Multianalyte method for the determination of regulated emerging and modified mycotoxins in milk: QuEChERS extraction followed by UHPLC–MS/MS analysis

Jesús M. González-Jartín [a], Inés Rodríguez-Cañás [a], Amparo Alfonso [a*], María J. Sainz [b], Mercedes R. Vieytes [c], Ana Gomes [d], Isabel Ramos [d], Luis M. Botana [a*].

[a] Departamento de Farmacología, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002, Lugo, Spain.
[b] Departamento de Producción Vegetal y Proyectos de Ingeniería, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002, Lugo, Spain.
[c] Departamento de Fisiología, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002, Lugo, Spain.
[d] Cooperativa Agrícola de Vila do Conde, R. da Lapa 293, 4480-848, Vila do Conde, Portugal

* Corresponding author:
Amparo Alfonso
Tfn: + 34 982 822 213
Fax: + 34 982 822 233
E-mail: amparo.alfonso@usc.es

* Alternative corresponding author:
Luis M. Botana
Tfn: + 34 982 822 233
Fax: + 34 982 822 233
E-mail: luis.botana@usc.es

Jesús M. González-Jartín: jesus.gonzalez@usc.es

Inés Rodríguez- Cañás: ines.rodriguez.canas@rai.usc.es

Amparo Alfonso: amparo.alfonso@usc.es

M. J. Sainz: mj.sainz@usc.es

M. R. Vieytes: mmercedes.rodriguez@usc.es

Ana Gomes: ana.gomes@cavc.pt

Isabel Ramos: isabel.ramos@cavc.pt

Luis M. Botana: luis.botana@usc.es

ABSTRACT

A simple method for the quantification of 40 mycotoxins in milk was developed. This method is based on a QuEChERS extraction followed by the ultra-high liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) detection, and allows the simultaneous analysis of regulated, emerging, and modified compounds. A sample treatment procedure was optimized to include a concentration step for the analysis of some compounds such as aflatoxin M_1. The method was in-house validated in terms of limits of detection (LODs), limits of quantification (LOQs), linearity, recoveries, and precision. LOQs lower than 10 ng/mL were obtained, and recoveries ranged from 61% to 120% with a precision, expressed as the relative standard deviation, lower than 15%. Therefore, acceptable performance characteristics were obtained fulfilling European regulations. The method was successfully applied for the quantification of mycotoxins in raw milk. It can be highlighted high occurrence of beauvericin and enniatins were found in low amounts.

KEYWORDS: Mycotoxin, milk, QuEChERS, UHPLC-MS/MS

1. Introduction

Agricultural products intended for animal feed such as cereals, concentrates, silages and green forages can be contaminated with mycotoxins. In fact, more than 80% of livestock feeds contain at least one mycotoxin (Rodrigues & Naehrer, 2012). The chronic intake of contaminated food and feed may lead to health problems such as immunosuppression, carcinogenicity, hepatotoxicity, nephrotoxicity, and reproductive disorders in both humans and animals. However, toxic effects vary considerably depending on the animal species. Ruminants typically show greater tolerance to mycotoxins, probably due to the modification to which these compounds are submitted by rumen microflora (Fink-Gremmels, 2008b). However, some mycotoxins such as fumonisins (FBs) pass the rumen barrier unaltered, while others, including deoxinivalenol (DON), aflatoxins (AFs) and zearalenone (ZEN), are modified to several metabolites which, in some occasions, are more toxic than parent mycotoxins (Fink-Gremmels, 2008a; Kiessling, Pettersson, Sandholm, & Olsen, 1984). Once these compounds are absorbed from the gastrointestinal tract, they are metabolized and subsequently excreted through different vias including milk. For instance, the aflatoxin B_1 (AFB_1) is hydroxylated in the liver to aflatoxin M_1 (AFM_1), and subsequently excreted in milk and urine. Other mycotoxins such as FBs, ZEN and its metabolites, ochratoxin A (OTA), and deepoxy-deoxynivalenol (DOM-1) have been also detected in animal milk (Fink-Gremmels, 2008a; M. E. Flores-Flores & Gonzalez-Penas, 2017).

Cow milk is included as an important element in a healthy and balanced diet, especially for children, which are the largest group of consumers (Pereira, 2014). Mycotoxins are not eliminated by technological treatments employed for milk processing such as pasteurization, sterilization or spray drying (Campagnollo et al., 2016). Therefore, its essential to keep the amount of mycotoxins in milk under tolerable levels. Consequently, world-wide regulations establish maximum levels of AFM_1 in this product, varying from 0.05 μg/kg in the European Union to 5 μg/kg in Indonesia. Although the presence of other mycotoxins has been reported in this matrix, few countries have established maximum limits for them (EC_1881/2006; van Egmond & Jonker, 2004).

50 The low amount of AFM_1 that EU legislation has established as maximum tolerable level makes
51 necessary to employ sensitive, selective and reliable analytical methods. In order to ensure milk
52 safety, it is necessary first to extract toxins from the matrix and then analyze the extract by an adequate
53 technique. In this sense, enzyme-linked immune sorbent assay (ELISA), and liquid chromatography
54 coupled with ultraviolet or fluorescence detection have been widely used for mycotoxin detection
55 (Huang et al., 2014). However, the ultra-performance liquid chromatography coupled to tandem mass
56 spectrometry (UHPLC-MS/MS) is the most employed analytic technique nowadays to study the
57 presence of mycotoxins in several matrices since allows the simultaneous detection of multiple
58 compounds maintaining adequate performance characteristics (Beltran et al., 2013; Gonzalez-Jartin,
59 Alfonso, Rodriguez, Sainz, Vieytes, & Botana, 2019).
60 Milk is a complex matrix characterized by a high fat and protein content. These components may lead
61 to negative impact on the accuracy, precision, and robustness of the method increasing the limits of
62 detection (LODs). The elimination of interference substances is a critical step in the sample extraction
63 procedure usually involving several clean-up steps (Wang & Li, 2015). Different procedures have
64 been applied to remove the co-extracted compounds from milk including liquid-liquid extraction,
65 solid-phase extraction (SPE) with immunoaffinity sorbents, matrix solid-phase dispersion (MSPD),
66 dispersive solid-phase extraction (D-SPE), liquid-phase microextraction (LPME), and solid-phase
67 microextraction (SPME) (Aguilera-Luiz, Plaza-Bolanos, Romero-Gonzalez, Vidal, & Frenich, 2011).
68 However, extraction procedures based on the QuEChERS (quick, easy, cheap, effective, rugged, and
69 safe) approach are the most used nowadays. This methodology, initially developed for the analysis
70 of pesticides, allows the simultaneous extraction and clean-up of samples. In addition, unlike
71 traditional methods, QuEChERS can be applied to the analysis of several groups of mycotoxins at the
72 same time and in different matrices (Perestrelo et al., 2019). QuEChERS methodology is based on an
73 initial extraction step with acetonitrile or mixtures of acetonitrile and water. Next, a liquid-liquid
74 partitioning is induced by the addition of inorganic salts, usually anhydrous magnesium sulphate
75 ($MgSO_4$) and sodium chloride (NaCl). In this way, mycotoxins are transferred into the organic phase,

76 while some polar components of the matrix remain in the aqueous layer (González-Curbelo, Socas-
77 Rodríguez, Herrera-Herrera, González-Sálamo, Hernández-Borges, & Rodríguez-Delgado, 2015). A
78 further d-SPE can be employed to reduce other matrix compounds from the organic phase. With this
79 aim, C18, graphite, and Primary Secondary Amine (PSA) can be employed. However, for toxin
80 analysis this clean-up step is sometimes omitted since some compounds may be trapped by the sorbent
81 reducing the recovery (Arroyo-Manzanares, Garcia-Campana, & Gamiz-Gracia, 2013; Eom et al.,
82 2017).

