

1 Analysis of mycotoxins in Spanish cow milk. Flores-Flores and González-Peñas.
2 Mycotoxins in milk can have toxic effects on human and animal health. Surveillance of
3 mycotoxin occurrence in milk is recommended. We have analyzed aflatoxins M1, B1, B2, G1
4 and G2, ochratoxins A and B, nivalenol, deoxynivalenol, deepoxy-deoxynivalenol, 3 and 15
5 acetyldeoxynivalenol, diacetoxyscirpenol, neosolaniol, fusarenon X, T-2 and HT-2 toxins,
6 fumonisins B1, B2 and B3, sterigmatocystin and zearalenone in 191 Spanish milk samples..
7 Mycotoxins, extracted with acidified acetonitrile were analyzed by LC-MS/MS (triple
8 quadrupole). None of the analyzed mycotoxins had a concentration level higher than their
9 detection limit. Aflatoxin M1 never exceeded the level established by the European Union.
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12 **Short communication**

13 **Analysis of mycotoxins in Spanish milk**

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28 **ABSTRACT**

29 This research paper aimed to show a survey on the presence of 22 mycotoxins in 191 Spanish
30 cow milk samples. Mycotoxins could be carried over into animal milk and having toxic
31 effects on human and animal health. The interaction of different mycotoxins may be additive
32 or synergetic. Therefore, surveillance of mycotoxin co-occurrence in milk is recommended.
33 Aflatoxins M1, B1, B2, G1 and G2, ochratoxins A and B, nivalenol, deoxynivalenol,
34 deepoxy-deoxynivalenol, 3 and 15 acetyldeoxynivalenol, diacetoxyscirpenol, neosolaniol,
35 fusarenon X, T-2 and HT-2 toxins, fumonisins B1, B2 and B3, sterigmatocystin and
36 zearalenone were analyzed. Samples were treated by liquid-liquid extraction with acidified
37 acetonitrile, followed by an acetonitrile-water phase separation using sodium acetate. The
38 analysis was carried out by high-performance liquid chromatography coupled to a triple
39 quadrupole mass spectrometer. None of the analyzed mycotoxins had a concentration level
40 higher than their detection limit (0.05-10.1 $\mu\text{g L}^{-1}$). The Aflatoxin M1 in the samples never
41 exceeded the level established by the European Union.

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44 **Key words:** Milk; mycotoxin;; co-occurrence

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46 Foods of animal origin may be contaminated with mycotoxins when they are based on or
47 prepared with products derived from animals whose diet contained mycotoxins (Capriotti et
48 al., 2012). Mycotoxins can appear in animal feed due to the contamination of agricultural
49 commodities by filamentous fungi, especially those belonging to the genera *Aspergillus*,
50 *Penicillium*, and *Fusarium* (Binder, 2007; Rodrigues and Naehrer, 2012) and therefore, the
51 European Union has recommended limits for some of the mycotoxins and established legal
52 limits for aflatoxins in products for animal consumption (European Commission 2006, 2013).
53 Ruminant metabolism usually degrades mycotoxins into less toxic compounds; however,
54 some of them can remain unaltered and they can be absorbed and accumulated in animal
55 tissues or biological fluids, including the milk (Flores-Flores et al. 2015). Special attention
56 has been paid to aflatoxin M1 (**AFM1**). It is formed as a degradation product in the hepatic
57 metabolism of aflatoxin B1 (**AFB1**) in ruminants (Wu et al. 2009) and is excreted into milk.
58 AFM1 has been classified as probably carcinogenic for humans (group 2B) (IARC, 2002):
59 The European Community has set a maximum allowable limit of AFM1 in milk ($0.05 \mu\text{g kg}^{-1}$)
60 (European Commission, 2010); whereas the levels of other mycotoxins in milk are not
61 regulated. Approximately, 9.8 % of the milk samples analyzed worldwide exceeded the
62 maximum limit set in the EU for AFM1, and it was also reported the presence of low levels of
63 other mycotoxins in milk (Flores-Flores et al. 2015). Huang et al. (2014), detected the
64 simultaneous presence of up to 4 mycotoxins in the analyzed milk samples: 15% were
65 contaminated with 2 mycotoxins, 45% with 3 mycotoxins and 22% with 4 mycotoxins. This
66 multi-exposure can change the toxic effects of mycotoxins on human and animal health due to
67 additive, synergistic or even antagonistic phenomena (Smith et al. 2016), even when levels
68 considered to be nontoxic of individual mycotoxins are present (Wan et al. 2013).

