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Evaluation of Fusarium mycotoxins and fungicide residues in barley grain produced in Uruguay

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ABSTRACT

This work aims to survey the current barley grain situation in terms of the concentration level of *Fusarium* mycotoxins and fungicides used to control Ramularia Leaf Spot and Fusarium Head Blight in barley grain. For this purpose, a total of 89 barley grain samples from different commercial pads in Uruguay were analyzed by liquid and gas chromatography coupled to mass spectrometry. The results obtained showed that 74% of the grain samples analyzed contained fungicide residues but below their corresponding European Union Maximum Residue Limit (EU MRLs). The most frequently found fungicides were chlorothalonil, azoxystrobin, carbendazim, and fluxapyroxad which were detected at concentration levels from 0.01 to 0.36 mg kg^{-1}. With regards to mycotoxins, deoxynivalenol was detected in 88 out of the 89 samples, while zearalenone was confirmed in eight samples. The EU MRL exceedance for deoxynivalenol and zearalenone represents the 31% and 3% of the analyzed samples, respectively. The co-occurrence of deoxynivalenol and zearalenone was also confirmed. Despite that, in general, the concentration levels of mycotoxins and fungicides comply with the MRL, the results highlight the need to perform a strict monitoring program and risk assessment to ensure human and animal food safety.

1. Introduction

Barley production in Uruguay has fluctuated in the last decade, reaching a minimum of 61,900 ha in the 2010/11 growing seasons and a maximum of 190,000 ha in 2016/17. The bulk of high-quality barley is used for the production of malt for export, while low-quality grain is used for animal feeding [1]. Thus, malting quality could be affected by biotic and abiotic factors, including leaf spot diseases, which are one of the most relevant biotic factors. Leaf spots diseases cause grain yield loss ranging between 20 and 33%. Ramularia Leaf Spot (RLS) is the most aggressive disease reaching losses up to 70% in both yield and quality. These losses have been associated with a 90% reduction rate in the yield of grains higher than 2.5 mm in size [2,3].

Besides RLS, Fusarium Head Blight (FHB) represents a significant threat to the barley industry, causing significant losses in yield and grain quality [4]. FHB is prevalent in various *Fusarium* species, most of them producing mycotoxins such as zearalenone (ZEN) and deoxynivalenol (DON), which are known to be highly toxic to animals and humans [3].

The impact of these two diseases may not only reduce yield and grain quality but also, due to the fact that they require fungicide sprays for their control, may result in fungicide residues on the grain at harvest. Once the crop is planted, the control is only based on chemical strategies [5]. RLS has an asymptomatic phase, and the symptoms may appear explosively resulting in difficulty of the management of the crop, based solely on disease thresholds. Therefore, chemical control of RLS depends on the predictions of risk disease prior to crop flowering, along with the detection of the pathogen and not on the appearance of the disease symptoms [6,7]. Succinate dehydrogenase inhibitors - SDHIs (i.e., fluxapyroxad and isopyrazam), demethylation inhibitors in sterol biosynthesis – DMIs (i.e., prothioconazole), and protectant multisite inhibitors such as chlorothalonil, are used to minimize the damaging effects of RLS [3,7]. Chlorothalonil has been prohibited in the European Union [8], which represents a limitation for the export market. In addition, insensitivity to SDHIs and DMIs has been detected in *Ramularia collo-cygni* populations [9,10], which represents a threat for effective management of this disease.

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In the case of FHB, preventive management is recommended; including the combination of crop rotation, the use of resistant cultivars, and chemical control. Fungicide sprays must be performed just before infection occurs, based on forecasting systems [3,11]. The recommended fungicides for FHB control are triazoles, such as prothioconazole, tebuconazole, metconazole, and carbendazim [3,12,13]. Recent reports on *Fusarium graminearun* establish a reduced sensitivity for metconazole and tebuconazole [14,15]. The use of strobilurin fungicides, alone or in mixtures, is not recommended since it has shown a good FHB control, but an increased grain contamination by DON, affecting grain quality [16,17]. The presence of mycotoxins and/or fungicide residues in cereals is a serious concern for the food and feed industry [18,19].

Barley is a raw material in the brewing industry for malt and beer production. Thus, the grain quality determines the final product. During the process of malting and brewing, mycotoxins can be transferred into the final product, causing a significant risk for beer consumers [20]. Moreover, the presence of mycotoxins in animal feed leads to mycotoxicoses and immune suppression that may result in severe economic losses [21]. These effects depend not only on their concentration, but also on their synergistic effects with other coexisting mycotoxins in the grain [22].

