SDRP Journal of Food Science & Technology 2016

Open Access

Research

Occurrence of fungicide residues on Argentinean blueberry fruit and juice samples.

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Received date: 28-03-2016; **Accepted date:** 26-04-2016; **Published date:** 29-04-2016

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CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

ABSTRACT:
Azoxystrobin, boscalid, cyprodinil, fludioxonil and pyraclostrobin are fungicides commonly used in Argentina against different fungus contaminations in the blueberry field. The presence of these fungicides was investigated in 50 samples of blueberry fruit and 15 samples of blueberry juice purchased in Argentina. Fungicide residues were determined by solid-phase microextraction (SPME) coupled to gas chromatography with micro-electron capture and nitrogen phosphorous detector. The average concentrations of azoxystrobin were 48 μg/kg in blueberry fruit. Average of boscalid were 43 and 239 μg/kg, of cyprodinil 1581 and 852 μg/kg, of fludioxonil 1077 and 2842 μg/kg, and of pyraclostrobin 578 and 3414 μg/kg, in fruits and juice respectively. The higher concentrations of fungicides were found in those cases where the time between the application of the compound and the fruit's commercialization was shorter than the fungicide's half-life.

Key words: Fungicides – Blueberries – Time between fungicide application and harvest – SPME/GC

INTRODUCTION

Highbush blueberry (*Vaccinium corymbosum* L.) is a small fruit that belongs to the Ericaceae family. Entre Ríos province is the main production area of Argentina, with more than 50% of the blueberry fields located in Concordia Department (lat 31°23′32″S, long 58°1′1″W), and represents more than 50% of the blueberry local production, followed by Tucumán and Buenos Aires (Bruzone, 2010). Approximately the 90% of the Argentinean production is exported as fresh fruit, principally to United States, Canada and European Union in their winter season.

This fruit has high levels of nutrients that are beneficial for health and that is the reason for their consumption increased in the last years (Lau, Shukitt-Hale & Joseph, 2007; McDougall, Ross, Ikeji & Stewart, 2008). These nutrients combined with a high water activity and low pH value, make blueberries particularly susceptible to fungal spoilage and the use of fungicides is a common practice in the field.

Azoxystrobin [methyl(2*E*)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate - $C_{22}H_{17}N_3O_5$], boscalid [2-chloro-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide - $C_{18}H_{12}Cl_2N_2O$], cyprodinil [4-cyclopropyl-6-methyl-*N*-phenylpyrimidin-2-amine - $C_{14}H_{15}N_3$], fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1*H*-pyrrole-3-carbonitrile - $C_{12}H_6F_2N_2O_2$], and pyraclostrobin [methyl 2-[1-(4-chlorophenyl)pyrazol-3-yloxymethyl]-*N*-methoxycarbanilate - $C_{19}H_{18}ClN_3O_4$] are the most important fungicides used in blueberry fields to avoid fungus contamination and spoilage (Tournas & Katsoudas, 2005; Luan et al. 2007; Vásquez et al. 2007; Wharton & Schilder, 2008; Rivera et al. 2009; Munitz et al. 2013a).

Depending on some factors such as their physico-chemical properties and blueberry post-harvest treatments, some of these fungicides can be also found in the final commercial product. The concentration of these pesticides in the berries is limited by legislation. For example, in Europe, the Regulation (EC) N° 396/2005 establishes maximum residue limits (MRLs) of 5.0 mg/kg for azoxystrobin, 10.0 mg/kg for boscalid, 5.0 mg/kg for cyprodinil, 3.0 mg/kg for fludioxonil, and 3.0 mg/kg for pyraclostrobin. However, despite of being a direct exposure source to consumers, MRLs of these fungicides for blueberry juice are still scarcely regulated. Thus, in order to establish future legal measures, it is mandatory to perform both monitoring and toxicological studies to assess the background levels of these chemicals in different juices and to evaluate the chronic exposure of consumers to these contaminants, respectively.

The time between the last application of the fungicide and the harvest, that is normally recommended by the commercializers of agrochemical compounds and used in the field, is a very important parameter that needs to be considered before consuming blueberries, because otherwise, could be associated with health problems (Agrosiembra, 2012; SENASA, 2012).

Although there are a lot of new reports about pesticide residues in unprocessed and processed products (Gérez et al., 2015), there has not been developed a study of contamination of fungicides in blueberry fruit and juice samples produced and/or marketed at least in Argentina. And there are not studies about the relationship between the fungicide application and the time until harvest, and the occurrence of these compounds in the final product.

The aim of the present study was to evaluate the concentration of these five fungicides in blueberry fruit and juice commercialized in the main supermarkets of Argentina.