69 Therefore, the continuous surveillance of mycotoxin co-occurrence in milk is needed in order
70 to obtain data for better risk assessment and to protect consumer and animal health. This
71 paper shows a survey on the presence of 22 mycotoxins in 191 Spanish cow milk samples.
72 Methanol (LC-MS grade), formic acid (mass spectrometry grade, purity > 98%), ammonium
73 formate (analytical grade) and sodium acetate (anhydrous, HPLC grade > 99.0%) were
74 purchased from Sigma-Aldrich (USA) and acetonitrile (HPLC grade) from Merck (Germany).
75 Deionized water (> 18 MΩ/cm resistivity) was purified in an Ultramatic Type I system from
76 Wasserlab (Spain). All mycotoxins (purity ≥ 98%) were obtained from Sigma-Aldrich (USA)
77 in solution except for ochratoxin A which was purchased in powder form.
78 Three mixed stock solutions (1, 2 and 3) were prepared by dilution of appropriate volumes of
79 each mycotoxin standard solution in 10 mL of acetonitrile as previously described (Flores-
80 Flores and González-Peñas 2015, 2017). Mixed stock solution 1 contained nivalenol (**NIV**),
81 deoxynivalenol (**DON**), deepoxy-deoxynivalenol (**DOM-1**), fusarenon X (**FUS-X**),
82 neosolaniol (**NEO**), 3-acetyldeoxynivalenol (**3-ADON**), 15-acetyldeoxynivalenol (**15-**
83 **ADON**), diacetoxyscirpenol (**DAS**), HT-2 toxin (**HT-2**) and T-2 toxin (**T-2**). Mixed stock
84 solution 2 contained AFB1, aflatoxin B2 (**AFB2**), aflatoxin G1 (**AFG1**), aflatoxin G2
85 (**AFG2**), AFM1, ochratoxin A (**OTA**), ochratoxin B (**OTB**), zearalenone (**ZEA**) and
86 sterigmatocystin (**STC**). Mixed stock solution 3 contained fumonisin B1 (**FB1**), fumonisin B2
87 (**FB2**) and fumonisin B3 (**FB3**). Mixed stock solutions 1 and 2 were stored at -20 °C. Mixed
88 stock solution 3 was prepared and used daily due to the instability of fumonisins in
89 acetonitrile. For a better understanding of this paper, the mycotoxins contained in mixed stock
90 solution 1 will be referred to as mycotoxin group 1 and mycotoxins from mixed stock
91 solutions 2 and 3 will be referred to as mycotoxin group 2. Due to the toxicity of these
92 compounds, all of them were handled in solution using gloves and a face shield. In addition,
93 low-light conditions were established during handling so as to prevent photo-instability.

94 One hundred and seven full cream milk samples were purchased from supermarkets in Spain
95 between September 2013 and April 2016 due to the fact that Spanish families purchase more
96 than 50% of the liquid milk consumed in this type of establishment (MAGRAMA 2015,
97 2016). Between 2010-2014 (INE, 2016), added together, Galicia, Castile and Leon,
98 Andalusia, Catalonia, Asturias, Cantabria, Navarra, Castile-La Mancha and Basque Country
99 make up 93% of the total production of cow milk in the country in 2014. Samples were from
100 26 collection centers located in the high milk production regions (figure 1). Samples were
101 opened and analyzed during 1-2 days.

