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Natural occurrence of emerging *Fusarium* mycotoxins in malting barley

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Abstract

Beauvericin (BEA), enniatins (ENNs A, A1, B and B1) and moniliformin (MON) are secondary metabolites produced mainly by F. avenaceum that contaminate cereals and their by-products worldwide. In recent years, researchers have paid more attention to these mycotoxins and their potential hazards to human and animal health. The present study is based on two annual surveys of commercially grown UK spring malting barley varieties collected between 2007 and 2011. Liquid chromatography double mass spectrometry (LC-MS/MS) was used to quantify the emerging Fusarium mycotoxins. A total of 223 samples of commercially grown malting barley were analysed from UK fields. The LC-MS/MS method produced a recovery rate in the range of 87%-99% with a relative standard deviation (RSD) of 10% for BEA and ENNs and in the range of 83%–89% (RSD 7%) for MON; the detection limit for quantified mycotoxins was in the range of 0.1-0.9 µg kg-1 for ENN A and MON. The most prevalent mycotoxin detected in 100% of the samples was ENN B, with mean concentrations ranging from 3072.9 to 3498.0 µg kg-1 in 2007 and 2009, while in 2010 and 2011, mean concentrations of 1940.5 and 1977.9 µg kg-1 were recorded in barley samples, followed by ENN B1 and A1. MON was detected only in 2010 and 2011, with incidence rates of 10.1% and 15.5% and a mean concentration of 5.1 to 45.3 µg kg-1. However, No BEA or ENN A was detected in this study. Analysis of the seasonal and regional distribution from 2007 to 2011 showed significant interactions between year and region, year and crop and year and variety in the levels of ENN A1, B1 in samples collected from five regions of the UK (Scotland, South, North, Midlands and East of England). There were significant differences between years in the level of ENN B which the highest was in 2007 in the East of England. MON was analysed in 2010 and 2011, and revealed significant interactions between region and crop, and between crop and variety which was higher in the Midlands. The findings can support the call by the European Food Safety Authority (EFSA) to establish international legislation for these mycotoxins in cereals and their products in a bid to protect humans and animals from potentially associated harmful effects.

Keywords: Emerging Fusarium mycotoxins, Enniatins mycotoxin, Malting barley, liquid chromatography-MS/MS

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1-Introduction

BEA, ENNs (A, A1, B and B1) and MON are a group of secondary metabolites produced by *F. avenaceum* and *F. tricinctum*; the former is also produced by *F. poae* (Gruber-Dorninger, 2017). These mycotoxins have been identified as 'emerging' because of an increased risk of consumer exposure to them via the cereal processing chain (Santini *et al*., 2012). Thus, in 2009, the EFSA launched a call to monitor these mycotoxins and provide data on their occurrence in food and feed products (Verstraete, 2009). Consequently, the ENNs A, A1, B and B1 and BEA and MON from the group of *Fusarium* mycotoxins have received more research interest in the last decade than they had previously. ENNs and BEA are bioactive compounds defined by their cyclic hexadepsipeptide structure, and they elicit a wide range of toxicological effects (Gruber-Dorninger *et al*., 2017). They have similar toxic actions because of their ionospheric properties, and in vitro studies have demonstrated that ENNs and BEA can display additive or synergistic cytotoxic effects in several cell lines because of their similar chemical structures (Juan-Garcia *et al*., 2015). The primary toxic action of ENNs and BEA results in the formation of stable lipophilic complexes with essential mono- and divalent (Ca^{2+}, Na^{+}, K^{+}) cations and their transport through biological membranes to disrupt normal physiological concentrations (Escrivá *et al*., 2015). Furthermore, ENNs can change monovalent ion transport across membranes and disrupt the ionic selectivity of cell walls, resulting in debilitating mitochondrial membranes and consequent uncoupling of oxidative phosphorylation (Manyes et al., 2014). The principal action of MON is the inhibition of thiamine pyrophosphatase–dependent enzymes, such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, which impair the tricarboxylic acid cycle and consequently reduce the cellular energy supply (Gruber-Dorninger et al., 2017). Interestingly, the European Commission (EC) has not yet set legislative limits for BEA, ENNs and MON, although there are studies showing the presence of this group of emerging toxins in barley grain. For instance, ENNs A1, B and B1 were detected in 52 barley samples with an incidence of up to 100%. ENN B was detected with the highest concentration of over 1.40 mg kg^{-1} in the Czech Republic (Bolechová et al., 2015). In 30 barley samples from Germany, the incidence rates of BEA and ENNs A, A1, B and B1 were 13%, 33%, 60%, 83% and 93%, respectively, while the concentration of ENN B reached up to 60 mg kg-1 (Habler and Rychlik, 2016). In contrast, in warmer Mediterranean countries, ENN A1 is the most common mycotoxin in barley grain. For example, in Morocco, approximately 87.5% of barley samples were contaminated with ENN A1, B and B1, and 50% with BEA. ENN A1 was detected in the highest concentration of 200 mg kg^{-1} (Zinedine et al., 2011). ENNs A, A1, B and B1 were found in 80% of Tunisian barley samples, with the highest concentration of ENN A1 up to 149.0 mg kg^{-1} (Oueslati et al., 2011). Von Bargen et al. (2012) used a SAX column to clean up isotopically labeled $13C_2$-MON standard samples and single mass transition in the MRM/MSM to detect MON in wheat and co-products, reporting a LOD of 0.7 µg kg^{-1} and a LOQ of 2.5 µg kg^{-1}. Recently, *F. avenaceum* was isolated with an incidence of up to 100% from more than 200 samples of commercial barley grain in the UK (Nielsen et al., 2014). However, BEA, ENNs and MON were not quantified in those samples. Therefore, it is of value to determine the occurrence and concentrations of these