MATERIALS AND METHODS

Samples

During 2014-2015, a total of 50 samples of blueberry fruit packaged on clamshells of 125 g and 15 samples of blueberry juice packaged on 0.5 l bottles were randomly purchased from supermarkets and grocery stores of Concordia city (Entre Ríos province) and Buenos Aires, Argentina.

The sample size should be of 1 kg for fruit and 0.5 l of juice to fit the European Norm EC (2002), so 8 packages of fruit and 1 bottle of juice were bought to have a representative sample. Samples were kept in their original packages, properly identified, and stored under freezing condition (-18 ± 1°C) until the analyses.

Standards, solutions and reagents

Azoxystrobin, boscalid, cyprodinil, fludioxonil and pyraclostrobin standards of high purity (>98%) were supplied by Sigma-Aldrich (Seelze, Germany). The stock solutions (1 g/l) were prepared by dissolving the standards in methanol HPLC grade (99,9%) purchased by Sintorgan (Buenos Aires, Argentina), and stored under freezing condition (-18 ± 1°C) in dark bottles sealed with PTFE/silicone caps. The working solutions were prepared daily by diluting with deionized water obtained from an E-pure water purification system (Barnstead/Thermolyne, Bedford, MA, USA). Sodium hydroxide was purchased from Biopack (Buenos Aires, Argentina).

Apparatus

The chromatographic conditions for azoxystrobin, boscalid and pyraclostrobin were those validated by Munitz et al. (2013a, 2014), and for cyprodinil and fludioxonil those validated by Munitz et al. (2013b); and they are briefly described as follows: gas chromatographic (GC) analyses were carried out on an Agilent 6890N gas chromatograph equipped with a micro-electron capture detector (μECD) and a nitrogen phosphorous detector (NPD), a split-splitless injection port, a 0.75 mm ID liner and a fused silica capillary column HP-5MS (30 m x 0.25 mm i.d. x 0.25 μm film thickness). The carrier gas helium was maintained at a constant flow of 1 ml/min. Desorption of the fibers into the injection port was carried out in the splitless mode at 240 °C for pyraclostrobin and 250 °C for the others pesticides. The desorption time was 6.5 min for azoxystrobin, boscalid and pyraclostrobin, and 5.0 min for cyprodinil and fludioxonil, respectively. The oven temperature was programmed as follows: initial temperature of 80 °C (6 min), increased at a rate of 80 °C/min up to 280 °C (10 min) for azoxystrobin, boscalid and pyraclostrobin. Cyprodinil and fludioxonil needed other oven conditions: initial temperature of 80 °C (5.3 min), increased at a rate of 55 °C/min up to 260 °C (2 min), and increased at a rate of 10 °C/min up to 280 °C (4 min). The detector temperature was 300°C. The SPME holder for manual use and fibers coated with 100 μm PDMS were purchased from Supelco (Bellefonte, PA, USA). The fiber was conditioned by heating in the GC injection port following the manufacturer recommendations. The extraction procedure was performed in 8.5 ml amber glass vials containing 8 ml of solutions that were stirred at 1500 rpm for azoxystrobin, boscalid and pyraclostrobin, and at 2000 rpm for cyprodinil and fludioxonil, respectively, with a magnetic stirrer.

Fungicide extraction

The extraction procedure could be summarized as follows: 10 g portion of fruit or 3 g of juice was weighted in a 25 ml centrifugal tube and extracted with 10 ml water. The solution was vigorously shacked in vortex mixer during 1 min. The pH was adjusted to 5.0 for pyraclostrobin and 7.0 for the other fungicides with sodium hydroxide. Then, samples were centrifuged at 3000 rpm during 15 min. Then, the supernatant was filtered with 0.2 µm filter in a vacuum maninfold. The extraction was repeated one more time. The procedure was made in triplicate.

RESULTS AND DISCUSSION

Quality assurance of the method

The presence of matrix effect was observed because of the difference between the standard and spiked samples curves slopes. The ANOVA test showed that there were statistically significant differences ($p<0.01$) for both cases. For that reason, the standard addition method was recommended for quantification studies. The performance characteristics of the analytical method obtained from spiked blank samples are presented in Table 1. The results showed a good linearity with regression coefficient of 0.9965 for azoxystrobin, 0.9975 for boscalid, 0.9997 for cyprodinil, 0.9998 for fludioxonil and 0.9990 for pyraclostrobin, respectively.

The repeatability of the method was determined by performing ten replicates of a single extracting solution from cyprodinil and fludioxonil spiked blueberry samples. The relative standard deviations (RSDs) obtained were lower than 10% in all cases. These values indicate that the precision of the method was satisfactory for control residue analysis (Table 1).