83 Although there are some methods available for the analysis of mycotoxins in milk, they are focus on
84 the detection of aflatoxin M_1 and *Fusarium* toxins such as ZEN, DON, and FBs (M. E. Flores-Flores
85 et al., 2017; Huang et al., 2014). However, cows are exposed to a high variety of mycotoxins from
86 other fungal species such as *Aspergillus* and *Penicillium* that can not be monotored with the currently
87 available methods (Fink-Gremmels, 2008a). In this context, this work aimed to develop a new method
88 for the simultaneous analysis of regulated, emerging and modified mycotoxins from *Fusarium*,
89 *Aspergillus*, *Penicillium* and *Alternaria* species in milk by the combined use of the QuEChERS-based
90 extraction and UHPLC-MS/MS detection.

91 **2. Materials and methods**

92 **2.1 Chemicals and reagents**

93 Anhydrous $MgSO_4$, NaCl, methanol, acetonitrile and acetic acid (glacial, 100%), were supplied by
94 Panreac Quimica S.A. (Barcelona, Spain). Ultrafree-MC, Durapore membrane centrifugal filters
95 (0.22 μm pore size) were from Millipore (Billerica, MA). Formic acid was purchased from Merck
96 (Madrid, Spain), C18 from Macherey-Nagel (Düren, Germany), and ammonium formate from Fluka
97 (Buchs, Switzerland). Water was purified in a Millipore Milli-Q Plus system (Millipore, Bedford,
98 MA). Solid standards provided by Sigma (Madrid, Spain) were: DON, ZEA, FB_1, AFB_1, AFB_2,
99 AFG_1, AFG_2, ENNA, $ENNA_1$, ENNB, $ENNB_1$, roquefortine C (RC), and fusaric acid (FA).
100 Analytical standards of T-2 toxin, HT-2 toxin, neosolaniol (NEO), FB_2, α-zearalenol (α-ZEA), β-

101 zearalenol (β-ZEA), 3-acetyldeoxynivaleol (3-Ac-DON), 15-acetyldeoxynivaleol (15-Ac-DON), and
102 deoxynivalenol-3-glucoside (DON-3-Gluc), AFM_1, zearalanone (ZOL), α-zearalanol (α-ZOL), β-
103 zearalanol (β-ZOL), citrinin (CIT), deepoxy-deoxynivalenol (DOM-1), hydrolyzed Fumonisin B_1
104 (Hydro-FB_1), sterigmatocystin (STC), mycophenolic acid (MPA), T-2 triol, diacetoxyscirpenol
105 (DAS), fusarenon X (FX), alternariol (AOH), alternariol methyl ether (AME), were from Romer Labs
106 (Tulln, Austria). OTA was obtained from Laboratorios CIFGA S.A. (Lugo, Spain), and BEA standard
107 was from Enzo (Barcelona, Spain). Milk samples were obtained from dairy farms in northern of
108 Portugal.

109 **2.2 Chromatography and mass spectrometric conditions**

110 The instrument consisted in a 1290 Infinity UHPLC system interfaced to a 6460 Triple Quadrupole
111 mass spectrometer (Agilent Technologies, Waldbronn, Germany). Separation was done using a 100
112 mm × 2.1 mm (inside diameter), 1.8 µm, Waters ACQUITY HSS T3 column (Waters, Milford, MA).
113 Mobile phase A was water containing 0.1% formic acid and 5 mM ammonium formate, and mobile
114 phase B was methanol. An elution gradient of 13 min was made as follows: The percentage of B was
115 started at 0%B, increased to 14%B within 0.5 min, and maintained for 1.5 min. Thereafter, the eluent
116 B was raised to 60%B within 1 min and then kept for 0.5 min. Next, the gradient was linearly
117 increased to 100%B within 4.5 min and maintained for 2 min. Finally, the gradient was changed to
118 0% in 0.5 min and, in order to equilibrate the column, this percentage was hold for 2.5 min. The flow
119 rate of the mobile phase was kept at 0.3 mL/min, the temperature was maintained at 40 °C, and the
120 injection volume was set at 5µL. The Agilent 6460 triple quadrupole mass spectrometer was equipped
121 with an electrospray ionization source (ESI) using Agilent Jet Stream Technology. The ion source
122 parameters were set as follows: capillary voltage, 4000 V; nozzle voltage in positive, 1500 V; nozzle
123 voltage in negative, 0 V; nebulizer, 45 psi; sheath gas, 12 L/min and 400 ºC, gas temperature, 8 L/min
124 and 350 ºC. The fragmentor voltage (FV), cell accelerator voltage (CAV), collision energy (CE) and
125 mass transitions were optimized for each metabolite using MassHunter Optimizer software (Table 1).

126 **2.3 Sample Preparation**

127 Ten mL of sample (raw milk) were added to a falcon tube and extracted with 10 mL of formic acid
128 (0.5%) in acetonitrile by employing a vortex mixer for 5 min. Next 8 g of $MgSO_4$ and 1.2 g of NaCl
129 were added to induce phase partitioning. After mixing for 1 min by using a vortex mixer, samples
130 were centrifugated at 3134 × *g* for 10 min and the upper phase (acetonitrile) was transferred to a new
131 tube. From this extract, 1 mL was evaporated to dryness employing a centrifugal evaporator and
132 reconstituted with 1 mL of the sample solvent acetonitrile/water/acetic acid [49:50:1 (v/v/v)]. On the
133 other hand, 2 mL of the extract were submitted to an additional d-SPE clean-up step using 900 mg of
134 $MgSO_4$ and 300 mg of C18 by shaking for 1 min and then centrifuged for 5 min (3134 × *g*). An
135 aliquot of 1500 µL of the supernatant was evaporated to dryness and reconstituted with 150 µL of the
136 sample solvent, that is a 10 times concentrated sample. Before injection, both aliquots were filtered
137 through 0.22 µm using centrifugal filters. The non-concentrated sample was used for the analysis of
138 AME, BEA, CTA, CTN, FA, Hydro-FB_1, OTA, Roq C, STG, FBs and ENNs, while the other toxins
139 included in the method were quantified in the sample submitted to the d-SPE clean-up step.

140 **2.4 Method validation**

141 **2.4.1 Calibration curves and performance characteristics**

142 In order to determine the range of toxin concentrations over which the quantitation method can be
143 applied, the linearity was assessed by constructing calibration curves in solvent and milk extract at
144 different concentrations, in general, from 0.78 to 200 ng/mL for all analytes except for aflatoxins. In
145 this case, concentrations were between 0.012 to 3 ng/mL. The correlation coefficient (R) was
146 determined using a linear regression model.

147 The lowest concentration of analyte that can be detected (LOD) and the lowest concentration of
148 analyte that can be quantified (LOQ) were calculated based on the signal-to-noise following the
149 guidance of the European Union Reference Laboratory (EU-RL) (Wenzl, Haedrich, Schaechtele,
150 Robouch, & Stroka, 2016). The LOD was calculated based on the noise at the retention time of each

151 toxin by applicating the following equation: $LOD = 3.9 \times \frac{Sb}{m}$. Where Sb corresponds to the standard
152 deviation of the noises of 10 blank samples, and m is the slope of the calibration curve. The LOQ was
153 calculated as 3.3 times the LOD. The repeatability of the detection method was calculated as the
154 percentage of variation of the slope of three lineal 1/X weighted calibration curves (repeatability
155 within-batch), while the repeatability between-batch was calculated with the analysis of calibration
156 curves over 3 days.