mycotoxins in UK barley to obtain data on potential consumer exposure. The main objectives of this study were as follows: i) to develop an LC-MS/MS assay for the detection and quantification of BEA; ENNs A, A1, B and B1; and MON in barley; and ii) to use this method to determine the incidence and quantity of BEA, ENNs and MON in barley grain collected between 2007 and 2011 from different regions in the United Kingdom.

2. Materials and methods

2.1 Chemicals and reagents

Organic solvents used for mycotoxin extraction and LC-MS/MS analysis (HPLC grade acetonitrile and methanol) were purchased from Sigma-Aldrich based in Steinheim, Germany. Anhydrous magnesium sulphate (with 99% purity), sodium chloride, ammonium formate solution and formic acid (>98%) were purchased from the same company. The certified standard materials of BEA; ENNs A, A1, B and B1; and MON were purchased from the New York–based company Alexis Biochemicals (with a purity level of 99%). All solvents were filtered through a cellulose filter of 0.2 µm (sourced from Membrane Solutions, Plano, TX, USA) before use. Whatman No. 1 type filter papers (Whatman Article No. 28413917, Sigma-Aldrich) were used to filtrate the extracted samples.

2.2 Preparation of standard solutions of mycotoxins

All stock solutions of BEA and ENNs A, A1, B and B1 standards were prepared individually. We had five bottles of mycotoxin standard materials; each mycotoxin bottle contained 1 mg of powder. We added 1000 µl of ACN (HPLC grade) to each mycotoxin bottle, placing them in a vortex for a few seconds before reaching a concentration level of 1 mg ml-1 per mycotoxin bottle (equivalent to 1000 µg 1000 μl^{-1}). Mixed mycotoxin standard (BEA and ENNs A, A1, B and B1) with a concentration of 10 µg ml^{-1} was used for the preparation of standard curves of mycotoxins with 4-fold series dilution ranging between 4.0 - 0.0039 µg ml^{-1}.

2.3 Malting barley grain samples

A total of 223 samples of commonly grown varieties of malting barley were previously collected from UK fields during the harvest seasons between 2007 and 2011 as part of the SAFEMalt project (Nielsen et al., 2014). The survey samples were barley flour and packed in polyethylene bags with a label describing each sample, which were stored in the freezer at a temperature of -20 °C until use.

2.4 Extraction method and analysis of BEA and ENNs by LC-MS/MS

An extraction method for (BEA and ENNs A, A1, B and B1) used a mixed solvent consisting of 10 ml of 0.1% formic acid HCOOH and 10 ml ACN added to 5 g of a milled grain sample in a 50 ml Flacon bottle, and shaken in the FastPrep 5G system (MPBio, USA) for 2 minutes, and 1 g of NaCl and 4 g of MgSO4 were added to the mixture and centrifuged at 5000 rpm for 5 minutes. Without disturbing the pellet, 1 ml of the supernatant was transferred to a clean HPLC vial. For the detection and quantification of the five target mycotoxins (BEA and ENNs A, A1, B and B1), the LC-MS/MS analysis was performed on an Agilent 1200 Infinity LC system (Agilent Technologies, Germany) with a binary pump, coupled with the Agilent 6490 MS/MS ESI (Agilent Technologies). The chromatographic separation of BEA and ENNs was conducted at 24 ± 1 °C on a reverse phase C18 column OOG-4252-40 (5 µm 250 × 3.0 mm). The mobile phase consisted of 0.1% (v/v) formic acid and 1 mM ammonium formate in methanol. An isocratic pump system was used to provide a mobile

