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Moniliformin Analysis in Maize Samples from North-West Italy Using Multifunctional Clean-up Columns and the LC-MS/MS Detection Method

Authors:

Valentina Scarpino, Massimo Blandino*, Michele Negre, Amedeo Reyneri, Francesca Vanara

Affiliation:

Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, via Leonardo Da Vinci 44, 10095 Grugliasco (TO), Italy.

* Corresponding author: Tel: +39-011-6708895; fax +39-011-6708798.

E-mail address: massimo.blandino@unito.it

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ABSTRACT: A fast clean-up method has been developed to purify maize extracts and to detect moniliformin (MON) in maize samples from North-West Italy over a four-year period (2008-2011). The method is based on the use of MycoSep[®] 240 Mon clean-up columns (Romer Labs[®]). Samples were extracted using acetonitrile/water (84:16, v/v), and the extracts were purified in the previously described clean-up columns. The LC-MS/MS analysis has been carried out by means of hydrophilic interaction chromatography (HILIC), combined with negative electrospray mass spectrometry (ESI⁻ - MS). The developed method has a recovery rate of 76-91% (RSD%: 6-14%), a limit of detection (LOD) of 1 µg kg⁻¹, and a limit of quantification (LOQ) of 4 µg kg⁻¹. One hundred and eight different naturally contaminated maize samples were analyzed for their MON content. The average percentages of positive samples was 93% with the following ranges (µg kg⁻¹): 33-2606 (2008); < LOD-527 (2009); < LOD-920 (2010); < LOD-409 (2011).

KEYWORDS: Moniliformin, maize, mycosep clean-up columns, LC-MS/MS, *Fusarium*

1 ABBREVIATIONS

EFSA, European Food Safety Authority; ESI, electrospray ionization; GDD 2 Accumulated growing degree days: HILIC. hydrophilic interaction 3 chromatography; HPLC, high-performance liquid chromatography; LOD, limit of 4 detection; LOQ, limit of quantification; MON, moniliformin; MS, mass 5 6 spectrometry detection; RSD, relative standard deviation; SAX, strong ion exchange; SPE, solid phase extraction. 7

8

9 **INTRODUCTION**

10 Moniliformin (MON) is a worldwide *Fusarium* mycotoxin which often occurs in 11 cereals and maize (Sharman *et al.* 1991). MON is a small (98.0081 g mol⁻¹) (Betina 12 1989), highly polar, acidic molecule. Due to the low pK_a value (< 1.7) of the free 13 acid (semisquaric acid), MON does not occur as an acid in nature but as a water 14 soluble sodium or potassium salt (Steyn *et al.* 1978).

15 It was first isolated by Cole et al. in 1973 (Cole *et al.* 1973) and structurally 16 characterized as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-17 dione (Figure 1) by Springer et al. in 1974 (Springer *et al.* 1974). MON can 18 produce plant growth regulation and phytotoxic effects in plant systems (Cole *et al.* 19 1973, Vesonder *et al.* 1992). MON is also toxic to several animal species, causing 20 myocardial changes, muscular weakness, respiratory distress, cyanosis, coma, and 21 death (Kriek *et al.* 1977). The action mechanism probably involves selective

inhibition of the pyruvate and α -ketoglutarate dehydrogenase enzyme systems (Burka *et al.* 1982). MON toxicity mainly affects cockerels (LD₅₀ = 4.00 mg kg⁻¹, oral) and ducklings (LD₅₀ = 3.68 mg kg⁻¹, oral) (Kriek *et al.* 1977).

