows a completely different fragmentation pattern compared to the other azoles with neither $m/z = 70$ nor $m/z = 69$ as product ion, which could be attributed to the silicon atom in the structure.

Due to the availability of standards for all 13 azoles unequivocal analyte identification was possible using quantifier and qualifier transitions and comparing retention times with standards in matrix solutions.

3.2.1.3. Liquid chromatography. The chromatographic conditions were optimized with respect to sensitivity. To achieve a very efficient ionization the analytes should elute from the column with a relatively high percentage of organic solvent to ensure complete evaporation of the LC solvents. Further no analyte should elute with the solvent front of the column which would lead to ion suppression in the electrospray process. This was achieved for all azoles with a Synergy Hydro RP column, acidified water as solvent A and a gradient starting at 50% A. Fig. 2A shows an overlay of the 13 single traces obtained by measuring the 13 specific quantifier MS/MS transitions at LOQ level in a spiked wine sample. Fig. 2B shows the quantifier transitions of standards spiked in wine of triadimenol and tebuconazole. These two azoles where detected in the sample shown in Fig. 2C. The signals eluting at 1.8 min with the solvent front are no azole compounds. Apart from imazalil eluting at 2.3 min all azoles are well separated from the solvent front. The use of compound specific MS–MS transitions does not require a complete resolution between the analytes. Sample matrix (wine versus water) has no influence on the separation.

Table 3
Results of the 103 wine samples tested

Azole detected	Occurrence	Range of levels determined (ng/mL)
Cyproconazol	2/103	<LOQ
Epoxiconazole	3/103	6.5–30
Flusilazole	9/103	0.5–0.75
Myclobutanil	25/103	0.5–35
Penconazole	26/103	0.25–1.2
Tebuconazole	55/103	0.25–33
Triadimenol	33/103	0.5–2.2

3.2.1.4. Quantitation. The only work-up step centrifugation has no effect on the azole concentrations (data not shown). This is also demonstrated by identical accuracy, precision and slope values of calibration standards and curves acquired in water or wine matrix (Table 2). As it is not a problem to get robust quantitative results and satisfactory data, the use of stable isotope labeled internal standards is not necessary. Furthermore an isotope labeled standard is not available for many azoles. The limits of quantitation ranged between 0.25 and 7.5 ng/mL. For all azoles tested the LOQ was lower than the maximum residues levels (MRL) of the European Union by at least a factor of 4 (Table 2) enabling the use of the method for screening of beverages, especially wine, without any sample enrichment step.

3.2.1.5. Application to wine samples. One hundred and three randomly chosen wine samples from the following 11 different countries were analyzed (in brackets the number of samples from the specific country): Germany (50), Italy (19), Spain (6), France (5), Chile (7), Czech Republic (1), Greece (2), Switzerland (1), South Africa (9), Australia (2), USA (1). The samples consisted of 49 red, 42 white and 12 sparkling

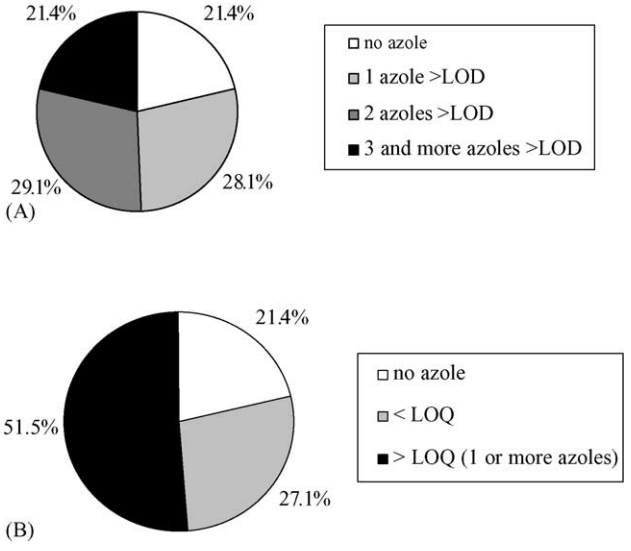

Fig. 3. Pie charts showing results of residue determination in 103 wine samples. (A) Number of different azole compounds found in one sample; (B) Findings were sorted on the basis of the ability to quantify one compound found or not.

wines. Among the grape varieties where e.g. Bacchus, Cabernet Sauvignon, Merlot, Riesling, Sauvignon Blanc as well as various blends. In more than 75% of the samples azoles were detected, but all concentrations were below the known MRL (Table 3 and Fig. 3). Tebuconazole is the most commonly used fungicide and was detected in 53% of the samples. Azole fungicide residues were observed in wine samples of every country but a statistical analysis of the data is not possible since the samples for each country do not represent a valid cross section. In one wine sample produced under ecological control no azoles were detected. Interestingly, about 50% of the wine samples contained more than one respectively 21% more than two azole fungicides (Fig. 3). Applying more than two azole fungicides to one acre in one season is against "good agricultural practice". In the winemaking progress, e.g. for Bordeaux blends, a variety of grapes from different vineyards, sometimes from more than one producer, are blended to one product. Thus, the more than two azoles in one wine sample could be explained by the usage of different fungicides on different acres and a mixture of the grapes of different origin in the cellar. Nevertheless, in the case of wine samples containing more than two azoles the levels of azoles are clearly below the MRL.

4. Conclusion

The method described shows good accuracy and precision and can be utilized as screening method for azoles in wine samples. With centrifugation as the only sample work-up step and a short LC-gradient as many as 100 samples can be measured in a day. Good chromatographic behavior of the analytes on the column used, results in good peak shapes and LOQs well below the maximum residue levels.

Wines from 11 different countries were tested and at least one sample from every country tested positive for an azole fungicide. Tebuconazole was the most frequently determined azole. In 50.5% of the 103 samples 2 or more azole compounds could be detected.

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