phase with a flow rate of 0.6 ml min^{-1}. The injection volume was 10 µl, and the total run time was 5 minutes. For the mass spectrometer, the ESI interface was used in positive ion mode with a source temperature of 100 °C, desolvation temperature of 450 °C, cone nitrogen gas flow of 60 L/h, desolvation gas flow of 450 L/h and capillary voltage of 3.5 kV. BEA and ENNs A, A1, B and B1 were analysed in MRM (Table 1).

2.5 Sample preparation and LC-MS/MS analysis for MON

A total of 160 samples collected between 2010 and 2011 were analysed for MON determination. MON was analysed in only 89 samples from 2010 and 71 samples from 2011, as these were the most recent years with a greater number of samples than those available for 2007, 2008, and 2009, as well as limitations. Twenty-five grams of barley flour samples were added to 100 ml of ACN/H2O (84:16 v/v), which was mechanically shaken at 100 rpm for 30 minutes. The extract was then filtered through filter paper (Whatman No. 1), and 5 ml of this filtrate was passed through MycoSep 240 clean-up columns (Romer Labs®, Inc., Union, USA, RL082416), forcing the extracts to filter upwards through the packing material of the column. One milliliter of the purified extract was transferred to the HPLC vial. LC-MS/MS, the mobile phase consisted of water buffered with the following: (i) 100 mM ammonium formate (pH 6.4) and (ii) ACN delivered at 0.5 ml min-1 with isocratic elution. The total run time was 4 minutes. The ESI interface was used in negative ion mode with the following parameters: source temperature, 100 °C; desolvation temperature, 450 °C; cone nitrogen gas flow, 60 L h-1; desolvation gas flow, 450 L h-1; and capillary voltage, 3.5 kV. MON was analysed in MRM mode (Table .1).

2.6 Variation during sample recovery (%), efficiency, primary sample extraction and intra-day quantification

The efficiency, accuracy, and recovery of BEA; ENNs A, A1, B and B1; and MON were verified in the validation process. Samples were spiked with three different concentrations of 50 µg kg-1,100 µg kg^{-1} and 200 µg kg^{-1} of BEA, ENNs and MON to validate the extraction procedure and LC-MS/MS method from spike recovery experiments for each of BEA; ENNs A, A1, B and B1; and MON, and are important methods for validating and assessing the accuracy of analytical techniques for particular sample types. The accuracy of the method was evaluated as a percentage of recovery, and precision was verified by three determinations on the same day (repeatability). Recoveries were calculated as the ratio of concentrations detected in spiked sample contents, and the arithmetic mean ± standard deviation (SD) was calculated. (Table 1). Sensitivities for BEA, ENNs and MON were assessed by identifying the LOD and LOQ. BEA, ENN and MON concentrations in samples were calculated based on plotted external standard calibration curves.

2.7 Statistical analysis

Summary statistics for each mycotoxin were calculated using Excel (Microsoft Office, 2003). The LOQ was determined as five times the baseline noise (signal/noise) and calculated to be 2 µg kg^{-1} for ENNs A1 and B and MON and 4 µg kg^{-1} for ENN B1. The calculated statistics included the percentages greater than 2 µg kg^{-1} for ENN B and A1, 3 µg kg^{-1} for BEA and ENN A and 4 µg kg^{-1} for MON; mean; 95th percentile; and maximum concentration for each mycotoxin detected. Undetected samples were assigned half the LOQ value in the calculation of mean values, which resulted in 0.4 for ENNs A1 and B and

MON and 0.8 µg kg^{-1} for ENN B1. To model the mycotoxin concentration of samples, samples with a mycotoxin concentration below the LOQ were assigned a value of (LOQ)/2, log10 transformed and analysed using a normal distribution. Analysis of an unbalanced design using Genstat® Version 14.1 for Windows (VSN International Ltd., UK) regression was carried out on mycotoxin data from 2007 to 2011 to determine the significance ($p< 0.05$) of sampling year, region, crop and malting barley variety. For regression analysis, samples below the LOQ were removed from the dataset and concentrations log10 transformed to normalise the variance.