Table 1: Performance characteristics of the analytical method

Analyte	Matrix	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Recovery[a] (%)	RSD%[b] (n=10)
Azoxystrobin	Blueberries	2	6	100 - 106	<9
Boscalid	Blueberries	2	4	98 - 104	<10
Cyprodinil	Blueberries	2	4	99 - 101	< 6
Fludioxonil	Blueberries	1	2	98 - 100	< 9
Pyraclostrobin	Blueberries	26	86	96 - 106	< 7

[a]Calculated spiking samples at three concentrations (n=3)
[b]Calculated at seven concentrations (0.05, 0.1, 0.25, 0.5, 1, 3 and 5 mg/kg for pyraclostrobin; and 0.005, 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 mg/kg for the others)

LOD and LOQ were calculated as three and ten times the signal-to-noise ratio, respectively (Table 1). These values satisfy the MRL established by the EC and means that the method is sufficiently sensitive.

Blueberry fruit and juice samples were spiked with 0.05 ppm of azoxystrobin, boscalid, cyprodinil and fludioxonil; and 0.5 ppm of pyraclostrobin, and ten replicates of each concentration were performed. The comparison of two independent samples was performed using the Statgraphics Centurion XV. Averages, medians, standard deviations and distributions of blueberry fruit and juice spiked samples were compared. There were not significant differences between both matrixes. For this reason, the same analytical method was used to blueberry juice.

Table 2 and 3 shows the concentration of each fungicide found in the different samples of blueberry fruit and juice, respectively. High frequency of each fungicide (azoxystrobin 40%, boscalid 70%, cyprodinil 84%, fludioxonil 76%, and pyraclostrobin 54%) and co-occurrence of some of them in the same sample have been found for blueberry fruit. Something similar occurred with the frequency of each fungicide in blueberry juice (boscalid 100%, cyprodinil 80%, fludioxonil 73%, and pyraclostrobin 60%). Azoxystrobin was not found in these samples.

Table 2: Concentration of different fungicides in blueberries (□g kg^{-1})

Sample	Azoxystrobin	Boscalid	Cyprodinil	Fludioxonil	Pyraclostrobin
1	ND	133	104	136	ND
2	75	117	27	58	ND
3	117	19	3382	2405	ND
4	109	90	558	473	ND
5	ND	105	209	362	597
6	ND	86	2154	3671	843
7	71	21	2495	3409	1505
8	ND	26	3864	3251	ND
9	72	ND	24	53	ND
10	ND	5	80	88	1041
11	72	22	7066	ND	1320
12	69	ND	5174	ND	623
13	ND	ND	47	106	ND
14	ND	30	58	128	1177
15	ND	1	43	2613	1719
16	ND	3	ND	ND	ND
17	ND	42	79	127	ND
18	72	ND	4482	268	ND
19	99	ND	48	2742	639
20	ND	142	116	3914	938
21	ND	ND	6241	1900	ND
22	ND	74	350	238	1054
23	ND	102	60	59	531
24	ND	ND	ND	ND	1520
25	ND	ND	183	815	ND
26	ND	32	ND	ND	1972
27	225	167	53	470	1443
28	261	ND	ND	ND	ND
29	71	121	5086	4470	1222
30	ND	51	ND	ND	ND
31	202	ND	1739	3084	ND
32	ND	101	2009	ND	ND
33	ND	108	68	109	1027
34	175	32	ND	177	438
35	ND	88	835	1288	710
36	77	10	444	3077	ND
37	ND	ND	210	ND	ND
38	ND	5	1036	89	1228
39	ND	16	2090	392	ND
40	106	44	5589	1039	1832
41	121	132	7001	4300	ND
42	260	17	6217	3621	600
43	ND	5	2285	201	ND
44	ND	ND	58	ND	811
45	ND	ND	83	832	1054
46	ND	58	ND	2200	ND
47	ND	95	4277	ND	1310
48	89	ND	3018	753	993
49	72	37	ND	ND	772
50	ND	10	94	929	ND

ND = not detected

Averages and medians were calculated replacing the not detected values by zero, and are shown in Tables 4 and 5. The half life of each fungicide in two blueberry varieties (Emerald and Jewel), and the time between the last application of the fungicide and the harvest, are shown in the same tables (Munitz et. al. 2013a, 2013b, 2014). The fungicides manufacturers recommend 1 day for fludioxonil and cyprodinil in blueberries (Syngenta, 2010). BASF (2013) does not make any recommendation about azoxystrobin, boscalid or pyraclostrobin in blueberries, but recommend 3 days for boscalid and pyraclostrobin in grapes. However, the commercializers recommend 6 days for boscalid and pyraclostrobin, and 90 days for azoxystrobin, and these are the periods used in the blueberry fields.

