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Residues of Azoxystrobin from Grapes to Raisins

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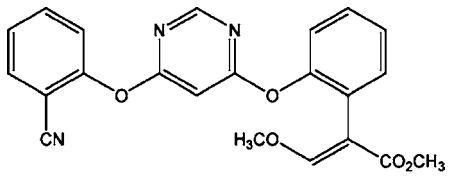
Azoxystrobin, a fungicide of the strobilurin group, has an European Union maximum residue level (MRL) of 2 mg/kg for grapes. This work aimed to assess residues on fresh and washed grapes and on raisins following processing with (i) alkali treatment and sun drying and (ii) sun drying only. QUADRIS 25% SC was applied according to good agricultural practice for two consecutive years on a typical cv. Thomson seedless and a seed-producing clone. Samples were collected 0, 15, and 21 days postapplication and analyzed using gas chromatography/electron capture detection; recoveries were 86 ± 12% for grapes and 99 ± 15% for raisins. Residues on grapes were 0.49−1.84 mg/kg, and washing removed 75% of the residue. Residues in raisins produced from seedless grapes were 0.51−1.49 (treatment 1) and 1.42−2.08 mg/kg (treatment 2), with residue transfer factors sometimes >1, even following alkali treatment, which reduced residues considerably. To avoid trade problems, a higher MRL for raisins is necessary.

KEYWORDS: Azoxystrobin; residues; grapes; raisins; processing

INTRODUCTION

Azoxystrobin [methyl (*E*)-2-{2-[6-(2-cyanophenoxy)pirimidin-4-yloxy]phenyl}-3-methoxyacrylate], with the structure shown in the figure below, is a systemic fungicide of the strobilurin group with protective action and is registered in Greece for grapes to control *Plasmopara viticola* and *Uncinula necator*.



It has been evaluated in the European Union (EU) in the framework of 91/414/EEC Council Directive (*1*) as a new molecule and was approved in 1998 as an active substance of minimum purity 930 g/kg containing 25 g/kg *Z* isomer (*2*). It is a compound with no particular toxicological concerns, with an acceptable daily intake (ADI) for man of 0.1 mg/kg b.w./day. On the basis of critical good agricultural practice (GAP) in Europe, which refers to uses in Northern countries, the value of 2 mg/kg was set as the EU maximum residue level (MRL) for grapes (*3*). According to the current EU legislation, in the case of dried or processed products for which maximum levels are not explicitly fixed, the MRL applicable is that for the fresh product taking into account, respectively, the concentration caused by the drying process or the concentration or dilution caused by processing (*4*). Trials carried out in Greece by the manufacturer on the Black Korinth variety of grapes have shown that residues in raisins are higher than in the fresh commodity (unpublished proprietary data).

Procedures used in industrial food processing and domestic cooking frequently have dramatic effects on residue levels (*5*−*7*). In most cases, large decreases in residues occur, as shown by Schattenberg et al. (*8*) in a study to determine the effects of normal household preparation on pesticides in many food commodities. However, residues may concentrate in processed commodities following certain procedures. The ratio of the residue in the processed commodity to that in the raw commodity is referred to as the transfer factor (TF). If this ratio is greater than 1, the residue is said to concentrate upon processing. Studies on the effect of processing and the determination of TFs have been recognized among the main priorities in the process of MRL setting (*9*) and for enabling a more realistic exposure assessment and risk management (*10*).

The aim of this work was to assess the magnitude of residues of azoxystrobin on fresh grapes of the typical cv. Thomson seedless (Sultana) and a seed-producing clone and on raisins produced from these after the two types of processing commonly used, i.e., sun drying alone and sun drying after treatment with a solution containing 3% K_2CO_3 and 1% ethyl oleate.