MON is produced by several Fusarium species on several crops, and has been 25 found in different geographical areas. The main Fusarium species that are able to 26 produce MON are listed in Table 1. MON contamination is higher in maize than in 27 other substrates and, in South Europe, it is commonly produced in maize infected 28 by F. proliferatum and F. subglutinans. Both species can be found globally, but the 29 optimum temperature for growth of F. subglutinans is lower than that of F. 30 proliferatum, thus the former is more common in temperate areas (Kostecki et al. 31 1999). In a study of the incidence, geographic distribution and toxigenicity of 32 Fusarium species in South African maize, F. subglutinans was found to 33 34 predominate in relatively cool and humid climates (Rabie et al. 1982). Since 1982, when it was first reported as a natural contaminant in Transkeian maize (16-25 mg 35 kg⁻¹) (Thiel et al. 1982), MON has been found in maize in different parts of the 36 world. This mycotoxin was detected in maize ears in Poland, from 1985 to 1991, 37 and the average content over these six years was 131 mg kg⁻¹ (Chelkowski et al. 38 1987, Chelkowski 1989, Lew et al. 1996). It has also been found to occur in 39 Austrian maize, with levels of up to 20 mg kg⁻¹ (Lew *et al.* 1991), as well as in 40 Canada, Germany, and New Zealand (Lamprecht et al. 1986, Scott et al. 1987, 41 Thalman et al. 1985). In these surveys the original samples were hand-selected in 42 order to collect visibly Fusarium infected kernels, thus high levels of MON 43

contamination were reported. Instead, the results of surveys on MON
concentrations and distribution in naturally contaminated maize grain from field or
commercial lots, which are reported in Table 2, have shown lower levels of MON
concentration.

48 Several approaches have been developed for the selective extraction, sample49 purification, chromatographic separation and detection of MON.

50 Liquid Chromatography has been the main chromatographic technique used in the analysis of MON and due to the highly polar properties of this mycotoxin the most 51 common procedure used in the years was the application of ion-pairing mobile 52 53 phases to achieve a good chromatographic separation with reversed-phase columns (Shepherd and Gilbert 1986, Munimbazi and Bullerman 1998, Sewram et al. 54 1999). More recent approaches to improve the chromatographic separation were 55 56 the use of hydrophilic interaction chromatography (HILIC) (Sørensen et al. 2007) and the Gemini C6-Phenyl column as reversed-phase column (Von Bargen et al. 57 2012). 58

Until now, the several analytical methods that have been reported in literature have used commercial SPE-columns for the purification of MON extracts. These mainly have been strong anion exchanger (SAX) columns (Munimbazi and Bullerman 1998, Filek and Lindern 1996, Parich *et al.* 2003, Sørensen *et al.* 2007, Von Bargen *et al.* 2012), but also nonpolar C₁₈-columns (Shepherd and Gilbert 1986), or a combination of the two (Sharman *et al.* 1991). However, Jestoi et al. (Jestoi *et al.*

65 2003) did not apply any sample purification steps, except filtering and66 concentration, in order to reduce the loss of MON during the analysis.

Since these purification methods are time consuming and require several steps, they
could lead to analytical errors and a detriment of analytical repeatability. In
addition, not applying a purification step can lead to the instrumentation becoming
dirty, thus impairing the analysis.

The purpose of the current study was to develop a fast, reliable and repeatable 71 clean-up method, using MycoSep[®] 240 Mon clean-up columns (Romer Labs[®]) for 72 the first time to purify maize extracts prior to the analytical determination of MON. 73 Moreover the European Food Safety Authority (EFSA) is currently working on 74 establishing a scientific opinion on the risks to public health related to the presence 75 of MON in feeds and food (EFSA 2010). Since there is this need to obtain major 76 77 information about the incidence of this mycotoxin in the most important cereal areas in the EU, this procedure has been applied to maize samples collected over a 78 79 four-year period (2008-2011) in North-West Italy in order to obtain data about the presence, diffusion and level of MON contamination. A first attempt has been 80 made to individuate the conditions which could lead to a higher contamination of 81 this mycotoxin. 82

83

84 MATERIALS AND METHODS

85

86 Chemicals

87 All the chemicals and analytical standards were purchased from Sigma Aldrich (St.

Louis, MO), or VWR (Milan, Italy). The solvents were gradient grade or LC-MSgrade.

The MON standard was purchased as sodium salt and a 93 mg L^{-1} stock solution of MON in acetonitrile/water (84:16, v/v) was prepared and stored at 4 °C. This stock solution was used to prepare standard solutions through dilution with acetonitrile/water (84:16, v/v).