102 Also, eighty-four raw milk samples were collected in March 2016. All of them were from
103 dairy farms located either in Navarra, La Rioja, Basque Country or Catalonia. One of the raw
104 samples was taken from a cow with signs of disease of unknown origin. None of these
105 samples suffered any treatment procedure after collection, with the exception of the addition
106 of azidiol (sodium azide/chloramphenicol) a preservative compound frequently used by milk
107 testing laboratories in Spain (Llopis et al. 2013) as a preservative. Samples were analyzed
108 within the week that they were collected and maintained at 4 °C until analysis. Prior to
109 chromatographic analysis, milk samples were treated following the procedures previously
110 developed by our group (Flores-Flores and González-Peñas 2015, 2017). Briefly, 1 mL of
111 milk was poured into a tube for the analysis of mycotoxin group 1 and another 1 mL was
112 poured into a second tube for the analysis of mycotoxin group 2. Each tube was extracted with
113 acidified acetonitrile. After centrifugation, the upper phase of each tube was transferred to
114 another clean tube and water-acetonitrile phases separation was induced by the addition of
115 sodium acetate. Next, each acetonitrile phase was dried and the residue from each one of the
116 tubes was reconstituted with LC mobile phase. In addition, the two groups of mycotoxins
117 were analyzed in separate runs with different separation conditions, as explained below.

118 An Agilent Technologies (Germany) 1200 LC system was used. The chromatographic column
119 was an Ascentis Express C18, 2.7 μm particle size, 150 mm x 2.1 mm from Supelco
120 Analytical (USA) maintained at 45 °C. The mobile phase consisted of solution A (5 mM
121 ammonium formate and 0.1% formic acid in water) and solution B (5 mM ammonium
122 formate and 0.1% formic acid in methanol:H₂O 95:5) in gradient conditions. Fifteen μL and
123 20 μL were injected for mycotoxin group 1 and 2, respectively. Flow rate was 0.4 mL min⁻¹.
124 Detection was carried out using a 6410 Triple Quad LC-MS/MS System from Agilent
125 Technologies (Germany) equipped with an electrospray ionization interface. MS operation
126 conditions were: capillary voltage at 4000 V, drying gas was high purity nitrogen at 350 °C, 9
127 L/min and 40 psi. In addition, ultra-high purity nitrogen (99.999%, Praxair, Spain) was used
128 inside the collision cell. Selected reaction monitoring was used for data collection. MS
129 parameters for identifying each one of the mycotoxins were those previously reported by our
130 group (Flores-Flores and González-Peñas 2015, 2017).
131 Validation of the two developed methodologies has been previously described (Flores-Flores
132 and González-Peñas 2015, 2017). Limits of detection (**LOD**) and quantification (**LOQ**),
133 linearity, precision, accuracy, recovery, matrix effect, and stability were studied for both
134 methods. Recovery values were between 53.8 and 94.4% for all the mycotoxins, except for
135 fumonisin B1 which was 42.1%. RSD (%) values (in intermediate precision conditions) were
136 lower than 15% for all the mycotoxins. Matrix effect appeared for all of the mycotoxins, and
137 matrix-calibration curves were constructed for all of them. Detection limits were between
138 0.02 and 10.14 $\mu\text{g L}^{-1}$ for all the mycotoxins (table 1). For AFM1, the only mycotoxin for
139 which a maximum limit of 0.05 $\mu\text{g kg}^{-1}$ has been established in the UE, a LOD of 0.025 $\mu\text{g L}^{-1}$
140 was achieved.
141 All the samples were analyzed as analytical sequences, including quality control samples (two
142 in every ten) prepared by spiking milk at the LOQ and the highest levels in the quantification

143 range of each one of the mycotoxins. In the case of mycotoxin presence in the sample, the
144 sample was re-analyzed along with calibration samples prepared by spiking milk samples,
145 using the same procedure as when validation studies were performed.