MATERIALS AND METHODS

Chemicals and Apparatus. Azoxystrobin formulated material (QUADRIS 25% SC) and analytical standard of 91% purity were kindly provided by Syngenta. All solvents were of pesticide residue analysis grade. Stock standard solutions of azoxystrobin at a concentration of 1000 μg/mL were prepared in toluene. K_2CO_3 and ethyl oleate were

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commercial products used widely for drying grapes of the Thompson seedless variety. Fungicide determinations were carried out using a Hewlett-Packard (Avondale, PA) 5890 Series II gas chromatograph, with electron capture detection (ECD) operated at 300 °C, a Hewlett-Packard 7673 autosampler, and a split−splitless injector operated in the splitless mode (220 °C, 60 s, 1 μL). The carrier and makeup gases were helium (1.2 mL/min) and nitrogen (50 mL/min), respectively. An HP-5MS (5% phenylmethylpolysiloxane) fused-silica column with dimensions 30 m × 0.25 mm i.d. × 0.25 μm film thickness was used. The oven temperature program was as follows: 80 °C, hold 1 min; 15 °C/min to 260 °C; 3 °C/min to 290 °C, hold 5 min.

Field Trials and Sampling. Treatments with QUADRIS 25% SC were applied for two consecutive years by knapsack sprayer at the end of veraison (mid-August) using the application rate recommended in Greece, i.e., 0.025 kg active substance (a.s.)/hL, equivalent to 0.255 kg a.s./ha. In the first year, the pesticide was applied to 40 vines of the Thomson seedless variety (Sultana) growing in the Research Foundation's vineyards in Athens in a well-drained clay soil. Six of the vines were of the typical seedless variety, producing small berries, while the remaining 34 were of a seed-producing clone with approximately three times larger berry size. In the following year, the trials were carried out in a vineyard in the region of Korinth in the North Peloponnese on nine vines of the typical Thomson seedless variety producing small berries.

In the first year, samples were collected at times 0 (4 h postapplication, when the spray mixture had dried) and 15 days postapplication only, due to the low yield obtained. In the second year, samples were collected at 0, 15, and 21 days postapplication. The recommended preharvest interval (PHI) was 21 days. In the first year, relatively small samples were collected as follows: at time zero, 0.88 kg of the seedless variety and 1.5 kg of the seed-producing clone, and at 15 days postapplication, 6.5 kg of the seedless variety and 1.6 kg of the seed-producing clone (all of the remaining bunches). Because of the high variability found between individual samples, larger samples were collected in the second year, i.e., 2.9 kg at day zero, 9.2 kg at 15 days, and 21.6 kg (all of the remaining bunches) at 21 days.

Sample Preparation and Sample Processing. Samples were placed in polyethylene bags, transported to the laboratory immediately after picking, and were subjected to the following handlings on the same day.

Day 0 Samples. After the bunches were cut into smaller pieces, the whole sample was taken and divided into five replicate samples. These were weighed, the stems were removed, the berries were homogenized, and the sample was stored at −20 °C until analysis.

Day 15 Samples. Approximately a fifth of each sample was taken and treated as the day 0 sample. The remaining grapes were subdivided into five replicate samples, weighed, and subjected to the commonly applied treatment for processing grapes of the Thomson seedless variety to sultanas. This treatment, which is referred to below as treatment 1, consisted of submerging each replicate sample for 3 min in 10 kg of tap water containing 3% (300 g) K_2CO_3 and 1% (100 g) ethyl oleate (solution pH 11.05 at 26.6 °C) and exposing it to direct sunlight for 15 days to dry. After they were dried, the raisins were weighed, homogenized after removal of the stems, and stored at −20 °C until analysis.

Day 21 Samples (Year 2 Only). Approximately a fifth of each sample was taken and treated as the day 0 sample. A further similar-sized portion was separated, subjected to washing by submerging in a sufficient volume of water to just cover the sample (8 L) for 10 min, and divided into five replicate samples. These were weighed, the stems were removed, and the berries were homogenized and stored at −20 °C until analysis. The remaining grapes were divided into two parts. One of these was subjected to processing treatment 1 described above to produce raisins. The second, after subdivision into five replicate samples, was left under direct sunlight to dry for 21 days. This is referred to below as treatment 2.