94

95 Samples

96 One hundred and eight maize grain samples, collected over 4 years in farm fields in North-West Italy (Torino and the Cuneo Province), were analyzed for natural MON 97 98 contamination. The number of maize grain samples collected each year was 16, 16, 40 and 36 in 2008, 2009, 2010 and 2011, respectively. The fields were cultivated 99 under irrigation with full length maturity hybrids, planted in each growing season 100 in the period between the last decade of March and the first decade of April. The 101 normal agronomic techniques of each area were adopted. The considered fields 102 were characterized by a natural infestation of European Maize Borer (ECB, 103 Ostrinia nubilalis Hübner) each year and in each site, since no insecticide was 104 applied to control ECB or other insects during the ripening period. 105

One hundred ears (included the ears used for the evaluation of ECB incidence and severity at harvest) were collected by hand for each maize kernel sample in each field at the end of maturity (moisture content of grains of between 23 and 27%) from five subplots and shelled using an electric sheller. The kernels from each plot were mixed thoroughly to obtain a random distribution; 5 kg samples were then taken to analyze the MON content and dried at 50°C for 3 days.

112 ECB damage incidence was calculated as the percentage of ears per plot with kernel injuries or apical and basal tunnels in the cob due to larvae activity. The 113 ECB damage severity was calculated as the percentage of kernels per ear with 114 115 injuries due to larvae activity. A scale of 1 to 7 was used in which each numerical value corresponded to a percentage interval of surfaces exhibiting visible kernel 116 117 damage due to larvae activity according to the following schedule: 1 = no injuries, 118 2 = 1-5%, 3 = 6-10%; 4 = 11-20 %, 5 = 21-35%, 6 = 35-60%, 7 > 60% (Blandino et al. 2009). The ECB damage severity scores were converted to percentages of 119 120 ears exhibiting symptoms and each score was replaced with the mid-point of the interval. 121

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128 Chemical Analyses

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130 Sample Preparation and Extraction

Maize samples were ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, 131 Haan, Germany) and the flour was used directly for the extraction. Twenty five g 132 ground maize samples from a MON-free sample were spiked in order to evaluate 133 the recovery rate of the analytical method. Three replicas with 100 µL of pure 134 solvent, or 0.93; 9.3; 93 mg MON L^{-1} ; or 1000 μ L of 93 mg MON L^{-1} were used to 135 obtain spiked MON levels of 0, 3.72, 37.2, 37.2, and 3720 µg kg⁻¹ for LC-MS/MS 136 137 determination. The experiments were performed on four different days to establish day to day variations. The spiked maize samples were incubated at room 138 temperature 2 h prior to extraction, which allowed the solvent to evaporate and 139 140 MON to enter the material.

141 Twenty five g maize flour was extracted by mechanical shaking at 100 rpm for 1 h
142 (shaker mod. M102-OS, MPM Instruments, Milan, Italy) with 100 mL
143 acetonitrile/water (84:16, v/v). The extracts were filtered through Whatman no. 1
144 filters (Brentford, UK) and subjected to clean-up and purification.

145

146 *Clean-up*

- 147 Two clean-up methods were tested. The first method was performed with Strata
 148 SAX (Strong Anion Exchange) columns (500 mg) (Phenomenex, Torrance, CA)
- 149 applying the clean-up procedure described by Sørensen et al. (Sørensen et al.

2007). The columns were activated with 2 mL methanol, 2 mL water, and 2 mL 0.1 150 151 M HCl. Two mL of concentrated maize extracts were then added to the SAX columns. The cartridge was washed with 2 mL methanol-water (50:50) and 2 mL 152 0.1 M HCl. MON was eluted with 2 mL 1 M HCl. The eluate was dried under 153 nitrogen, diluted in 100 µL acetonitrile-water (85:15) and transferred to an HPLC 154 vial. The second clean-up method was performed with MycoSep[®] 240 Mon clean-155 up columns (Romer Labs[®], Tulln, Austria). The clean-up procedure was adapted 156 from the Romer Labs[®] procedure. The cleanup MycoSep[®] columns were pushed 157 into test tubes containing 5 mL of the sample extracts, forcing the extracts to filter 158 159 upwards through the packing material of the columns. The interferences adhered to 160 the chemical packing in the columns and the purified extracts, containing MON, passed through the columns. The evaporation to dryness under nitrogen step was 161 162 excluded because it provoked a loss of MON of up to 40%. The use of silanized vials, which were adopted to avoid the adsorption of MON on the glass, did not 163 improve the recovery. The purified extracts (1.5 mL) were transferred to HPLC 164 vials and analyzed by means of LC-MS/MS according to the method described 165 166 below.