157 **2.4.2 Evaluation of matrix effect**

158 In order to calculate the signal suppression/enhancement (SSE), calibration curves were constructed
159 in solvent and toxin-free extract (blank sample) at seven concentration levels for each analyte with
160 concentrations ranged from 3.12 to 200 ng/mL except for AF whose levels ranged from 0.047 to 3
161 ng/mL. The SSE factor was calculated according to the following equation $SSE(\%) =$
162 $\frac{\text{slope of spiked extracts curve}}{\text{slope of standards curve in solvent}}$.

163 **2.4.3 Accuracy and precision**

164 The accuracy and precision of the method were evaluated at three contamination levels using the
165 recovery from samples spiked before extraction. Blank samples were spiked at low, medium, and
166 high contamination levels, 0.05, 0.1, and 0.15 ng/mL of AFs and 2.5, 5 and 10 ng/mL of other toxins,
167 respectively. Recovery and precision at low and high contamination levels were calculated using tree
168 replicate samples (n=3), while six replicate samples (n=6) were used for the medium contamination
169 level. In the case of DON-3-Gluc, FX, PAT and T-2 triol, the recovery and precision were only
170 evaluated in the medium contraction level (n=6) at 20 ng/mL. After extraction, solvent based
171 calibration curves were employed to calculate the amount of each analyte in the extracts. In this way,
172 apparent recoveries (R_A), calculated as $R_A(\%) = \frac{\text{area spiked sample}}{\text{area standar}} \times 100$, and the standard
173 deviation (RSD) were determined for each toxin. Thereafter, in order to determinate the accuracy of
174 the method, the recovery of the extraction (R_E) was calculated by applying the SSE factor to the R_A

175 as follows $R_E = \frac{R_A}{SEE} \times 100$. The RSD of the recovery was used to calculate the precision. The
176 repetitively (intra-day precision) was calculated for the three concentration levels by analyzing
177 samples on the same day, whereas the within laboratory reproducibility (inter-day precision) was
178 evaluated at the medium concentration level for three consecutive days.

179 **3. Results and discussion**

180 Although several mycotoxins can be carried over to milk, the methods currently used for the analysis
181 of this matrix are focused on the detection of AFM_1 and *Fusarium* toxins (M. E. Flores-Flores et al.,
182 2017). Due to the diet of cows, these animals are exposed to several emerging mycotoxins produced
183 by other fungal species that can eventually reach the milk (Myra Evelyn Flores-Flores, Lizarraga,
184 López de Cerain, & González-Peñas, 2015). Therefore, the purpose of this work was the development
185 of a new method to detect, at the same time, not only the main types of naturally occurring regulated
186 and emerging mycotoxins but also their modified forms that can appear in milk due to the
187 metabolization processes undergone in the animal. In this sense, an UHPLC-MS/MS analysis method
188 was optimized and then an extraction procedure was developed.

189 **3.1. Optimization of UHPLC-MS/MS conditions**

190 The optimization of a suitable chromatographic method is essential to detect several compounds at
191 the same time. The UHPLC-MS/MS conditions employed in this study were previously developed
192 for the analysis of mycotoxins in beer (Gonzalez-Jartin et al., 2019). However, some modifications
193 were made regarding to the original method. The number of target compounds was increased from
194 23 to 40 and, consequently, source parameters were re-optimized. For instance, the nozzle voltage in
195 positive was changed from 0 to 1500 V, which enhance the signal of some compounds (Fig. S1). The
196 CAV, originally set at 2, was optimized for each compound as shown in Table 1. DON derivatives 3-
197 Ac-DON and 15-Ac-DON coelute at the retention time of 5.72 min, with the product ions previously
198 optimized it was no possible to differentiate these compounds. Now, the ions *m/z* 213 and *m/z* 137
199 were chosen to quantify 3 AC-DON and 15 AC-DON (Fig. S2), respectively, allowing their

200 identification without chromatographic separation (Berger, Oehme, & Kuhn, 1999; Berthiller, 201 Schuhmacher, Buttinger, & Krska, 2005; Habler & Rychlik, 2016). Another important parameter in 202 chromatography is the solvent in which samples are diluted for the analysis. In this case, the 203 acetonitrile/water/acetic acid mixture 49/50/1(v/v/v) was chosen since it was shown as the best 204 compromise solvent (Gonzalez-Jartin et al., 2019). In this way, 40 mycotoxins can be detected (Fig. 205 S3).

206 The acquisition mode for detection and quantitation was performed in dynamic multiple reaction 207 monitoring mode (dMRM), monitoring two transition ions for each analyte. In this way, dwell times 208 are maximized since transitions are monitored only when the analytes elute which increase the 209 sensitivity of the method (Z. X. Jia, Zhang, Shen, & Ma, 2016). In this way, targeted compounds are 210 uniquely identified by the retention time as well as precursor and product ions. Therefore, the method 211 is suitable for the confirmatory analysis of contaminants (Decision, 2002).

212 Firstly, performance characteristics of the analytical method were tested in solvent including the 213 LOD, LOQ, and the repeatability within and between-bach (Table 2). The LOQs, calculated based 214 on the guidance of the EU-RL, ranged from 0.007 ng/mL for AFG_1 to 14.61 ng/mL for PAT and, in 215 general, were below to 2 ng/mL (Wenzl et al., 2016). Calibration curves were constructed over the 216 range (0.012−200 ng/mL) and analyzed by triplicate preparation on the same day and over three 217 different days. The percentage of variation of the slope within the same day (within-bach 218 repeatability) was low, with a maximum variation of a 3.08%, while the variation over three different 219 days (between-bach repeatability) was lower than 10%. Therefore, the detection method was 220 appropriate for the analysis of several mycotoxins.

221 **3.2. Optimization of extraction and clean-up procedure**

222 An extraction method was developed since the currently available procedures are scarce and they are 223 focused on regulated toxins. These methods employ an extraction with acidified acetonitrile or 224 methanol followed by a centrifugation step. For the clean-up, some strategies such as freezing the

225 sample or more typically an SPE cartridges have been evaluated to remove co-extractive compounds.
226 (Aguilera-Luiz, Plaza-Bolaños, Romero-González, Martínez Vidal, & Frenich, 2011; Beltran et al.,
227 2013; M. E. Flores-Flores et al., 2017; Huang et al., 2014; Sorensen & Elbaek, 2005; Tsiplakou,
228 Anagnostopoulos, Liapis, Haroutounian, & Zervas, 2014; Xia et al., 2009). The LC/MS methods
229 available for the analysis of mycotoxins in milk are included in Table S1. Aguilera-Luiz, Plaza-
230 Bolaños, Romero-González, Martínez Vidal, & Frenich (2011) developed a method for the detection
231 of 6 regulated mycotoxins (AFs and T-2 toxin) and several pesticides using a C18 cartridge. Huang
232 et al. (2014) and Xia et al. (2009) optimized methods based on a liquid-liquid extraction with
233 acetonitrile and a SPE clean-up step with Oasis cartridges, these methods can be employed to quantify
234 AFM_1, OTA ZEN and their modified forms. Similarly, Sørensen and Elbæk (2005) developed a
235 liquid-liquid extraction procedure using hexane and acetonitrile, followed by a SPE procedure with
236 an Oasis cartridge for the detection of 18 mycotoxins including regulated and modified compounds;
237 the extraction must be performance at pH of 2.0, which makes it necessary to adjust the pH before
238 extraction, and again before the SPE step. There are other methods based on a liquid-liquid extraction
239 that do not use cleaning steps with SPE columns. For instance, Flores-Flores & González-Peñas
240 (2017) developed a method for the detection of 15 mycotoxins, the method is based on an extraction
241 with formic acid in acetonitrile and sodium acetate to increase the separation of the aqueous and
242 organic phases from the extract. Tsiplakou, Anagnostopoulos, Liapis, Haroutounian, & Zervas (2014)
243 used acidified methanol and freeze the sample for 12 hours to increase the separation of the phases,
244 this method allows the detection of 11 compounds. The detection limit of these two last methods is
245 higher than those using SPE columns; for instance, the LOQ of the AFM_1 is 0.05 ng/mL, which
246 corresponds to the legal limit. QuEChERS extraction-based methods are increasingly used as they
247 allow the simultaneous extraction and clean-up of multiple compounds from different matrices. In
248 this sense, tree QuEChERS procedures have been applied for the analysis of mycotoxins in milk. A
249 procedure using mixtures of acetonitrile/water/ acetic acid for the extraction, followed by the addition
250 of $MgSO_4$ and sodium acetate anhydrous to induce phase partitioning reached recoveries near to