Currently, there is high social pressure by consumers to produce food free of contaminants that might threaten human and animal health. Evidently, food safety has become an essential requirement for consumers, producers, and authorities responsible for food quality control. International organizations such as the European Union (EU) and the *Codex Alimentarius* aim to control these contaminants, by the establishment of Maximum Residue Limits (MRLs) to ensure the safety of agro-food products [23,24].

Several studies are focused on the validation of methodologies for pesticide residues and determining mycotoxin in barley grain [25,26]. However, there is little information about the presence of contaminants in barley obtained under production conditions [27,28]. Therefore, this work aims to survey the current barley grain safety in terms of the concentration level of *Fusarium* mycotoxins and fungicide residues in grains collected from commercial pads in Uruguay.

2. Materials and methods

2.1. Selection of contaminants

Deoxynivalenol and zearalenone were selected as the most frequently detected *Fusarium* mycotoxins that cause severe damage in cereals worldwide [19]. Moreover, ten of the most frequently used and recommended fungicides, due to their efficiency on RLS and FHB control were selected [3,7,12,13]; chlorothalonil, carbendazim, fluxapyroxad, isopyrazam, pyraclostrobin, trifloxystrobin, azoxystrobin, prothioconazole, epoxiconazole and triticonazole (Table 1). All these fungicides are currently approved for the production of barley in Uruguay.

2.2. Standards and reagents

Analytical standards were purchased from HPC Standards (Bosdorf, Germany). The purity of all the standards was higher than 98%. HPLC-grade acetonitrile (MeCN), ethyl acetate (EtAc), methanol (MeOH), and formic acid (98–100%) were supplied by Merck (Darmstadt, Germany). Ammonium acetate and ammonium formate were from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl), anhydrous magnesium sulfate ($MgSO_4$), sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), and sodium citrate dibasic sesquihydrate ($C_6H_6Na_2O_7{\cdot}1.5H_2O$) were from Scharlab (Barcelone, Spain). Water used for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was obtained from a Direct-Q3 Ultrapure Water System from Millipore (Billerica, MA, USA).

Individual stock standard solutions for the target compounds were prepared based on the solubility properties of each compound; in pure MeOH or MeCN, and stored at −18 °C. Twenty milligrams of each standard weighed were dissolved in 10.00 mL of the appropriate solvent to obtain a 2000 mg L^{-1} stock solution. Working solutions (1 and 10 mg L^{-1}) were prepared by appropriate dilution of the stock solutions in MeOH and used for the construction of matrix matched calibration curves.

2.3. Apparatus and instruments

Automatic pipettes suitable for handling volumes of 1–10 μL, 100–1000 μL and 1–10 mL, were from Eppendorf (Hamburg, Germany). Analytical balances weighing 1 mg were from Shimadzu (Kyoto, Japan). The centrifuge, suitable for use with the centrifuge tubes employed in the procedure and capable of achieving at least 3000×*g*, was an SL16 by Thermo Electron (Langenselbold, Germany). The orbital shaker used was a Stuart SSL1 (Staffordshire, UK).

LC-MS/MS was performed with an Agilent 1200 LC system coupled with a 4000 QTRAP® LC-MS/MS System from SCIEX™ run in the Scheduled® MS/MS-mode. MS/MS detection was performed in the multiple reaction monitoring mode.

The system was equipped with a Turbo V™ Ion Source operating in both positive and negative ionization modes. The ionization voltage was 5500 and -4500 V for the positive and negative mode, respectively. The probe temperature was 500 °C, the nebulizer gas was synthetic air at 50 psi, and the curtain gas was nitrogen at 20 psi. LC-separation was performed on a ZORBAX Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column from Agilent. The mobile phase consisted of 0.1% acetic acid and 5 mM ammonium acetate in water (solvent A) and 5 mM ammonium acetate in MeOH: 0.1% acetic acid in H_2O (95:5, *v/v*), (solvent B). The

Table 1
Maximum Residue Limits (MRLs) established by European Union (EU), *Codex Alimentarius*, Brazil and Argentine the chemical group, and the octanol-water coefficient expressed as log Kow for the selected contaminants in barley grain.