167 This one step cleanup required less than one minute per sample, while the method 168 based on SAX clean-up, because of the long time required to evaporate the HCl 169 aqueous solution, was much more time consuming.

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171

172 *LC-MS/MS*

173 LC-MS/MS analysis was carried out on a Varian 310 triple quadrupole mass spectrometer (Varian, Italy) equipped with an electrospray ionization ESI source, a 174 212 LC pump, a ProStar 410 AutoSampler and dedicated software. LC separation 175 was performed on a 100 mm \times 2.1 mm i.d., 3.5 μ m, 100 Å ZIC[®]-HILIC (Merck, 176 SeQuant, Milan, Italy) column. The mobile phase consisted of water buffered with 177 178 100 mM ammonium formate (pH 6.4) (A) and acetonitrile (B) delivered at 200 μ L/min. The gradient was 5 to 50 % A in 7 min. Mass spectrometric analyses 179 were performed in the negative -ion mode. The nebulising gas was N_2 (20 psi), the 180 drying gas was air (250 °C, 25 psi), the needle voltage was -3000 V, the shield 181 182 voltage was -600 V, the detector voltage was 1250 V, the capillary voltage was -20 V and the collision energy voltage was 12 V. The deprotonated molecule (m/z =183 97.0) was fragmented to its product ion (m/z = 41.0) and used for quantification 184 and identification purposes. 185

186

187 *Calibration*

188 Ten different MON concentrations were prepared for calibration in 189 acetonitrile/water (84:16, v/v), between 0.93 and 930 μ g L⁻¹. A linear regression 190 was used to obtain the regression curve.

191

192 **RESULTS AND DISCUSSION**

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194 LC-MS/MS Analyses

195 Previous studies on the chromatographic separation of MON reported the use of different types of columns. The chromatography of the current study was based on 196 197 the Sørensen et al. (Sørensen et al. 2007) method and the authors own observations 198 during the development of the LC-MS/MS method. The MON retention time with the HILIC gradient program was 3.9 min with a runtime of 17 min (Figure 2). This 199 runtime was necessary to elute, with 50% water buffered with 100 mM ammonium 200 201 formate, stronger retained contaminant compounds than MON. MON is a strong acid and hence produces more negative than positive ions. Negative ion polarity 202 203 usually generates less background noise than the positive mode, therefore 204 improving sensitivity. ESI was therefore adopted, in negative mode, in the current study using a triple-quadrupole instrument. The presumed MON fragmentation 205 206 pathway is shown in Figure 3. Unfortunately, in tandem mass spectrometers MON generates only one strong product ion in the collision cell of the instrument. Thus, 207 only one MRM, the fragmentation of m/z 97 to m/z 41 can be programmed (Jestoi 208 209 et al. 2003). To improve the sensitivity and specificity of the analysis of MON and to avoid the use of the only possible transition in tandem mass spectrometry, a 210 recent approach was the use of a high resolution instrument (Von Bargen et al. 211 212 2012).

213

214 Clean-up

215 Several surveys have shown the columns most frequently adopted for SPE clean up procedures, to purify maize and cereal grain extracts, are SAX columns (Sharman 216 217 et al. 1991, Munimbazi and Bullerman 1998, Filek and Lindner 1996, Sørensen et al. 2007). When these clean-up procedures were applied to ours samples, low 218 recoveries were observed (< 50%), with one working day being necessary for five 219 samples. The MycoSep[®] column clean-up was instead much less time consuming 220 (less than 1 min per sample) and the matrix effect was reduced. This phenomenon, 221 known as suppression, is caused by the co-eluting matrix components, which 222 223 interfere with the ionization of the analyte (Tang and Kebarle 1993, Gilar et al. 2001). 224

When the whole Romer Labs[®] procedure was applied low recoveries were obtained. For this reason it was hypothesised that the concentration step by evaporation to dryness under nitrogen could be a significant factor in the loss of MON, even when silanized vials were used. Higher recoveries were obtained by excluding this concentration step from the Romer Labs[®] procedure.