251 100% for several mycotoxins. However, this method was validated using commercial dairy product
252 samples and do not detail their fat content (W. Jia, Chu, Ling, Huang, & Chang, 2014). It was
253 described that QuEChERS methods using citrate and acetate buffer did not reach adequate recoveries
254 for AFM_1 when applied to full-cream milk (Aguilera-Luiz, Plaza-Bolanos, et al., 2011). In addition,
255 European Regulations set the tolerable levels for AFM_1 in raw milk, which has a high fat content.
256 Therefore, an unbuffered QuEChERS extraction protocol was optimized to analyse raw milk. With
257 this aim, white samples were artificially contaminated with a mixture of mycotoxins at 25 ng/mL for
258 DON, ZEN, FB_1, OTA, T-2, BEA, ENNA, and 0.375 ng/mL for AFB_1, AFG_1, and they were extracted
259 using different conditions. A general extraction procedure was initially applied which consisted in
260 extract 10 mL of milk with 10 mL of acidified acetonitrile, induce the phase separation using 4 g of
261 $MgSO_4$ and 1 g of NaCl, dry 200 µl of extract and reconstitute it with the same volume of
262 acetonitrile/water/acetic acid mixture 49/50/1(v/v/v). The first step was to identify the amount of acid
263 needed to maximize the extraction of toxins. In this sense, the acetonitrile was acidified with 0.5, 1,
264 1.5, 2 and 2.5% of formic acid. As shown in Fig. 1A, the use of 0.5% of formic acid led to increased
265 R_A of AFs while there were no differences in the recovery of other toxins. Therefore, 0.5% of formic
266 acid was chosen for the following step. The possibility of concentrate the extract was evaluated since
267 it would allow reaching lower LOQs of the method but may also entail an increase in the matrix effect
268 leading to troubles in the quantification process. In order to find the concentration level that allows
269 decreasing the LOQs, as in the previous case, a white sample was artificially contaminated, and the
270 extract was concentrated between 0 and 14 times. The R_A was calculated, and as shown in Fig. 1B,
271 when the sample was concentrated more than 10 times, the R_A starts to decrease probably due to the
272 signal suppression caused by matrix. Therefore, a sample 10 times concentrated was chosen for the
273 analysis. Next, the ratio of salts used for the induction of the separation of the aqueous and organic
274 phases was studied. As shown in Fig. 1C, the use of a ratio 6:1 and 8:1 (w/w) of $MgSO_4$/NaCl showed
275 increases in R_A in comparison with the ratios 4:1, which is commonly used in QuEChERS extraction
276 procedures (González-Curbelo et al., 2015). For instance, in the case of the AFB_1, a R_A of 45% was

277 obtained with the ratio 4:1, while this percentage was increased to 57% with the ratio 8:1. Therefore,
278 the ratio 8:1 (w/w) of $MgSO_4$/NaCl was chosen for the analysis. Finally, different amounts of C18
279 were evaluated for the clean-up procedure. As shown in Fig. 1D, the use of higher amounts of C18
280 led to higher R_A, which means a decreasing of the matrix effect. However, for some compounds such
281 as FBs, OTA, BEA and ENN_S the use of C18 led to reductions in the R_A; therefore, these compounds
282 may be absorbed by the resin. Similar observations have been previously done (Azaiez, Giusti,
283 Sagratini, Mañes, & Fernández-Franzón, 2014; Gonzalez-Jartin et al., 2019). In addition to these
284 mycotoxins, the recovery of AME, CTA, CTN, FA, Hydro-FB_1, Roq C, and STG was lower in the
285 concentrated samples (Fig. S4). Therefore, these compounds are analysed in the non-concentrated
286 extract to which the d-SPE clean-up step is not applied. The other mycotoxins included in the method
287 are analysed in the concentrate extract obtained as described in materials and methods.

288 **3.3. Method validation**

289 Once the main parameters that could affect the extraction process had been optimized, the analytical
290 procedure was validated. First, LODs and LOQs were evaluated in milk according to the EU-RL
291 guidelines by analyzing blank extracts (Wenzl et al., 2016). Low levels of mycotoxins are expected
292 in milk; therefore, LODs and LOQs should be as low as possible. As shown in Table 3, the LOQ of
293 AFM_1 was 0.013 ng/mL, that is 4 times lower than the maximum amount allowed in milk, 0.05 µg/kg
294 (EC_1881/2006). LOQ for other mycotoxins varied from 0.002 ng/mL for AFB_1 to 10.76 ng/mL for
295 CTN. In this sense, the sensitivity for all mycotoxins is in the range, or lower, to that shown by
296 previously published methods even when, with the proposed procedure, a large number of toxins can
297 be simultaneously detected (Table S1).

298 Matrix-match calibration curves were created to evaluate linearity and SSE (Table 3). In matrix, the
299 method shows a linear response in a wide range of concentrations varying from 0.002 to 0.3 ng/mL
300 for AFs, from 0.78 to 200 ng/mL for mycotoxins analyzed in the non-concentrated sample and from
301 0.07 to 20 ng/mL for other mycotoxins. In all cases, R values were higher than 0.995. Therefore, the

302 method can be applied for the analysis of samples with different levels of contamination. The slope
303 of calibration curves constructed in solvent and in milk extract were compared to calculate the SSE,
304 which is a measure of the matrix effect. SSE percentages higher than 100% indicate matrix
305 enhancement, while matrix suppression occurs if the obtained values are lower than this number.
306 Type B trichothecenes were the most affected compounds by matrix since significant signal
307 suppressions were observed, being the DON-3-Gluc the most affected mycotoxin with an SSE of
308 25%. Although the SSE for compounds like DON is high, the signal suppression observed is lower
309 than that obtained with other analysis methods (M. E. Flores-Flores et al., 2017). In the case of T-2
310 and DAS, type A trichothecenes, signal enhancement was observed. For other mycotoxins,
311 intermediate values were obtained, and therefore a low matrix effect. In the case of AFM_1 the SSE
312 was 95%, that is no matrix effect (Table 3).