3. Results

3.1 LC-MS/MS Method by developing new separation conditions for detection and quantification of BEA, ENN A, A1, B, B1, and MON.

The results of new separation conditions showed that BEA and ENNs A, A1, B and B1 were separated in the higher peaks and response values in the positive ionisation mode as a result of the formation of [M + NH4]+, which was fragmented between the cone and hexapole 1 to [M + H]+. The collision energies optimised for the main daughter ions of BEA and ENNs B, B1, A1 and A were 244.0, 196.1, 196.1, 210.2 and 210.3, respectively, based on RT (Table .1). This method was given a short RT between 2.57 and 2.92 minutes with baseline separation of the five compounds of BEA and ENNs A, A1, B and B1 (Figure 1). Although the RTs were very close between all mycotoxins, the separation remained apparent with a high resolution. Five compounds were recovered linearly on all three occasions, with little variation between different experiments and R^2 values ranging from 0.982 to 0.996 (Table 2). The specificity and selectivity of the BEA and ENNs depended on the chromatographic RT and the MRM transition. The peaks of these mycotoxins in the samples were confirmed by comparing the RT of the peak with the RTs of pure standards at a maximum tolerance of ±0.2 minutes. The sensitivity of the method was evaluated by the LOD and LOQ, which were in the range of 0.1 to 0.8 and 2.0 to 4.0 µg kg-1, respectively (Table 2). The accuracy of the method expressed as a recovery of each toxin was evaluated at three spiking levels (low level: 50 µg kg-1, intermediate level: 100 µg kg-1, high level: 200 µg kg^{-1}). MON was detected in the negative ionisation mode as [M - H]- ion, which was fragmented between the cone and hexapole 1 to [M - H]- (Table 1). The collision energy was optimised for the main MON daughter ion, which was 41.2 (Table .1). The liquid chromatography method of MON started with the development of two types of mobile phases as follows: (i) water buffered with 100 mM ammonium formate (pH 6.4) and (ii) ACN (HPLC grade). There was a shorter RT of no more than 3 minutes with baseline separation of MON, where the peak was confirmed by comparing the RT of the peak with the RTs of a standard level at the maximum tolerance at 2.47 minutes (Figure 1).

3.2 Incidence and concentration levels of ENN A1, B, B1 and MON in UK barley

BEA and ENN (A, A1, B and B1) mycotoxins were analysed in 223 malting barley samples from between 2007 and 2011, whereas MON was analysed in 89 samples in 2010 and 71 samples in 2011 (Table 3). The incidence rates of ENN A1, B and B1 in the analysed samples, ranged from 3.4% for ENN A1 to 100% for ENN B. The highest concentration level of ENN B was detected in 2009, with a mean concentration of 3498.0 µg kg^{-1}, followed by mean concentrations of 3177.2, 3072.9, 1977.9 and 1940.5 µg kg^{-1} in 2008,

2007, 2010 and 2011, respectively (Table 3). ENN B1 showed a 100% incidence rate in 2007, where 41.6% of samples had a concentration of over 150 µg kg^{-1}, while the mean and maximum concentrations were 212.9 and 843.4 µg kg^{-1}, respectively. MON was only detected in 89 samples from 2010 and 71 from 2011; these were more recent years and represented a greater number of samples than were taken in 2007, 2008 and 2009. Malting barley samples were contaminated with MON at incidence rates of 10.1% and 15.5%, with mean concentrations of 45.43 and 80.53 µg kg^{-1} in 2010 and 2011, respectively (Table 3).

Table (1): Optimized ESI-MS/MS parameters including Precursor ion, Retention time (RT), Cone voltage (V), Product ion 1, Collision energy (eV), Product ion 2 and Collision energy (eV) for BEA, ENNs and MON

mycotoxin Compound	Precursor ion	RT (min)	Cone voltage (V)	Transition (m/z)		Relative Collision energy	ESI interface	Linearity R^2
BEA	784.4	2.57	50	784 →	244	25	Positive	0.986
ENN B	640.4	2.67	50	640 →	196.1	25	Positive	0.994
ENN B1	654.4	2.74	50	654 →	196.1	30	Positive	0.982
ENN A1	668.5	2.84	50	668 →	210.2	27	Positive	0.985
ENN A	682.5	2.92	50	682 →	210.3	27	Positive	0.982
MON	97.0	2.47	50	97.0 →	41.2	12	Negative	0.996

● Product ion resulting from the fragmentation of precursor ions of a specific mass-to-charge ratio (m/z). Collision energy (CE) is an instrument parameter that is frequently optimized to increase fragment ion intensity, which is a key parameter determining the information content of beam-type collision-induced dissociation tandem mass spectrometry (MS/MS) spectra.