Thus, because of the higher recoveries and reduced time necessary, MycoSep[®] columns were used and the previously described clean-up procedure was applied to purify maize samples.

The percentage of recovery (Table 3) ranged from 76 to 91 % (Relative Standard
Deviation, RSD%: 6-14%), independently of the MON concentration.

No differences were observed in recovery rate between the three days, thus confirming the repeatability of the method.

The limit of detection (LOD) and the limit of quantification (LOQ) were 1 μ g kg⁻¹ and 4 μ g kg⁻¹, respectively.

The results attest the accuracy, repeatability and robustness of the method, whichhas here been applied to maize extracts for the first time.

241

242 Naturally Contaminated Samples

The developed analytical method was applied to 108 maize samples collected over a four-year period (2008-2011) in North-West Italy, in order to obtain data about the presence, diffusion and level of contamination of MON in the grain maize cultivated in this area.

The considered growing seasons showed remarkably different meteorological trends (Table 4). The years 2008 and 2010 were characterized by low growing degree days (GDDs) and high rainfall, in particular during the 2010 growing season, from the early milk stage to the harvest. The year 2009 had ordinary GDDs and rainfall from flowering to the end of ripening. The year 2011 was instead characterized by high GDDs and extremely limited rainfall from the milk stage to the harvest.

Table 5 summarized the percentages of positives samples, the arithmetic mean (\pm RSD) and the range of MON contamination for each sampling year. Overall, the average percentages of positive samples was 93%, with the following ranges (µg

257 kg⁻¹): 33-2606 (2008); < LOD-527 (2009); < LOD-920 (2010); < LOD-409 (2011).

258 The MON concentration means ($\mu g k g^{-1}$) (± standard deviation) for each year were:

259 1127 ± 784 (2008); 106 ± 135 (2009); 262 ± 243 (2010); 89 ± 99 (2011).

260 On the basis of the results of the MON contamination it is possible to state that this mycotoxin is diffused through the investigated areas and shows considerably high 261 levels for each sampling year. However, considering that there is a lack of MON 262 263 contamination diffusion data in literature and a lack of recent data performing to the most important cereal areas in the EU, it has not been possible to conduct an 264 exhaustive comparison. Nevertheless, on the basis of Table 2 it can be seen that 265 266 ours results are in agreement with the worldwide levels of MON contamination present in naturally contaminated maize commodities. 267

Table 6 summarized the mean data of ECB incidence and severity of ears collected for each sampling year. The samples collected in the year 2008 showed the highest ECB severity, and thus was followed by those harvested in 2010. The ECB pressure was lower in 2009 and 2011.

The collected data confirm an important link between MON contamination and ECB activity performing to the damage of maize ears, which was also observed also by Papst et al. (Papst *et al.* 2005) in Germany, in a comparative study on Bt maize and its isogenic counterparts. Moreover, Papst et al. (Papst *et al.* 2005) and Magg et al. (Magg *et al.* 2003) reported a significant correlation between the percentage of ECB damaged ears and the MON concentration in the kernel: the

278 MON content in ears manually infested with ECB larvae was 3 times higher than 279 the control, which has been protected through an insecticide application.