313 Accuracy and precision of the method were assessed based on the average and the RSD of the
314 recoveries (Table 4). Blank raw milk samples were spiked at three different concentration levels with
315 six replicates for the intermediate level and three replicates for the low and high levels. Samples were
316 extracted with the proposed procedure and quantified by using calibration curves constructed in
317 solvent. Obtained values were compared with the expected ones according to the spiked amounts. In
318 this way, the R_A (Table S2) was obtained and subsequently corrected with the SSE factor (Table 3)
319 to obtain R_E (Table 4). The precision of the method was calculated based on the RSD of the recoveries
320 for the repeatability (intra-day precision), and in the case of samples contaminated in a medium level
321 for the reproducibility (inter-day precision), which was calculated with values obtained from samples
322 analysed in three different days. The mean recoveries of all compounds ranged from 61.22% to
323 120.63%, and the inta-day and inter-day precision were 0.58-10.57% and 2.11%-15.80%,
324 respectively. According to the EU legislation the recovery of the AFM_1 should be between the 60 to
325 120%, and the inter-day precision lower than 22%. Therefore, the proposed method meets the
326 performance criteria for AFM_1 (EC_519/2014; EC_1881/2006). Maximum levels in milk for other
327 mycotoxins have not been established although it was reported the presence of other AFs, OTA, FB_1,

328 and metabolites of DON and ZEN (Myra Evelyn Flores-Flores et al., 2015). Fig. 2 summarize the
329 recovery for toxins with the higher probability of being carried into milk. For all compounds (Table
330 4), the proposed method meets the general analysis criteria regulated toxins with recoveries within
331 60-120% and RSD lower than 20% (EC_519/2014; EC_1881/2006).

332 As shown in table S1, most of the published methods for milk analysis use SPE columns which are
333 relatively expensive and time-consuming since this clean-up process required multiple steps (Du et
334 al., 2018). The proposed method is easy, cheap and less time-consuming since multiple samples can
335 be processed at the same time, avoid the use of SPE columns and freeze the extracts. Among the
336 methods validated using triple quadrupole instruments, the proposed one detect the highest number
337 of toxins. In addition, due to the concentration step, the LOQ of AFM_1 is lower than the reached one
338 with most of the available methods and accurate multi-mycotoxin trace level quantification is
339 feasible.

340 **3.4. Application to real samples**

341 To demonstrate the suitability of the validated method in the routine analysis it was used on 31
342 samples of raw milk obtained from dairy farms in northern of Portugal. The AFM_1 is the most studied
343 mycotoxin in milk; however, other AFs, T-2 toxin, OTA, FB_1, DOM-1 and ZEN and its metabolites
344 and have also been found in milk (Becker-Algeri, Castagnaro, de Bortoli, de Souza, Drunkler, &
345 Badiale-Furlong, 2016; Fink-Gremmels, 2008a; Myra Evelyn Flores-Flores et al., 2015). In the
346 present study, T-2 toxin, Roq C, ENNs and BEA were detected in samples (Table S3). The T-2 toxin
347 was found in one sample, the carry-over of T-2 toxin into milk is well stablished, however, up to now
348 there were no reports about their presence in samples intended for human consumption (Becker-
349 Algeri et al., 2016; Fink-Gremmels, 2008a). The Roq C was found in two samples, its presence in
350 silages supplied to dairy cattle is common, and this compound is also regularly found in cheese;
351 however, no data about their presence in milk was available (Dobson, 2017; Tangni, Pussemier,
352 Bastiaanse, Haesaert, Foucart, & Van Hove, 2013). It can be highlighted the high occurrence of

353 ENNs and BEA, these compounds were found in 21 and 28 samples, respectively. Although the
354 presence of these compounds had been previously detected in human breast milk, this is the first
355 report evaluating the presence of these emerging mycotoxins in cow milk (Rubert et al., 2014). BEA
356 and ENNs are emerging mycotoxins frequently found in cereals, data available regarding their
357 toxicity indicate adverse effects on gastrointestinal tract, immunity and steroidogenesis (Fraeyman et
358 al., 2018). The high occurrence of these compounds points out the need to perform further occurrence
359 surveys in this matrix.

360 **Conclusion**

361 A new method based on a QuEChERS extraction procedure followed by UHPLC-MS/MS detection
362 was developed for the analysis of mycotoxins in raw milk. The analysis method allows the
363 quantification of 40 mycotoxins in a run time of 13 min. The proposed QuEChERS extraction was
364 optimized to minimize the matrix effect caused by milk. The method was validated for raw milk and
365 shows good performance characteristics including linearity, repeatability, accuracy, and precision. In
366 addition, the method also displayed satisfactory sensitivity with a LOQ lower than the maximum
367 residue levels established in the European Union, which points out their suitability for the detection
368 and quantification of regulated mycotoxins. In addition, several emerging and modified mycotoxins
369 from the main mycotoxigenic genus (*Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*) can be analyzed
370 at the same time. It was found a high occurrence of the emerging mycotoxins BEA and ENNs in milk
371 samples obtained from different farms.

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378 **Appendix A**

379 Supplementary data

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528 **Figure captions:**

529 **Fig. 1 Optimization of the extraction process.** Apparent recovery (R_A) of samples extracted with 530 0.5, 1, 1.5, 2 and 2.5% of formic acid in acetonitrile (A). R_A after concentrate the sample 0, 4, 6, 8, 531 10, 12 and 14 times (B). R_A obtained when using a weight ratio of 4:1, 6:1 and 8:1 (w/w) of the 532 dispersive salts MgSO4/NaCl (C). R_A obtained without a clean-up procedure (0) and using 300 mg 533 of $MgSO_4$ and 100, 200 or 300 mg of C18 (D).

534 **Fig. 2 Recovery of mycotoxins from milk.** Recovery at a low (diagonal stripes columns), medium 535 (horizontal stripes columns) and high (dark columns) contamination level for some representative 536 mycotoxins.

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Figure captions:

Fig. 1 Optimization of the extraction process. Apparent recovery (R_A) of samples extracted with 0.5, 1, 1.5, 2 and 2.5% of formic acid in acetonitrile (A). R_A after concentrate the sample 0, 4, 6, 8, 10, 12 and 14 times (B). R_A obtained when using a weight ratio of 4:1, 6:1 and 8:1 (w/w) of the dispersive salts MgSO4/NaCl (C). R_A obtained without a clean-up procedure (0) and using 300 mg of $MgSO_4$ and 100, 200 or 300 mg of C18 (D).

Fig. 2 Recovery of mycotoxins from milk. Recovery at a low (diagonal stripes columns), medium (horizontal stripes columns) and high (dark columns) contamination level for some representative mycotoxins.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jesús M. González-Jartín: Investigation, Writing - Original Draft, Writing - review & editing.
Inés Rodríguez- Cañás: Investigation
Amparo Alfonso: Methodology, Writing - Review & Editing.
María J. Sainz: Conceptualization, Investigation.
Mercedes R. Vieytes: Methodology.
Ana Gomes: Investigation.
Isabel Ramos: Investigation.
Luis M. Botana: Funding acquisition, Supervision.