3.3 Modelling of seasonal and regional distributions and concentrations of ENN A1, B, B1 and MON in UK barley.

The statistical modeling aimed to determine the effect of agronomic factors on the concentrations above the LOQ of the ENN mycotoxin in UK malting barley collected from 2007 to 2011 and MON from samples collected in 2010 and 2011. The agronomic factors entered for selection were as follows:

- Year
- Region
- Variety

The impact of practice on the ENN A1, B and B1 and MON concentrations were analysed from logarithmic-transformed values, grouped by year, in an unbalanced design using Genstat regression. Figures (3 – 6) show the $\log_{10}$ of ENN A1, B and B1 and MON concentrations for each significant factor or interaction for the predicted means.

For ENN A1 and B1, there were significant interactions between year and region, between year and previous crop, and between year and variety. There was a highly significant interaction between year and region ($p< 0.001$), with a greater level of ENN A1 in the eastern part of the United Kingdom in 2007 (Figure 3A). The predicted mean concentration of ENN A1 of the positive samples was 46.37 µg kg^{-1}. However, overall trends of much lower ENN A1 were found towards the Midlands, the North and the South of the United Kingdom, as well as Scotland, with a consistent reduction trend in the regions in 2008, 2009, 2010 and 2011 (Figure 3A). For ENN B1, there was a significant interaction between year and region ($p= 0.032$), with no consistent trend across year or region with higher concentrations detected in the East. The predicted mean concentration of ENN B1 of

the positive samples was 300.26 µg kg^{-1} (Figure 3A). There was a highly significant interaction between year and variety in ENN A1 ($p< 0.001$) and lesser in ENN B1 ($p= 0.037$), which was also higher in variety cv. Pearl in 2007 (Figure 4B). In statistical modeling of ENN A1, there were significant differences between regions ($p< 0.001$) and variety ($p< 0.001$), with higher levels in the East of England and variety cv. Pearl (Figure 5A and 5B). In contrast, there appears to be a trend towards increasing amounts of ENN B in England during the 5 years of sampling (Figure 6A). Since there is no previous data for ENNs mycotoxins in UK barley, it is not possible to determine whether the high level