In temperate areas, F. verticillioides is favoured more by ECB larvae feeding than 280 281 other Fusarium species (Lew et al. 1991). In the years with high rainfall and low temperatures, the development of F. verticillioides on damaged areas of kernels, 282 caused by second generation ECB, could be less predominant than other fungi, 283 such as F. subglutinans and F. proliferatum, which commonly cause MON 284 contamination. On the basis of these assumptions, and the different meteorological 285 trends of the considered growing seasons (Table 4), the mean MON concentration 286 287 data collected for each year could explain why 2008 and 2010 had the highest MON contamination. 288

289 In conclusion, the obtained results indicate that the developed clean-up procedure, 290 which was here used for the first time, is very fast, highly accurate, repeatable and robust. Since this method allows a reliable quantification of MON in maize 291 samples as low as 4 μ g kg⁻¹, it was possible to quantify MON in more than 90% of 292 293 the analyzed samples. Moreover, the present work provides a first important series of data on natural MON contamination in maize kernels, referring to several 294 295 growing seasons with different meteorological trends. The first collected data suggest that the risk of MON contamination in the North Italian cropping area 296 297 increases for growing seasons with higher rainfall and lower temperatures, from the early milk stage to the harvest. Furthermore, an important link exists between 298 MON contamination in kernels and injuries caused by ECB larvae on maize ears. 299

300	The first MON results obtained in the current study could be very useful for EFSA
301	in order to assess the risks to human and animal health related to this mycotoxin.
302	These data need to be integrated with information from maize, other cereal grains
303	and derived products from the main cropping areas. Thus, the new clean-up
304	procedure could be utilized to quantify MON occurrence in an accurate but rapid
305	and efficient way.
306	

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Table 1. Fusarium species most frequently associated with MON production in

 maize throughout the world.

Fusarium species	Other mycotoxins produced ^a	References
F. proliferatum	FB, BEA, FP	Logrieco <i>et al.</i> 1995 - Miller <i>et al.</i> 1995
<i>F. verticilliodes</i> FB, FUS		Leslie <i>et al.</i> 1996 - Sanhueza <i>et al.</i> 2004
F. subglutinans	BEA, FP	Kostecki et al. 1999
F. avenaceum	BEA, ENN, FUS	Morrison et al. 2002
F. chlamydosporum	-	CFP/EFSA/CONTAM/2008/01
F. oxysporum	BEA	CFP/EFSA/CONTAM/2008/01
F. tricinctum	BEA	CFP/EFSA/CONTAM/2008/01

^{*a*} BEA = beauvericin; FB = fumonisin B_1 , B_2 and B_3 ; FP = fusaproliferin; FUS = fusarin C; ENN = enniantins.

Country	Year	Cereal	Samples (n°)	LOQ ^b (µg kg ⁻¹)	n > LOQ	Min (µg kg ⁻¹)	Max (µg kg ⁻¹)	Sampling procedure	Clean-up	References
Germany	na ^a	Maize	58	na	25	na	> 650	na	na	Talman <i>et</i> <i>al</i> . 1985
Worldwide	1985 - 1989	Maize	64	50 ^c	27	< 50	3160	Field samples of maize from 10 different countries	SAX columns ^d	Sharman <i>et al</i> . 1991
Austria	1991 - 1992	Wheat	48	10 ^c	29	< 10	880	Wheat from Austrian fields	na	Adler <i>et al.</i> 1995
China	na	Maize	104	na	47	52	1116	Maize from Chinese fields	na	Yu <i>et al.</i> 1995
Austria	na	Maize	na	20	na	50	2000	Maize from Austrian fields	SAX columns	Filek and Lindner 1996
South Africa	1997	Maize	4	5 ^c	2	< 5	17	Maize samples from Transkei	RP-C18 columns ^e	Sewram <i>et</i> <i>al</i> . 1999

Table 2. Results of surveys on MON concentrations and distribution in naturally contaminated cereal commodities.

USA	1998	Maize	100	25 ^c	83	< 25	774	Food-grade commercial maize samples	na	Gutema <i>et</i> <i>al.</i> 2000
Poland	na	Wheat	10	na	4	na	200	Wheat from 10 private farms	Columns containing Florisil [®]	Krysinska- Traczyk <i>et</i> <i>al.</i> 2001
Austria	na	Maize	na	39 ^c	na	160	1030	Maize from Austrian fields	SAX columns	Parich <i>et al.</i> 2003
	2001	Barley	22	20	22	< 20	750	Cereals from		
Finland	2002	Wheat	14	20	10	< 20	810	Southern and Central	na	Jestoi <i>et al</i> . 2004
		Oats	1	20	1	-	84	Finland fields		2004
	2000	Barley	75	130	53	< 130	380	Cereals from		
Norway	-	Wheat	83	130	76	< 130	950	Norwegian	SAX	Uhlig <i>et al</i> . 2004
	2001	Oats	73	130	38	< 130	210	fields	corumns	2004
Denmark	na	Maize	28	12	0	1	< 12	Whole maize plants	SAX columns	Sørensen <i>et</i> <i>al.</i> 2007