146 Raw milk samples contained azidiol, and therefore, the effect of the presence of this
147 compound in the response of mycotoxins in the detector was evaluated. Milk containing this
148 product was fortified at LOQ and the highest level in the quantification range of each
149 mycotoxin. The responses (peak areas) obtained were compared with those obtained for
150 mycotoxins in fortified commercial milk samples at the same concentration levels. The
151 experiment was carried out on three consecutive days. The relationship between the mean
152 areas in both types of samples at each one of the concentration levels (LOQ and the highest
153 level in each range) was less than 15% for all the mycotoxins. Thus, we considered no
154 additional matrix effects due to the azidiol. However, when analyzing raw milk samples, we
155 prepared the quality control samples using raw milk containing azidiol as matrix. Figure 2
156 shows examples of the chromatograms obtained for both groups of mycotoxins in raw milk
157 samples.

158 We did not find levels of mycotoxins higher than the respective LOD values in any of the
159 analyzed samples. Therefore, and with respect to AFM1, the only mycotoxin for which a
160 permissible maximum limit has been established in milk within the European Union, all tested
161 samples complied with legislation in terms of this mycotoxin.

162 Aguilera-Ruiz et al. (2011) analyzed 8 mycotoxins in 15 milk samples purchased in the
163 province of Almeria. Beltrán et al. (2013) analyzed 18 mycotoxins in different food matrices,
164 including 10 milk samples from the region of Valencia, and Beltrán et al. (2011) analyzed 6
165 mycotoxins in 2 raw milk samples from the city of Castellon. None of these studies found
166 mycotoxins levels. The LOQs from these studies were higher than the LOQs of the methods
167 used in this study. In addition, González-Osnaya et al. (2008) analyzed OTA in 16 samples of

168 whole milk purchased from the province of Valencia; none of them contained OTA above the
169 detection level of the method.

170 In a previous publication, we carried out a review regarding the presence of AFM1 levels in
171 milk collected in Europe (Flores-Flores et al. 2015). From 13566 analyzed milk samples, only
172 119 (0.9%) had AFM1 in levels higher than the maximum permitted by the EU. From a
173 geographical point of view, 117 out of 119 positive samples for AFM1 correspond to
174 countries in the Adriatic Sea region. In Spain, Rodríguez et al. (2003) and Cano-Sancho et al.
175 (2010) did not detect AFM1 levels higher than those established by EU legislation when
176 analyzed milk samples from Leon and Catalonia, respectively. Thus, the results obtained in
177 this study (none of the analyzed mycotoxins had a concentration level higher than their
178 detection limit and aflatoxin M1 never exceeded the level established by the European
179 Union). coincide with those found in the literature and they are encouraging and demonstrate
180 a low risk of mycotoxin contamination in Spanish milk. However, due to the importance of
181 milk due to its economic impact as well as its elevated consumption, especially by a
182 vulnerable group such as children, we recommend the carry-over of periodic surveillance
183 programs.

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Table 1. Linear range, LOQ (lowest level of the range) and LOD of the validated methods (Flores-Flores and González-Peñas 2015, 2017)

	Range ($\mu\text{g L}^{-1}$)	LOD ($\mu\text{g L}^{-1}$)		Range ($\mu\text{g L}^{-1}$)	LOD ($\mu\text{g L}^{-1}$)		Range ($\mu\text{g L}^{-1}$)	LOD ($\mu\text{g L}^{-1}$)
NIV	20.2 - 202.3	10.1	AFG2	0.15-1.50	0.075	FB2	2.50-25.00	2.500
DON	5.0 - 50.3	2.5	AFM1	0.05-0.50	0.025	FB3	2.50-25.00	0.625
DOM-1	3.0 - 30.3	1.5	AFG1	0.10- 1.02	0.025			
FUS-X	3.7 - 37.0	1.9	AFB2	0.04-0.40	0.020			
NEO	0.2 - 2.0	0.1	AFB1	0.04-0.40	0.020			
3-ADON	1.0 - 10.0	0.5	OTB	0.05-0.50	0.050			
15-ADON	2.0 - 20.2	1.0	FB1	10.14-50.70	10.140			
DAS	0.16 - 1.6	0.08	ZEA	0.51-5.09	0.510			
HT-2	0.8 - 8.0	0.4	STC	0.50-5.02	0.125			
T-2	0.1 - 1.0	0.05	OTA	0.20-1.00	0.200			