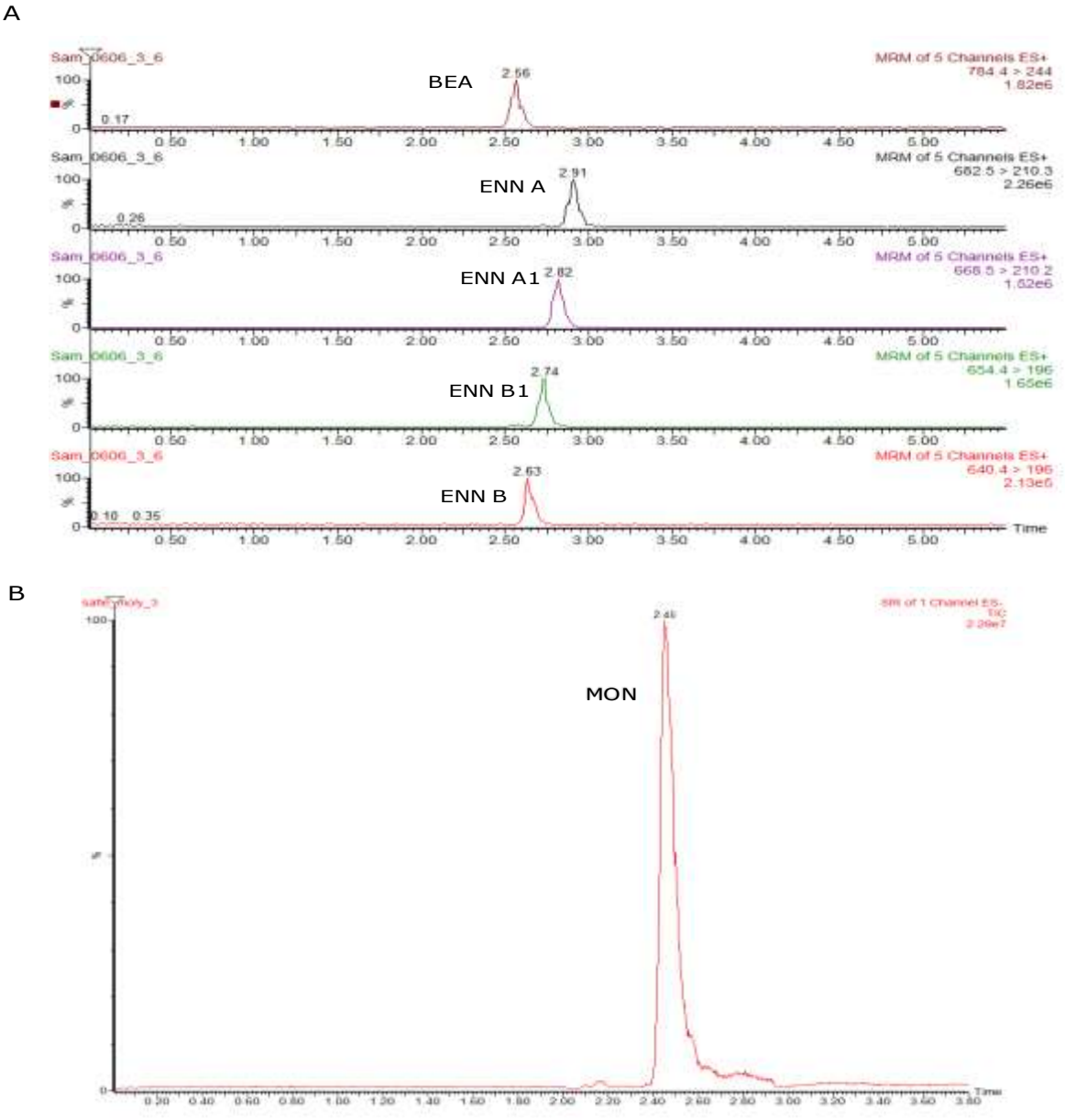

Figure (1): Selected ion recording (SIR) chromatograms of BEA, ENNs and MON on LC-MS/MS; chromatogram (A) represented as mixed mycotoxin standard of BEA and ENN A, A1, B and B1. (B) MON standard. The x-axis shows chromatographic time (min). The ion mass monitored for each of the five mycotoxins is shown in the upper right of each trace along with the maximum signal height. Each peak is annotated with the name, retention time and peak area of the compound

of ENN B is a recent occurrence where the survey samples were collected from 2007 to 2011, whereas in MON concentrations , there was a significant interaction between region (p= 0.011), with higher concentration in the Midlands of England compared to the rest of region (Figure 6A).

Table (2): Validation parameters of LC-MS/MS used for the detection and quantification of BEA, ENNs (A, A1, B and B1), and MON.

Mycotoxin	Spike level (µg kg^{-1})	Mean recovery (%) ±SD	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
Beauvericin	50	87±6.0	0.3	3.0
	100	92±8.0		
	200	94±2.0		
Enniatin B	50	86±8.0	0.6	2.0
	100	94±8.0		
	200	99±4.0		
Enniatin B1	50	89±3.0	0.8	4.0
	100	95±2.0		
	200	98±2.0		
Enniatin A1	50	88±10.0	0.1	2.0
	100	94±5.0		
	200	99±3.0		
Enniatin A	50	86±4.0	0.4	3.0
	100	94±2.0		
	200	96±2.0		
Moniliformin	50	83±7.0	0.9	2.0
	100	85±6.0		
	200	89±4.0		

Limit of detection (LOD); limit of quantification (LOQ); Standard deviation (SD)

Table (3): Concentration of enniatins A1, B, B1 and MON in UK malting barley samples collected between 2007 to 2011, described by mean, 95% confidence interval and maximum detected values.