^{*a*} Not available; ^{*b*} LOQ = Limit of Quantification; ^{*c*} LOD = Limit of Detection; ^{*d*} SAX = Strong anion exchange; ^{*e*} RP-C18 = reversed-phase (C18).

MON Concentration ^{<i>a</i>}	Recovery ^b	RSD ^c	
(µg kg ⁻¹)	(%)	(%)	
 3720	76	6	-
372	76	8	
37.2	82	14	
3.72	91	9	

Table 3. Recovery rate of the analytical method at four MON concentration levels.

^{*a*} Spiked MON levels (μ g kg⁻¹); ^{*b*} Mean recovery (%) obtained from three replicas for each MON concentration level in three different days; ^{*c*} RSD = Relative Standard Deviation.

Table. 4. Total rainfall, rainy days, relative humidity and growing degree days (GDD 10s) from June to October 2008-2011 in a representative site of the investigated maize growing area .

Veen	N <i>I</i> 41-	Rainfall	Rainy days	GDD $10s^a$
rear	Month	(mm)	(n °)	(°C d ⁻¹)
2008	May	121	16	204
	June	95	17	304
	July	63	8	382
	August	52	6	372
	September	57	8	228
	October	30	5	151
	May-October	418	60	1641
2009	May	30	10	292
	June	26	7	341
	July	121	8	391
	August	56	11	404
	September	62	8	273
	October	54	6	163
	May-October	349	50	1864
2010	May	117	12	214
	June	192	11	332
	July	37	8	420
	August	116	11	354
	September	51	12	240
	October	105	9	120
	May-October	618	63	1680
2011	May	42	7	286
	June	104	13	335
	July	59	7	364
	August	9	2	431
	September	24	3	346
	October	19	1	111
	May-October	257	33	1873

^{*a*} Accumulated growing degree days for each month using a 10°C base.

Data refer to the Carmagnola site (44° 50' N, 7° 40' E; altitude 245 m).

Year	Samples ^{a, b} (n°)	Mean MON Concentration ^c (µg kg ⁻¹)	MON Concentration Range (µg kg ⁻¹)	Positives ^e (%)
2008	16	1127 ± 784	33 - 2606	100
2009	16	106 ± 135	<LOD ^{d} - 527	81
2010	40	262 ± 243	< LOD - 920	98
2011	36	89 ± 99	< LOD - 409	92

Table 5. MON Concentrations in maize samples from North-West Italy over afour-year period (2008-2011).

^{*a*} N° Samples = Number of samples analyzed each year; ^{*b*} Naturally contaminated maize samples collected in fields in North-West Italy; ^{*c*} Concentrations not corrected for recovery; ^{*d*} Limit of detection (LOD) = 1 μ g kg⁻¹; ^{*e*} Positive samples = samples with MON concentration \geq LOQ.

Year	Samples (n°)	ECB incidence ^a (%)	ECB severity ^b (%)
2008	16	99 ± 2	25 ± 4
2009	16	68 ± 19	8 ± 4
2010	40	78 ± 16	13 ± 6
2011	36	40 ± 13	6 ± 2

Table 6. Mean data of ECB incidence and severity of ears collected in each sampling year.

^{*a*} ECB incidence was calculated as the percentage of ears with ECB damage, considering 100 ears per sample; ^{*b*} ECB severity was calculated as the percentage of kernels per ear with ECB damage, considering 100 ears per sample.

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Figure 1. Sodium and potassium salt structures of of 1-hydroxycyclobut-1-ene-3,4-

12 dione (MON).

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Figure 2. LC-MS/MS chromatogram of a maize sample (t_R MON = 3.9 min).



Figure 3. Presumed MON fragmentation pathway.