To assess the effect of growth on berry weight, batches of 100 fresh berries were weighed for each sample before homogenization. For calculation of the concentration factor (CF) of the produce after drying, i.e., kg of fresh produce required to produce 1 kg of dry product (*11*), a random batch of 100 raisins was weighed for each sample for comparison with the mass of 100 fresh berries.

Analysis. A multiresidue gas chromatographic (GC) method with ECD was used for the extraction of azoxystrobin residues from grapes and raisins (*12*). For grapes, this involved extraction of a 25 g homogenized sample with 50 mL of toluene and 25 mL of propan-2-ol using an Ultra-Turrax, removal of propan-2-ol by washing twice with water (125 mL of 2% Na_2SO_4 solution each time), and cleanup of the pesticide-containing toluene phase with 1 g of an adsorbent mixture of Celite and activated charcoal (1:3, w/w) after which the extract was filtered. For raisins, the sample was first mixed with distilled water (1:1 w/w) (*13*). This method gave extracts with 0.5 and 0.25 g sample/mL for grapes and raisins, respectively. Each sample was analyzed in duplicate. The residue concentrations in extracts were determined under the conditions described previously. The retention time of azoxystrobin was 22.9 min, and there were no interference peaks in this region of the chromatoghaph. Initial tests to assess the matrix effect on calibration (*14*), involving the use of standards in solvent and standards in grape or raisin extract, showed no significant matrix effect during GC analysis. However, matrix-matched standard solutions were used for analysis, since they are routinely used in our laboratory. Quantification was carried out using calibration curves with 3−5 levels, which covered the appropriate concentration range (typically 0.1−1.0 μg/mL). Recovery tests for grapes were carried out for six fortification levels in the concentration range of 0.02−2 mg/kg and for raisins for four fortification levels in the concentration range of 0.1−2 mg/kg.

RESULTS AND DISCUSSION

Recovery Tests. The mean percentage recovery for azoxystrobin in grapes was 86% ($n = 33$) with a relative standard deviation (RSD) of 12%. Individual recoveries ranged from 68 to 104%, and the limit of quantification (LOQ), established by carrying out replicate fortifications at this level, was 0.02 mg/kg with a mean recovery of 102% ($n = 5$) and RSD of 3.4%. For raisins, the mean percentage recovery was 99% ($n = 27$) with an RSD of 15% and individual recoveries ranging from 84 to 130%. The LOQ established for raisins and found to be adequate for this study was 0.1 mg/kg, with a mean recovery of 104% ($n = 8$) and RSD of 13.3%.

Effect of Growth on Berry Weight. No statistically significant difference ($p < 0.05$) was found between the masses of 100 fresh berries collected at 0, 15, and 21 days. Apparently, the berries were fully developed at treatment. The mean masses were 132 ± 18 (year 1) and 195 ± 14 g (year 2) for the seedless variety and 400 ± 29 g for the seed-producing clone.

Effect of Drying on Berry Weight and Residues. The CF of the produce was calculated for each replicate sample from the masses before and after processing to raisins according to the formula

$$\mathrm{CF} = \frac{\text{weight of fresh produce (kg)}}{\text{weight of dry produce (kg)}}$$

The mean values found for each experiment are given in **Table 1**.

An analysis of variance showed no statistically significant difference at the 95% confidence level between the means of the CFs for raisins produced during both years from the seedless variety with treatment 1 (PHIs of 15 and 21 days). The mean value was 4.1 ± 0.5, which compares well with the theoretical CF from grapes to raisins of 4.7 estimated by the Environmental Protection Agency (*11*) and the value of 4.0 reported by Cabras et al. (*15*) for cultivar Imperatrice. The samples collected 21 days postapplication but subjected to treatment 2 (no alkali processing) and the samples from the seed-producing clone both had lower mean CFs of 3.2 and 3.4, respectively. In the first case, this was due to less efficient removal of water, despite