Compounds	Chemical group	MRLs (mg kg^{-1})				log Kow
		EU	*Codex*	Brazil	Argentine	
Azoxystrobin	Strobilurins	1.50	1.50	0.60	0.10	2.50
Carbendazim	Benzimidazole	2.00	0.50	0.20	–	1.48
Chlorothalonil	Benzonitrile	0.40	–	0.50	–	2.94
Epoxiconazole	Triazole	1.50	–	1.00	0.05	3.30
Fluxapyroxad	Carboxamide	2.00	2.00	0.5	1.00	3.13
Isopyrazam	Carboxamide	0.60	0.60	–	0.50	4.25
Prothioconazole	Triazole	0.20	0.20	0.3	0.01	2.00
Pyraclostrobin	Strobilurins	1.00	1.00	1.5	0.50	3.99
Triticonazole	Triazole	0.01	–	0.1	–	3.29
Trifloxystrobin	Strobilurins	0.50	0.50	0.5	0.20	4.50
Deoxynivalenol	Trichothecene	1.25	–	–	–	–
Zearalenone	Zearalenone	0.10	–	–	–	–

*Octanol-water coefficient pH 7, 20 °C.

gradient program used was initially 90% of solvent A which was kept constant for 1 min and decreased to 0% at 8 min; maintained for 5 min and increased to 90% within 2 min. The final proportion (90%) was kept constant for 4 min. The analysis time for each mode was 19 min at a flow rate constant of 0.6 mL min^{-1}. The injection volume was 5 μL.

Based on reported studies [29,30] the analysis of chlorothalonil was performed on negative chemical ionization (NCI) gas chromatography-mass spectrometry. An Agilent Technologies (Santa Clara, CA) 7890 gas chromatograph with a 5977B MS system equipped with an HP5-MS column (30 m × 0.25 mm i.d. × 0.25 μm) was operated under NCI mode. Helium was used as carrier gas at a constant flow of 1 mL min^{-1}. Methane gas with a purity of not less than 99.999% was used as the reaction gas for NCI analysis. Default values of a 40% methane flow rate and filament current were set. The GC oven temperature program was as follows: 90 °C kept for 1 min, raised to 300 °C at a rate of 20 °C per min and held for 5 min. The temperature of the injection port and transfer line was 250 °C. The splitless injection volume was 1 μL with a solvent delay time of 5 min. The ion source temperature was set at 150 °C, MS quadrupole temperature at 150 °C, the electron multiplier voltage was set at 1800 V, and the ion source energy 70 eV. The analysis was performed in the selected ion monitoring (SIM) mode looking for *m/z*: 264, 266, and 268.

2.4. Sample collection

Eighty-nine representative samples of barley grain were obtained from different pads located in the northwest region of Uruguay during the growing season 2016/2017. The geographical distribution of the samples is shown in Fig. 1. These samples were produced under real farm conditions following good agricultural practices in Uruguay; including chemical control before heading and following the recommendations suggested on the labels of each formulation [31].

After harvest, the grain with an average moisture content of 14% was transported to the brewing industry. A representative sample of each commercial pad was collected in a paper bag and taken to the laboratory for analysis.

For this growing season, the aforementioned region showed an 85% land area used for the planting of barley in the country, which corresponds to the Soriano, Colonia, Rio Negro and Paysandú region [32]. Forty-four samples belong to Paysandú, 41 to Río Negro, and one to the Salto region. The varieties included in this study were Arrayan and CLE 280 from the Instituto Nacional de Investigación Agropecuaria and MUSA 19 and MUSA 936, from Maltería Uruguay S.A, which are commonly used for malt production in Uruguay. The selected cultivars are characterized periodically by the *Red Nacional de Evaluación de Cultivares* in Uruguay [34].

2.5. Sample treatment for fungicide and mycotoxin analysis

One kilogram of each sample was ground with an IKA®-WERKE Model M20 (Wilmington, USA) at 2 mm diameter sieve to obtain the barley flour and stored in the freezer at −80 °C until analysis. Before the analysis, a subsample was obtained by the quartering sampling method to obtain around 2.0 g of the sample following Codex guidelines [35].

2.6. Extraction method

Barley grain was extracted using a solid-liquid extraction method based on citrate buffered QuEChERS [36]. The sample amount was 2.0 g, which was homogenized with milli-Q water and extracted with MeCN:-H_2O:formic acid (80:19:1, *v/v*). The salting-out was based on the addition of $MgSO_4$, NaCl, $C_6H_5Na_3O_7{\cdot}2H_2O$, and $C_6H_6Na_2O_7{\cdot}1.5H_2O$. After the extraction step, the samples were directly injected without further clean-up in the LC-MS/MS, while for GC-MS three mL extracts were concentrated and dried under a gentle nitrogen stream and then re-dissolved in 0.1 mL of ethyl acetate for analysis.