Table 3: Concentration of different fungicides in blueberries juice (µg kg^{-1})

Sample	Azoxystrobin	Boscalid	Cyprodinil	Fludioxonil	Pyraclostrobin
1	ND	140	1073	3033	7529
2	ND	452	291	850	ND
3	ND	687	ND	ND	5439
4	ND	105	1750	5081	6236
5	ND	23	1411	4098	ND
6	ND	376	ND	4072	ND
7	ND	29	1298	3992	5902
8	ND	67	1702	ND	6693
9	ND	109	428	3902	6218
10	ND	197	377	4872	7388
11	ND	49	ND	3810	ND
12	ND	87	629	ND	2943
13	ND	173	1307	3883	2865
14	ND	638	1632	5045	ND
15	ND	449	882	ND	ND

ND = not detected

Table4: Average, median, maximum and minimum concentration of different fungicides in blueberries (µg kg^{-1}). Positive samples, half life time and time between application in the field and harvest.

		Azoxystrobin	Boscalid	Cyprodinil	Fludioxonil	Pyraclostrobin
Samples (n = 50)	Average	48	43	1581	1077	578
	Median	ND	22	196	253	564
	Max	261	167	7066	4470	1972
	Min	69	1	24	53	531
	N° possitive samples	20	35	42	38	27
Half life time (days)	Emerald	11.6	5.3	2.2	12.7	5.5
	Jewel	17.8	10.6	3.4	16.3	8.0
Time between application and harvest (days)		90	6	1	1	6

ND = not detected

Table5: Average, median, maximum and minimum concentration of different fungicides in blueberries juice (µg kg^{-1}). Positive samples.

		Azoxystrobin	Boscalid	Cyprodinil	Fludioxonil	Pyraclostrobin
Samples (n = 15)	Average	ND	239	852	2842	3414
	Median	ND	140	882	3883	2943
	Max	ND	687	1750	5081	7529
	Min	ND	23	291	850	2865
	N° possitive samples	0	15	12	11	9

ND = not detected

The concentration average for cyprodinil and fludioxonil in blueberry fruit are 1581 and 1077 µg/kg, respectively. However, the medians are quite small (196 and 253 µg/kg, respectively). The maximum concentrations found are 7066 and 4470 µg/kg for these two compounds, respectively. These high values could be related with the half life time that is 16.3 and 12.7 days for fludioxonil and 3.4 and 2.2 days for cyprodinil, in Jewel and Emerald blueberries, respectively; and the time recommended between application and harvest that is 1 day in both cases. The blueberry producers use in the field the doses of fungicides and the moment of application that are recommended by the fungicide manufacturers. There are not investigations and enough information about the period of time that should be let after each fungicide application and the harvest. It is different for each chemical compound and probably for each blueberry variety. The producers would not be leaving pass the necessary time before harvest for reducing the fungicide concentration to a half. On the other hand, the time normally used for azoxystrobin between application and harvest is 90 days which is very high in comparison with the half life time of this fungicide for both blueberry cultivars that are lower than 18 days (Munitz et al., 2014). For this reason azoxystrobin show the lower average concentration (48 µg/kg).

Referring to blueberry juice samples, azoxystrobin was not detected in all samples probably because of the long time between the application of this fungicide in the field and the harvest of the fruit, related with the half life time. Cyprodinil and fludioxonil are presented in high concentrations in all samples of blueberry juice. The average concentrations are 852 and 2842 µg/kg, respectively.

The average concentration of pyraclostrobin is 3414 µg/kg, higher than in fruit. This could be probably because fruits that is not good for consumption, because they have concentrations of fungicides higher than the LMRs for instance, are used in the juice elaboration process.

CONCLUSIONS

Besides the high occurrence frequency of fungicides found in blueberry fruit and juice samples, co-occurrence of some of them in the same sample was observed. Azoxystrobin, boscalid and pyraclostrobin concentration in blueberries did not exceed the LMR established in Europe. But seven samples of fruit presented concentrations of cyprodinil higher than the LMR, and nine of fludioxonil. The concentration of these in fruit and juice were high probably because of the short period of time between the application in the field and the commercialization or industrialization. The results show that a change in the time between application of the fungicide and the harvest of the fruit is needed.

ACKNOWLEDGEMENTS

The authors acknowledge to Agencia Nacional de Promoción Científica y Tecnológica, Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Universidad de Buenos Aires, Fundación de Investigaciones Científicas Teresa Benedicta de la Cruz and Universidad de Entre Ríos for financial support.

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