Table 1. Calculated CFs and Azoxystrobin Residue TFs from Grapes to Raisins

	PHI[a] (days)	residue in fresh grapes (mg/kg)	drying time (days)	CF[d] (mean ± SD, $n = 5$)	theoretical residue level in raisins[e] (mg/kg)	residue in raisins (mg/kg)	residue loss (%)	residue transfer factor
				year 1				
seedless variety	15	1.27	15[b]	3.8 ± 0.6	4.8	0.91	81	0.72
seed-producing clone	15	0.64	15[b]	3.4 ± 0.6	2.2	0.75	66	1.2
				year 2				
seedless variety	15	0.75	15[b]	4.1 ± 0.5	3.1	1.35	54	1.8
seedless variety	21	0.72	15[b]	4.4 ± 0.3	3.2	0.92	71	1.3
seedless variety	21	0.72	21[c]	3.2 ± 0.3	2.3	1.72	25	2.4

[a] PHI. [b] Alkali treatment followed by drying for 15 days. [c] Sun drying only. [d] CF. [e] The residue found in grapes multiplied by the CF.

Table 2. Azoxystrobin Residues in Fresh Grapes and Raisins

	sampling time (days)	treatment	commodity	residues (mg/kg) in replicates[a] 1	2	3	4	5	mean	RSD
				seedless variety						
year 1	0		grapes	0.91	1.76	0.82	0.77	0.92	1.04	38
	15		grapes	1.66	0.82	1.84	1.31	0.71	1.27	38
	15	alkali and drying	raisins	1.26	0.53	1.11	0.51	1.13	0.91	37
year 2	0		grapes	1.37	1.14	1.16	1.24	1.19	1.22	8.3
	15		grapes	0.73	0.78	0.69	0.85	0.72	0.75	8.8
	15	alkali and drying	raisins	1.43	1.13	1.40	1.28	1.49	1.35	10.6
	21		grapes	0.72	0.63	0.69	0.68	0.87	0.72	12.9
	21	washing	grapes	0.15	0.11	0.17	0.23	0.25	0.18	33.3
	21	alkali and drying	raisins	0.90	0.94	0.94	0.84	1.00	0.92	9.9
	21	drying,[b] 21 days	raisins	1.68	1.42	1.68	2.08	1.72	1.72	15.4
				seed-producing clone						
year 1	0		grapes	1.36	0.91	0.80	0.84	1.91	1.17	38
	15		grapes	0.24	1.14	0.67	0.49	0.64	0.64	49
	15	alkali and drying	raisins	0.53	1.06	0.60	0.78	0.78	0.75	27

[a] Mean of duplicate analysis of each replicate. [b] Sun drying only without alkali treatment.

the longer drying period, while in the second it may be attributed to the larger berry size and the presence of pips.

Residues in Grapes and Raisins. Table 2 gives the results determined for azoxystrobin residues in the different samples. The values are the means of the duplicate analyses carried out for each sample.

Initial Deposits (Day 0). A *t*-test assuming unequal variances showed no significant difference between the mean residues on the seedless variety for the 2 years of the study at the 95% confidence level, and the pooled data gave a mean concentration ± standard deviation (SD) for azoxystrobin of 1.13 ± 0.30 mg/kg. This was very similar to the deposits of 1.17 ± 0.47 mg/kg found for the seed-producing clone.

Residues at Harvest (Days 15 or 21). *Seedless Variety.* For the first year of the study, there was no reduction in residues during the PHI of 15 days. In contrast, a *t*-test for the second year data indicated a significant reduction ($p < 0.001$) in residues between day 0 and day 15, from 1.22 ± 0.09 to 0.75 ± 0.06 mg/kg. Because of the larger sample size and consequently lower RSDs for the mean residues in the second year, data from this year are considered to be more representative. Laboratory samples had a mean mass of 640 g in year 2 as compared to 210 g in year 1. There was no further reduction in residues between 15 and 21 days in the second year.

Seed-Producing Clone (Year 1 Only). Although the residues appeared to decrease with time to 0.64 ± 0.33 mg/kg, no significant difference was found between the means for days 0 and 15 ($p = 0.0748$) at the 95% confidence level, due to the large variance between subsamples. In both years, the residues in samples collected 15 or 21 days postapplication were always below the EU MRL.