		Mycotoxin concentrations µg kg^{-1}															
		ENN A1				ENN B				ENN B1				MON			
Year	N	% incidence	Mean	% 95th	Max	% incidence	Mean	% 95th	Max	% incidence	Mean	95th %	Max	% incidence	Mean	% 95th	Max
2007	12	41.7	125.1	56.3	264.3	100	3072.9	1634.8	10105.4	100	212.9	147.3	843.4	NQ	NQ	NQ	NQ
2008	30	30.0	36.4	0.1	136.3	100	3177.2	1093.8	9702.1	60.0	76.9	26.1	171.1	NQ	NQ	NQ	NQ
2009	21	14.3	13.3	3.5	21.2	100	3498.0	1973.4	16696.5	52.4	39.2	22.1	148.4	NQ	NQ	NQ	NQ
2010	89	3.4	6.1	0.5	9.0	68.5	1977.9	664.3	12972.2	19.1	43.7	12.9	260.1	10.1	45.3	1.9	194.40
2011	71	4.2	5.1	0.4	6.0	78.9	1940.5	668.2	12084.1	16.9	16.5	3.1	40.4	15.5	80.53	28.4	429.80

N: number of samples; LOQ: ENN A1 = 2.0 µg kg -1, ENN B = 2.0 µg kg -1, ENN B1 = 4.0 µg kg -1, MON= 2.0 µg kg -1. The limit of quantification (LOQ) was determined as five times the baseline noise (signal/noise) and calculated to be 2 µg kg for ENN A1, B and MON, and 4 µg kg for ENN B1. Mean based on a value of LOQ/5 (0.4 for A1, B, MON) and LOQ/5 (0.8 µg kg-1 for ENN B1), Number of samples (n). Not quantified (NQ).