284

285 Limit of Quantification (LOQ), Limit of Detection (LOD), nivalenol (NIV), deoxynivalenol
 286 (DON), deepoxy-deoxynivalenol (DOM-1), fusarenon X (FUS-X), neosolaniol (NEO), 3-
 287 acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol
 288 (DAS), HT-2 (HT-2), T-2 (T-2), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), aflatoxin G1
 289 (AFG1), aflatoxin B2 (AFB2), aflatoxin B1 (AFB1), ochratoxin B (OTB), fumonisin B1
 290 (FB1), zearalenone (ZEA), sterigmatocystin (STC), ochratoxin A (OTA), fumonisin B2 (FB2)
 291 and fumonisin B3 (FB3).

292

293 **Figure captions**

294 Figure 1. Distribution of analyzed commercial milk samples classified by collection center
295 code

296 Figure 2. Superposed quantification products ions obtained after analysis of mycotoxin group
297 1 (A and B) and 2 (C and D). (A and C) Raw milk sample (containing azidiol) fortified at
298 Limit of Quantification and (B and D) non-fortified raw milk sample (containing azidiol).
299 Group 1: (NIV), deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM-1), fusarenon X
300 (FUS-X), neosolaniol (NEO), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol
301 (15-ADON), diacetoxyscirpenol (DAS), HT-2 (HT-2), T-2 (T-2). Group 2: aflatoxin G2
302 (AFG2), aflatoxin M1 (AFM1), aflatoxin G1 (AFG1), aflatoxin B2 (AFB2), aflatoxin B1
303 (AFB1), ochratoxin B (OTB), fumonisin B1 (FB1), zearalenone (ZEA), sterigmatocystin
304 (STC), ochratoxin A (OTA), fumonisin B2 (FB2) and fumonisin B3 (FB3).

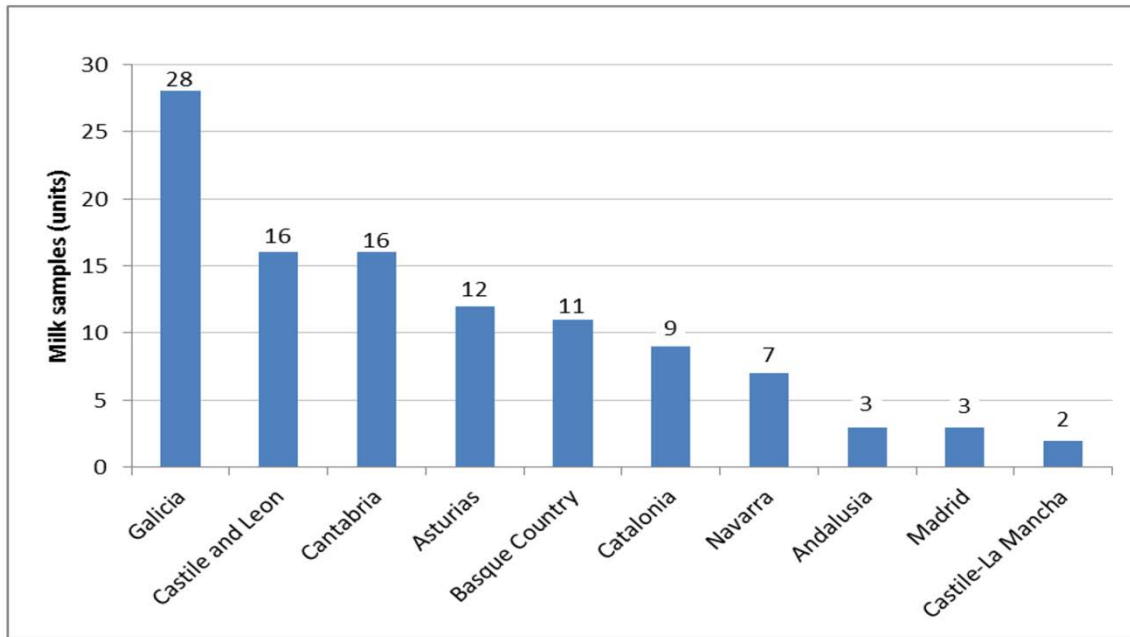
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308 Flores-Flores. Figure 1.