Residues after Washing (Day 21). Residues in washed grapes of the seedless variety were much lower than those in the unwashed commodity, with a reduction of 75% (mean residue 0.18 ± 0.06 mg/kg). Although azoxystrobin is a compound with translaminar and systemic properties and low water solubility (6 mg/L at pH ranging from 5 to 9, log $P_{o/w} =$ 2.5 at 20 °C), it would appear that deposits remained largely on the grape surface following treatment and were removed to a great extent by washing.

Residues in Raisins, Treatment 1. *Processing with Alkali Treatment and Sun Drying.* In the first year, the mean residues in raisins produced from fresh grapes of the seedless variety collected 15 days after application and containing 0.91 ± 0.36 mg/kg on average were lower than those in the fresh commodity (1.27 ± 0.48 mg/kg), but no statistical difference was found between them at the 95% confidence level due to the large variance between subsamples. In contrast, in the second year, the mean concentration in raisins was significantly ($p < 0.01$) higher than that in the fresh grapes (1.35 ± 0.14 mg/kg as compared with 0.75 ± 0.06 mg/kg). An increase in residues from 0.72 ± 0.09 in grapes to 0.92 ± 0.06 mg/kg in the raisins was also observed following collection of the grapes 21 days postapplication in the second year ($p < 0.01$). Residues of azoxystrobin in raisins produced from grapes of the seed-producing clone gave a mean concentration of 0.75 ± 0.21 mg/kg, which differed very little from the concentration of 0.64 ± 0.33 mg/kg in the fresh commodity, in agreement with the result for the seedless variety in the same year.

Residues in Raisins, Treatment 2. *Sun Drying without Alkali Processing (Day 21 Only).* The mean residue in raisins produced from fresh grapes collected 21 days after application was 1.72

± 0.26 mg/kg. The higher residues as compared with those of raisins produced after treatment with alkali solution are consistent with the finding that washing substantially decreases residues on the grapes.

Loss of Residues on Processing. Table 1 also gives the theoretical residues expected in raisins, i.e., the residues, allowing for loss of water, which would be found in the raisins if no loss of azoxystrobin occurred. The fact that the actual residue concentrations in raisins were lower than these theoretical values showed that in practice loss of residues occurred. Reductions resulting from alkali treatment followed by drying were 69 ± 13% for the seedless variety and 66% for the clone. This was close to the percentage loss of 75% that occurred on washing grapes harvested on day 21, and it therefore seems likely that the residue loss occurred during alkali treatment. This conclusion is supported by the fact that reduction in residues was only 25% for raisins production with drying only.

Residue TFs. TFs, given in **Table 1**, were calculated using the formula

$$\mathrm{TF} = \frac{\text{residues in processed product (mg/kg)}}{\text{residues in raw agricultural commodity (mg/kg)}}$$

The values found for the seedless variety with alkali treatment ranged from 0.72 to 1.8, with a value of 1.3 for grapes harvested at the recommended PHI of 21 days, showing that an increase of residues from grapes to raisins may occur during the commonly applied processing treatment. For the seed-producing clone, the value was 1.2. When grapes of the seedless variety were dried without any alkali treatment, the TF was higher. Given this increase in residues found on processing, a higher MRL is justified for raisins.

In conclusion, when grapes of the Thomson seedless (Sultana) variety were treated with azoxystrobin according to the GAP authorized in Greece, residues at harvest did not exceed the EU MRL of 2 mg/kg. Washing of berries removed a large amount of residues. The processing treatment with alkali and sun drying effectively removed a substantial amount of azoxystrobin residues. Commercial production of raisins is, however, also carried out with sun drying only. Because it has been found that residues in raisins might be higher than those in grapes, even following alkali treatment (TF up to 1.8), a higher MRL in raisins is necessary in order to ensure that legal residues in grapes (i.e., up to 2 mg/kg) do not give rise to violative residues in raisins and thus to trade problems. Azoxystrobin is a compound with no particular toxicological concerns (ADI 0.1 mg/kg bw/day, no need for acute reference dose), and such an increase in the MRL for raisins would not pose any risk for consumers.

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