Table 1

Analyte	Precursor *m/z* and mode	Product *m/z* and (CE)		FV	CAV	Retention time (min)
AFB_1	313 $[M+H]^+$	285 (24)	241 (44)	144	2	6.45
AFB_2	315 $[M+H]^+$	287 (28)	259 (32)	147	1	6.25
AFG_1	329 $[M+H]^+$	243 (28)	200 (48)	132	2	6.05
AFG_2	331 $[M+H]^+$	245 (32)	217 (40)	146	2	5.9
AFM_1	329 $[M+H]^+$	273 (25)	259 (29)	120	2	6.0
AME	271 $[M+H]^-$	256 (20)	228 (28)	125	6	8.4
AOH	257 $[M+H]^-$	213 (20)	215 (24)	155	2	7.34
BEA	801 $[M+NH_4]^+$	134 (70)	784 (14)	90	2	10.15
CTA	394 [M+H]+	379 (24)	350 (32)	83	2	7.5
CTN	251 $[M+H]^+$	205 (20)	233 (9)	110	2	6.7
DAS	384 $[M+NH_4]^+$	307 (4)	105 (36)	90	2	6.6
DOM-1	281 $[M+H]^+$	109 (16)	79 (48)	90	6	5.5
DON	297 $[M+H]^+$	249 (8)	203 (12)	74	2	5.0
15 AC-DON	339 $[M+H]^+$	261 (8)	137 (8)	75	2	5.72
3 AC-DON	339 $[M+H]^+$	261 (8)	213 (8)	75	2	5.72
ENNA	699 $[M+NH_4]^+$	100 (60)	228 (28)	170	2	10.5
$ENNA_1$	685 $[M+NH_4]^+$	210 (28)	668 (12)	145	2	10.35
ENNB	657 $[M+NH_4]^+$	196 (28)	640 (12)	90	2	10.1
$ENNB_1$	671 $[M+NH_4]^+$	196 (30)	654 (14)	130	2	10.25
FA	180 [M+H]+	162 (8)	134 (16)	88	2	6.1
FB_1	722 $[M+H]^+$	352 (40)	334 (40)	170	1	7.08
FB_2	706 $[M+H]^+$	336 (40)	318 (48)	165	1	8
HT-2	447 $[M+Na]^+$	345 (16)	285 (20)	108	2	7.14
Hydro-FB_1	406 [M+H]+	388 (9)	370 (17)	105	2	7.05
MPA	321 $[M+H]^+$	207 (16)	303 (0)	80	2	7.7
NEO	400 $[M+H]^+$	215 (16)	169 (28)	74	2	5.35
OTA	404 $[M+H]^+$	239 (22)	102 (75)	84	2	8.0
RC	390 $[M+H]^+$	193 (40)	322 (16)	150	2	7.15
STC	325 $[M+H]^+$	281 (40)	310 (24)	130	1	5.5
T-2	484 $[M+NH_4]^+$	215 (16)	197 (16)	84	2	7.6
ZEN	317 $[M-H]^-$	273 (20)	131 (28)	123	2	8.08
ZOL	319 $[M-H]^-$	275 (24)	107 (28)	120	2	8.2
α-ZEN	319 $[M-H]^-$	275 (20)	160 (28)	155	2	7.9
α-ZOL	321 $[M-H]^-$	277 (0)	303 (16)	175	2	7.85
β-ZEN	319 $[M-H]^-$	275 (20)	160 (28)	155	2	7.4
β-ZOL	321 $[M-H]^-$	303 (16)	259 (20)	175	2	7.35
DON-3-Gluc	503 $[M+HCOO]^-$	457 (12)	427 (20)	125	4	4.9
FX	355 $[M+H]^+$	175 (8)	71 (20)	90	4	5.4
PAT	153 $[M-H]^-$	109 (8)	81 (12)	60	2	4.9
T-2 triol	405 $[M+Na]^+$	303 (16)	125 (12)	110	2	6.7

Table 1: Precursor and Product Ions (*m/z*) of mycotoxins and MS detection conditions. Collision energy (CE), fragmentor voltage (FV), cell accelerator voltage (CAV).

Table 2

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Repeatability within-batch (%)	Repeatability between-batch (%)
AFB_1	0.002	0.008	2.15	5.83
AFB_2	0.003	0.009	2.09	4.71
AFG_1	0.002	0.007	1.85	5.52
AFG_2	0.006	0.021	2.11	6.09
AFM_1	0.007	0.023	2.76	5.62
AME	0.06	0.18	0.90	1.43
AOH	0.57	1.89	2.30	2.96
BEA	0.006	0.019	0.89	3.32
CTA	0.011	0.035	1.22	7.67
CTN	3.79	12.51	1.50	5.14
DAS	0.06	0.20	1.65	8.32
DOM-1	0.70	2.31	2.99	2.78
DON	0.20	0.67	0.89	6.82
15 AC-DON	0.43	1.43	1.49	5.65
3 AC-DON	0.35	1.15	1.80	6.25
ENNA	0.003	0.011	0.57	7.18
$ENNA_1$	0.007	0.023	0.51	6.22
ENNB	0.012	0.040	1.16	1.65
$ENNB_1$	0.018	0.058	0.68	5.45
FA	0.11	0.35	1.66	7.48
FB_1	0.19	0.63	1.23	6.92
FB_2	0.17	0.57	2.38	8.68
HT-2	0.25	0.84	0.56	6.93
Hydro-FB_1	0.25	0.82	0.99	7.63
MPA	0.05	0.17	2.64	7.00
NEO	0.56	1.85	0.16	5.23
OTA	0.05	0.16	1.79	6.57
RC	0.03	0.10	1.84	8.60
STC	0.02	0.05	1.08	6.93
T-2	0.08	0.26	2.58	5.35
ZEN	0.11	0.36	2.14	3.52
ZOL	0.11	0.36	2.08	4.95
α-ZEN	0.08	0.25	2.93	3.54
α-ZOL	2.49	8.22	2.62	9.96
β-ZEN	0.05	0.17	3.08	3.43
β-ZOL	0.54	1.77	2.36	5.35
DON-3-Gluc	0.09	0.28	1.43	6.32
FX	2.75	9.08	4.00	5.47
PAT	4.43	14.61	1.08	6.19
T-2 triol	0.60	1.98	2.50	8.65

Table 2: Performance characteristics of the analysis method in solvent

Table 3

Analyte	LOD (µg/L)	LOQ (µg/L)	Calibration range (µg/L)	R	SSE (%)
AFB_1	0.001	0.002	0.002-0.3	0.999	87.81
AFB_2	0.002	0.005	0.009-0.3	0.999	76.08
AFG_1	0.002	0.007	0.009-0.3	0.998	81.89
AFG_2	0.004	0.013	0.018-0.3	0.999	76.72
AFM_1	0.004	0.013	0.018-0.3	0.996	95.16
AME	0.04	0.14	0.8-200	0.999	103.21
AOH	0.21	0.68	0.62-20	0.997	84.29
BEA	0.59	1.95	3.12-200	0.999	88.03
CTA	0.10	0.34	0.78-200	0.999	84.05
CTN	3.26	10.76	12.5-200	0.996	97.65
DAS	0.01	0.03	0.07-20	0.998	120.85
DOM-1	0.62	2.06	2.5-20	0.999	44.19
DON	0.23	0.75	1.2-20	0.999	39.12
15 AC-DON	0.25	0.84	1.2-20	0.996	60.13
3 AC-DON	0.34	1.11	1.2-20	0.997	52.38
ENNA	0.02	0.08	0.78-200	0.999	66.48
$ENNA_1$	0.11	0.37	0.78-200	0.999	64.61
ENNB	0.08	0.27	0.78-200	0.999	89.54
$ENNB_1$	0.07	0.24	0.78-200	0.999	80.31
FA	0.87	2.88	3.12-100	0.999	83.29
FB_1	0.35	1.15	3.12-200	0.999	101.10
FB_2	0.31	1.04	3.12-200	0.998	116.20
HT-2	0.14	0.47	0.62-20	0.999	36.80
Hydro-FB_1	1.69	5.57	6.25-200	0.999	107.80
MPA	0.04	0.13	0.31-20	0.999	167.07
NEO	0.04	0.12	0.31-20	0.999	100.56
OTA	0.62	2.05	3.12-200	0.999	123.17
RC	0.28	0.94	0.78-200	0.999	74.41
STC	0.07	0.23	0.78-200	0.999	115.82
T-2	0.07	0.22	0.31-20	0.999	134.22
ZEN	0.02	0.08	0.12-20	0.999	139.11
ZOL	0.09	0.30	0.31-20	0.999	103.87
α-ZEN	0.06	0.21	0.31-20	0.999	77.27
α-ZOL	0.21	0.70	0.62-20	0.999	109.09
β-ZEN	0.18	0.59	0.62-20	0.996	93.82
β-ZOL	0.62	2.06	0.62-20	0.996	105.45
DON-3-Gluc	0.66	2.19	2.5-160	0.996	25.14
FX	1.53	5.04	5-160	0.998	61.04
PAT	1.43	4.73	5-160	0.996	94.55
T-2 triol	0.80	2.62	2.5-160	0.995	39.72