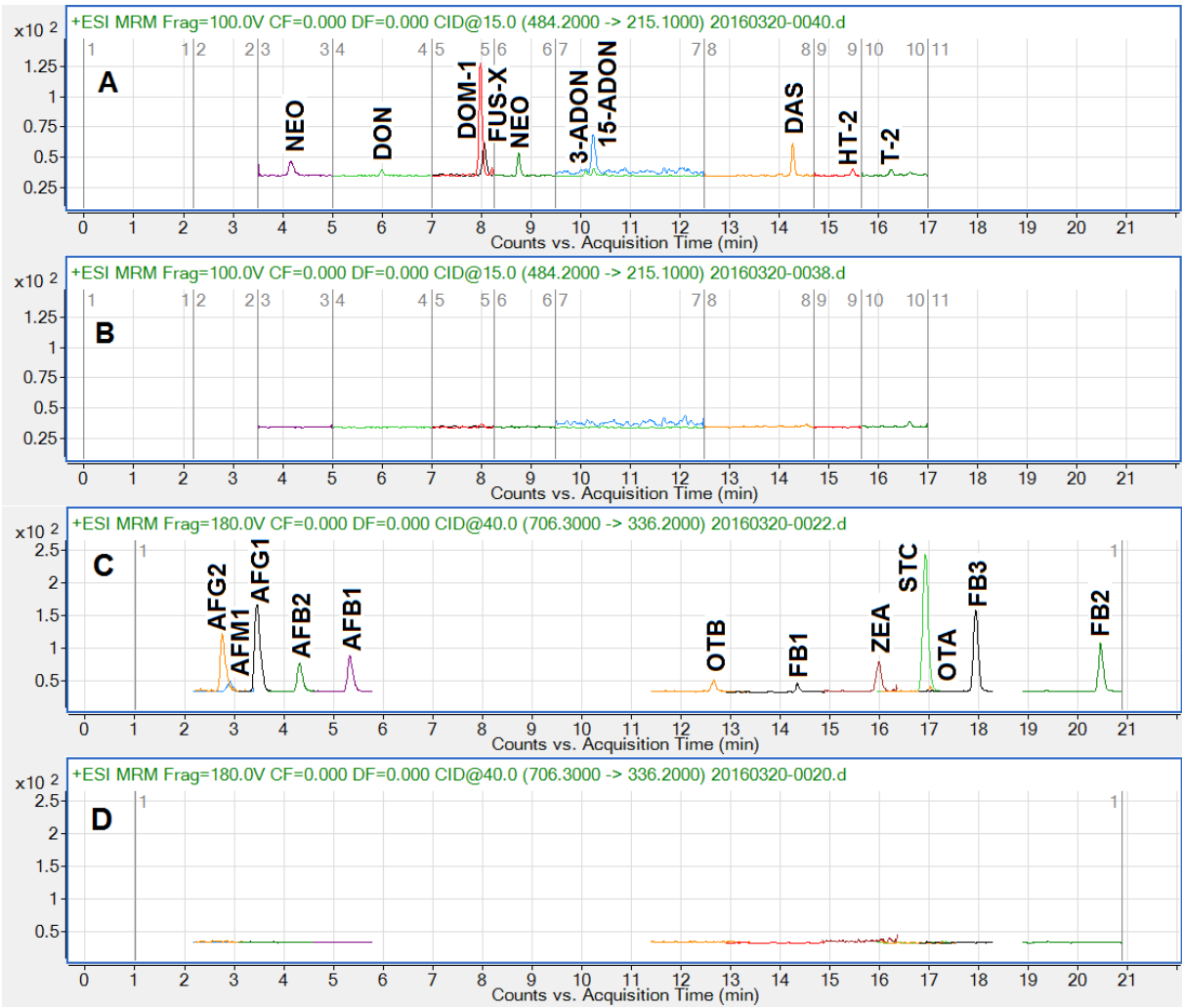
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312 Figure 2.



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