3. Results and discussion

3.1. Method performance

This method was previously validated by our group obtaining acceptable accuracy according to the guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed from the European Commission [37]. The limit of quantification (LOQ) was considered as the lowest concentration, which presented an acceptable recovery percentage and reproducibility (mean recoveries in the range 70–120% with a relative standard deviation (RSD) ≤20%) [37]. The LOQs were 0.010 mg kg^{-1} for the majority of the compounds, except for epoxiconazole, fluxapyroxad and zearalenone (0.020 mg kg^{-1}), prothioconazole and deoxynivalenol (0.1 mg kg^{-1}). However, all of them were lower than their MRLs. The detection limit of each compound was set at the lowest concentration where the signal to noise ratio was higher than three for the qualifier product ion transition in LC-MS/MS or of the lower *m/z* ion for GC-MS (Tables 2 and 3) [38].

3.2. Analysis of barley samples

This work emphasizes the simultaneous determination of fungicides and mycotoxins in barley samples. The monitoring of barley grain produced on the northwest of the country is a valuable indicator of the current performance of barley production. Therefore, the proposed method was applied to the analysis of 89 barley grain samples from different commercial pads in Uruguay.

Table 4 shows the number of samples analyzed of each variety and the summary of the results obtained for fungicides and mycotoxin analysis.

3.2.1. Fungicide residues

Sixty-six out of the 89 analyzed samples showed the presence of fungicide residues. Three samples presented three fungicides in different combinations: epoxiconazole, fluxapyroxad and chlorothalonil or

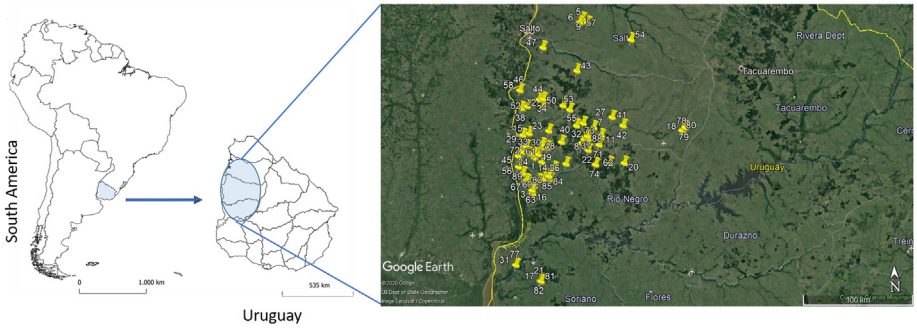

Fig. 1. (a). Geographical location of Uruguay in South America and the Google earth map showing the distribution of the different sampling sites (Google, n.d.) [33].

pyraclostrobin. Fig. 2 shows the extracted ion chromatogram of a real sample with the presence of the last mixture mentioned. Eleven of the samples contained two compounds, while 15 samples presented only one fungicide. Twenty-three samples did not show any fungicide residue at concentrations higher than their LOD, while 36 samples presented fungicide residues at concentrations below their LOQs.

The most frequently found fungicides were azoxystrobin, carbendazim, chlorothalonil, epoxiconazole, and fluxapyroxad, except for two samples containing azoxystrobin and one sample epoxiconazole, where the concentrations levels were higher than the Argentine MRL. The rest of the compounds comply with the different studied MRLs (Argentine, Brazil, Codex and, EU) [23,24,39–41]. Azoxystrobin was detected in 29 out of the 89 analyzed samples followed by chlorothalonil which was detected in 20 samples. Fluxapyroxad, carbendazim, and epoxiconazole, were found in 19, 12 and nine samples, respectively.

Trifloxystrobin, pyraclostrobin, prothioconazole, and isopyrazam were detected less frequently, whereas none of the samples presented triticonazole.

In summary, although several fungicides were detected in the grain samples, the concentrations found were below the corresponding EU or *Codex* MRL [23,24], (Table 2).

According to Komárek et al. [42], the presence of fungicides in barley samples could be explained as a combination of factors; such as pesticide lipophilicity, water solubility, vapor pressure, and Henry's Law constant, doses, number and application time as well as weather conditions.

The results obtained in the present study cannot be associated with a particular region or climate event as fungicide presence is spread along the studied area. However, they are in accordance with the commercial mixtures [43] available in the market that are recommended for the control of the most common diseases in barley.