Table 3: Performance characteristics of the analysis method in milk

Table 4

Analyte	Medium			Low		High	
	Recovery (%) (n=6)	Intra-day precision (%) (n=6)	Inter-day precision (%) (n=6)	Recovery (%) (n=3)	Intra-day precision (%) (n=3)	Recovery (%) (n=3)	Intra-day precision (%) (n=3)
AFB_1	83.18	3.22	9.11	101.91	3.36	79.14	1.29
AFB_2	84.99	6.22	2.11	109.60	1.64	84.97	1.47
AFG_1	76.02	6.84	7.60	101.23	5.68	79.30	4.30
AFG_2	95.57	5.19	6.71	105.74	7.11	97.19	6.46
AFM_1	102.71	5.28	7.23	96.68	2.80	93.71	1.33
AME	111.12	7.88	8.52	118.09	7.26	101.61	4.60
AOH	84.55	8.82	8.33	75.31	8.58	78.93	9.59
BEA	93.39	2.76	6.76	99.63	4.48	91.13	3.88
CTA	103.36	3.87	7.69	97.39	9.20	99.54	1.40
CTN	105.87	3.36	5.16	100.25	6.91	109.55	5.17
DAS	100.46	9.23	8.63	103.80	8.14	92.03	3.04
DOM-1	107.02	4.80	8.33	120.63	3.71	101.14	2.48
DON	90.46	6.90	6.10	98.58	7.47	88.83	0.58
15 AC-DON	112.00	0.90	5.53	105.23	7.64	113.08	2.22
3 AC-DON	113.56	2.71	3.70	103.55	8.33	115.45	4.61
ENNA	86.74	4.30	4.90	93.26	2.64	92.57	5.78
$ENNA_1$	94.17	4.00	4.06	98.67	1.20	100.58	6.74
ENNB	107.69	4.68	8.47	119.82	6.19	109.59	2.16
$ENNB_1$	99.58	3.29	7.24	112.21	7.16	103.46	1.04
FA	107.52	4.50	9.57	94.99	4.86	109.26	2.57
FB_1	119.96	3.17	13.23	109.45	9.24	108.20	1.75
FB_2	101.94	3.02	3.92	91.71	2.93	103.83	4.97
HT-2	103.58	3.96	5.32	116.49	3.52	107.56	0.73
Hydro-FB1	113.10	1.53	11.12	107.73	4.93	111.11	2.80
MPA	80.21	7.01	9.85	87.89	8.08	81.33	10.57
NEO	113.41	5.29	10.11	103.49	2.00	114.57	1.71
OTA	109.29	7.51	8.35	107.13	9.25	111.36	5.88
RC	109.10	6.85	8.86	116.43	6.80	110.10	4.30
STC	112.70	9.05	11.92	110.98	4.91	109.38	9.55
T-2	75.13	6.58	10.34	92.12	3.21	75.91	2.18
ZEN	61.22	5.44	9.18	70.84	7.40	65.43	6.07
ZOL	70.06	5.04	9.05	69.66	5.15	64.76	2.43
α-ZEN	78.19	7.17	8.91	75.35	3.18	70.69	2.31
α-ZOL	71.59	10.34	15.80	83.70	8.16	72.05	8.71
β-ZEN	73.71	6.40	6.45	83.86	9.18	83.13	6.84
β-ZOL	88.15	3.46	8.24	99.45	6.71	94.41	2.30
DON-3-Gluc	79.24	1.26	2.39	-	-	-	-
FX	100.37	4.99	7.35	-	-	-	-
PAT	62.73	1.29	8.41	-	-	-	-
T-2 triol	101.38	1.99	4.60	-	-	-	-

Table 4: Results for the recovery and precision arising from the validation of the analytical method

Figure 1

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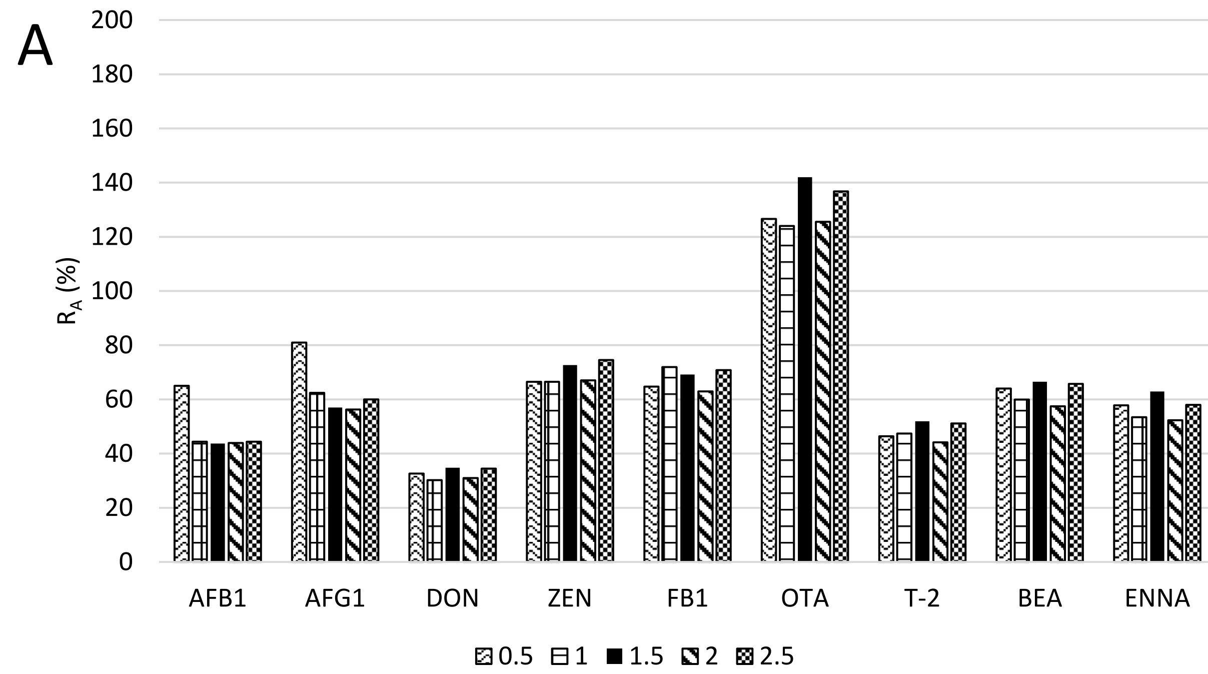