Several studies report that fungicide residue concentrations can be reduced during malt production, mainly during the steeping process [44]. The decrease in concentration is related to the octanol-water coefficient (log Kow) of the compounds. Those fungicides with an octanol-water coefficient higher than two can remain on malt [45]. Therefore, as all the fungicides selected in this study, except for carbendazim and prothioconazole, have an octanol-water coefficient higher than two there is a high probability of detecting these compounds in the malt [44,45]. However, the log Kow of the studied fungicides indicates that they do not tend to bioaccumulate as these values are lower than 5 [46], (Table 1).

In 2019, the European Union banned the use of chlorothalonil due to its high toxicity for humans [47]. This fungicide was widely used in the production of barley as it was the only chemical strategy for RLS control, while all the other managements processes reported resistance [8]. Since then, in Europe there is great concern for developing new strategies for RLS control. As an example, Ireland has adopted the use of Folpet [48]. In Uruguay, the aforementioned strategies (azole or SDHI fungicides) for RLS control have not reported resistance yet and they are still used, but chlorothalonil is widely applied too. Although Uruguay exports malt to the MERCOSUR region, we think that sooner or later the use of chlorothalonil will be limited or banned. Therefore, it is necessary to look for other strategies in the near future.

Hence, as barley grain in Uruguay is used mainly for malt production, the barley sector should be focused on the development of management practices that ensure low levels of fungicides in the grain.

The results of the fungicide residues are in accordance with Malinowska et al. [28] which evaluated the occurrence of plant protection residues in 37 barley samples. The detected fungicides were methyl thiophanate, carbendazim, azoxystrobin, cyprodinil, cyproconazole, propiconazole, and tebuconazole, none of them exceeding the MRL requirements of the EU and *Codex Alimentarius* [23,24]. Furthermore, Lozowicka et al. [27] reported the evaluation of 180 pesticides in 15 barley grain samples including eight fungicides; tebuconazole, epoxiconazole, azoxystrobin, carbendazim, trifloxystrobin, chlorothalonil, metconazole, and pyraclostrobin matched with the fungicides used in the present study. The analyzed barley samples did not contain any fungicide residues.

3.2.2. *Fusarium mycotoxins*

As regards to mycotoxins, DON was detected in 90% of the tested samples, in a concentration range from < LOQ to 3.74 mg kg^{-1}. A real sample containing DON along with three fungicides is presented in Fig. 2. These results showed that 31% of the samples exceeded the 1.25 mg kg^{-1} MRL in barley grain established by the EU [24], (Table 3).

Piacentini et al. [49] reports that the levels of *Fusarium* mycotoxins (DON and ZEN) decreased significantly during the production process. Besides this and taking into account the concentrations detected, further studies related to processing factors would be necessary to ensure food safety. Mainly, because Uruguay exports 74% of their produced malt to Brazil [50], where DON MRL for malt is 0.750 mg kg^{-1} [41].

Zearalenone was found in eight out of the 89 samples, representing 9% of the total samples in a concentration between 0.046 and 0.131 mg kg^{-1}. In this case, three of the samples presented concentrations higher than the EU MRL [24]. The co-occurrence of these two mycotoxins was confirmed as all the samples containing ZEN, presented DON. Moreover, in six of the samples containing ZEN, DON was detected at concentrations higher than the EU MRL [24]. Once again, the results cannot be classified according to a particular area or weather condition. Indubitably, more studies should be performed on conditioning factors (type of management, phenological stage or climatic conditions) to understand the occurrence of these mycotoxins in barley grain [51,52].

Additionally, future studies should be focused on potential synergistic effects of mycotoxins and an extensive revision of current regulation of their MRLs in barley grain. Furthermore, a risk assessment study would be valuable considering the high prevalence of DON in Uruguay.