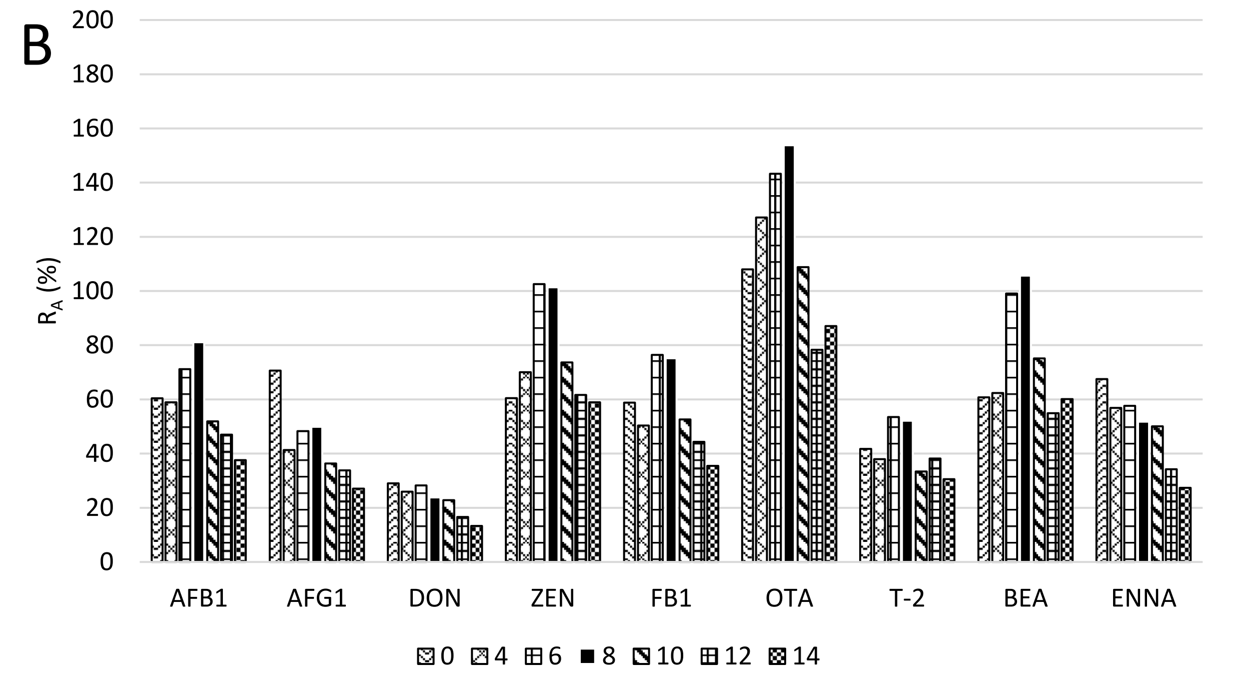

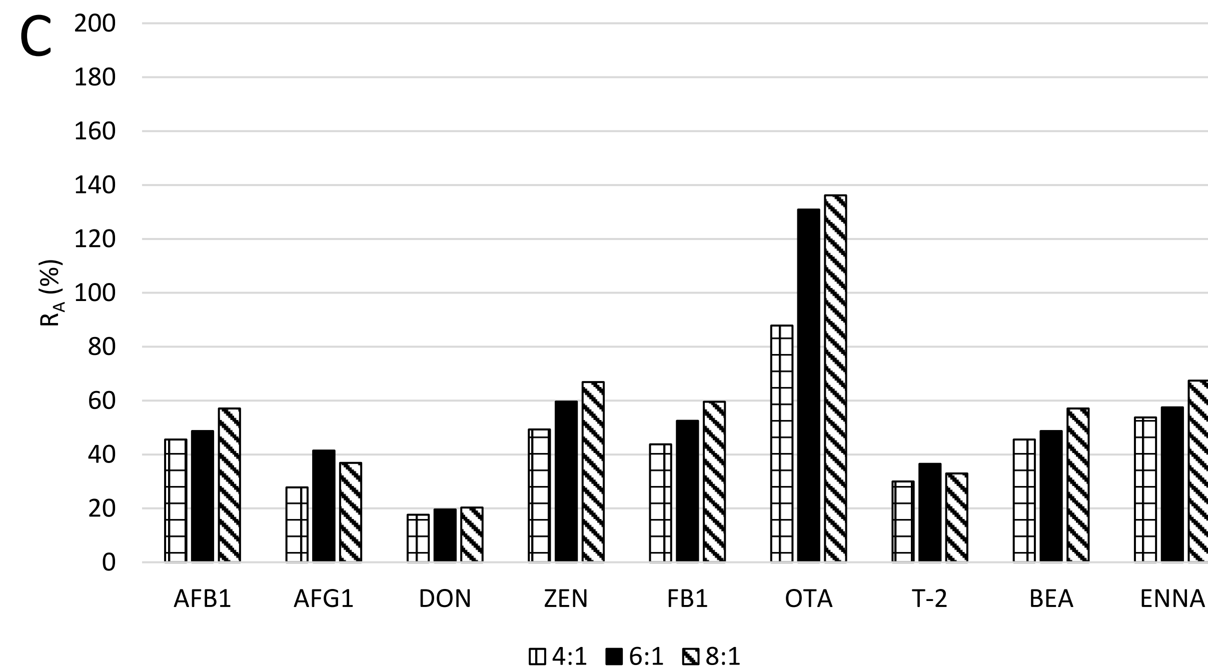

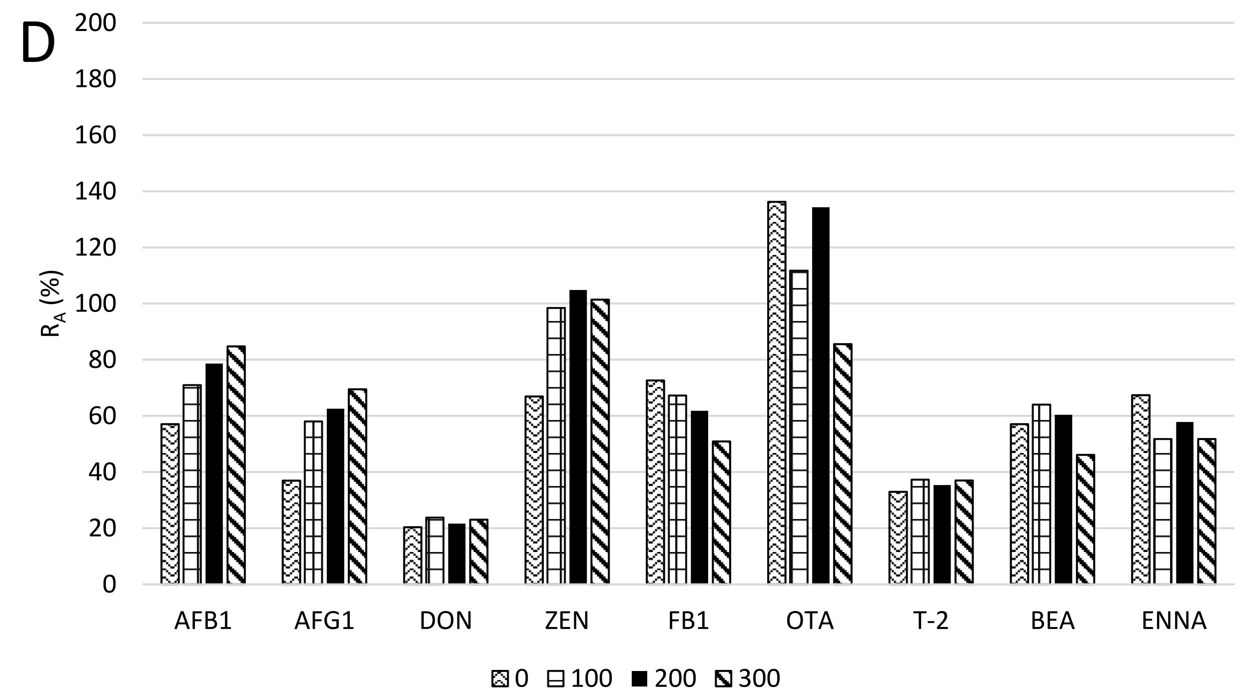

Figure 2

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