4. Conclusions

For the purpose of this study, a one-year monitoring program to evaluate the occurrence of fungicides and mycotoxins in barley grain under real farming conditions was assayed. Eighty-nine samples were

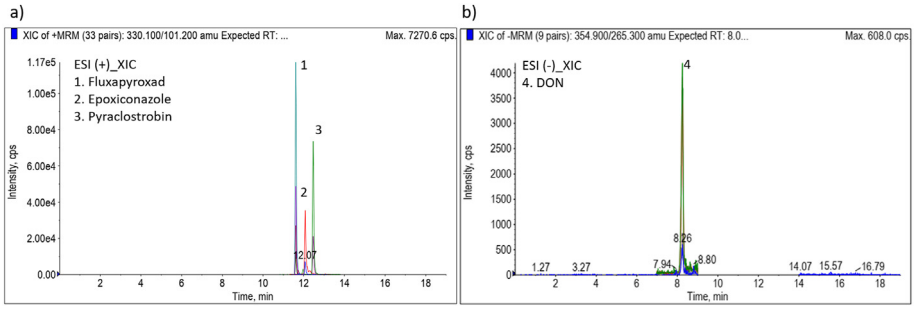

Fig. 2. Extracted ion chromatograms obtained by LC-MS/MS by electrospray ionization of a barley sample; a) fluxapyroxad (1), epoxiconazole (2), and pyraclostrobin (3) detected in the positive mode; b) DON (4) found in the negative mode.

Table 2
Summary of the main fungicides residues results obtained during the studied season; limit of detection and quantification (LOD and LOQ), and the amount of the analyzed samples classified as, negative samples, number of samples with fungicides at concentrations below or higher than their <LOQ, and the detected concentration range (mg kg^{-1}) in barley grain.

Fungicide	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Negative sample	Number of samples<LOQ	Number of samples >LOQ	Concentration range (mg kg^{-1})
Chlorothalonil	0.010	0.010	89	13	7	0.01–0.02
Azoxystrobin	0.0025	0.010	60	20	9	0.01–0.34
Carbendazim	0.0025	0.010	77	12	0	<LOQ
Epoxiconazole	0.020	0.020	81	0	8	0.05–0.59
Fluxapyroxad	0.0025	0.020	70	9	10	0.02–0.36
Isopyrazam	0.0025	0.010	83	4	2	0.02–0.03
Pyraclostrobin	0.0025	0.010	83	2	4	0.01–0.05
Triticonazole	0.010	0.010	89	0	0	–
Trifloxystrobin	0.0025	0.010	88	1	0	<LOQ
Prothioconazole	0.025	0.100	88	1	0	<LOQ

Table 3
Summary of the main mycotoxins results obtained during the studied season; limit of detection and quantification (LOD and LOQ), and the amount of the analyzed samples classified as, negative samples, number of samples with fungicides at concentrations below or higher than their <LOQ, and the detected concentration range (mg kg^{-1}) in barley grain.

Mycotoxins	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Negative sample	Number of samples<LOQ	Number of samples >LOQ	Concentration range (mg kg^{-1})
Deoxynivalenol	0.025	0.100	1	21	67	0.116–3.74
Zearalenone	0.020	0.020	81	0	8	0.046–0.131

Table 4
Number of samples analyzed of each variety and the summary of the results obtained for fungicides and mycotoxin analysis.

Region	Variety *(number of samples)*	Fungicides		Mycotoxins	
		Number of samples>LOQ	Concentration range (mg kg^{-1})	Number of samples>LOQ	Concentration range (mg kg^{-1})
Paysandú	Arrayan *(11)*	3	0.013–0.146	11	0.011–2.508
	Cle 280 *(3)*	0	–	3	889–1.370
	Musa 19 *(9)*	4	0.012–0.586	9	0.022–1.932
	Musa 936 *(20)*	8	0.014–0.143	20	0.077–3.618
Río Negro	Arrayan *(22)*	6	0.010–0.355	22	0.011–3.739
	Cle 280 *(2)*	0	–	2	0.060–0.484
	Musa 19 *(5)*	3	0.011–0.338	5	0.036–2.048
	Musa 936 *(16)*	4	0.011–0.500	15	0.030–3.079
Salto	Musa 936 *(1)*	0	–	1	2.735

analyzed and several fungicide residues were identified at concentrations below their respective MRLs. The most frequently detected fungicides in barley samples were azoxystrobin, chlorothalonil and fluxapyroxad. The presence of chlorothalonil demonstrates the importance of seeking new control strategies for RLS, since this fungicide is no longer acceptable for use in the European Union.

Regarding mycotoxins, the detected concentrations confirmed the importance of performing risk assessment as 31% of the samples exceeded the MRL. In all the samples that presented ZEA the co-occurrence with DON was found. Future studies should be focused on the occurrence of mycotoxins and the conditions that enable their production.

Moreover, due to the high prevalence of DON, a strict monitoring program on mycotoxins and the evaluation of the effect of barley processing in their concentrations would be necessary to ensure food safety.

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