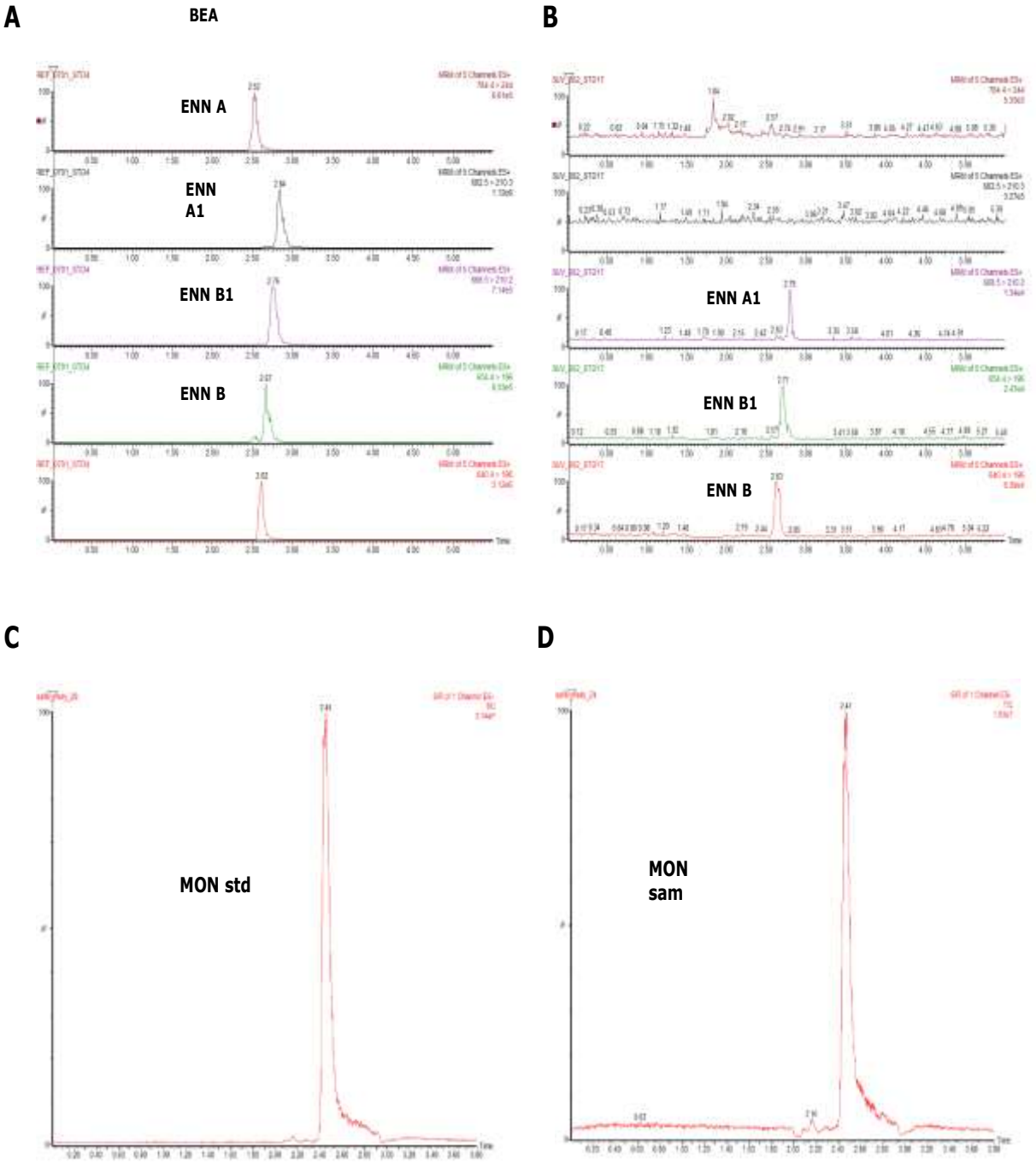

Figure (2): Selected ion recording (SIR) chromatograms of BEA and ENNs; (A) mixed mycotoxin standard of BEA, ENN A, A1, B and B1 with chromatographic time (min). (B) positive sample contaminated with ENN A1, B1 and B (C) standard of MON with chromatographic time (min). (D) positive sample contaminated with MON. Each peak is annotated with the name, retention time and peak area of the compound.

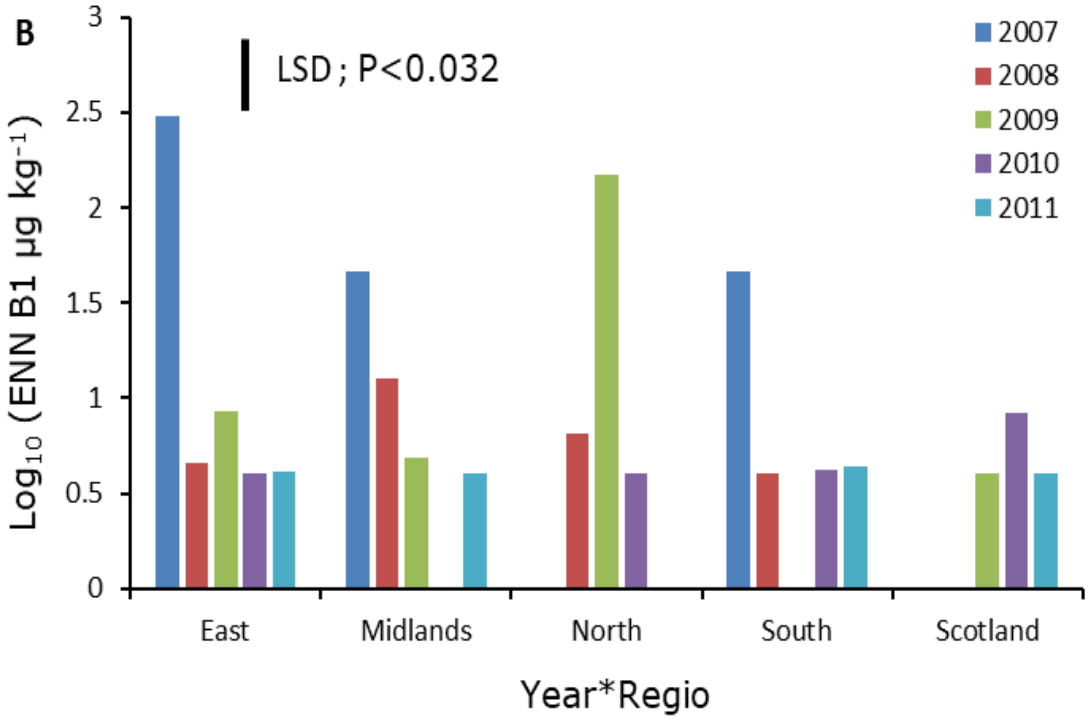

Figure (3): Regional and seasonal variations of enniatins concentrations (A) ENN A1 and (B) ENN B1, described as log10 of concentration (µg kg-1) in UK malting barley collected from 2007 to 2011. A) highly significant interaction ($p<0.001$, n= 116) between year and region in ENN A1 with higher concentration in the East of England compared to the rest of the region. B) a significant interaction ($p=0.032$, n= 116) between year and region in ENN B1 with higher concentration in the East in 2007.

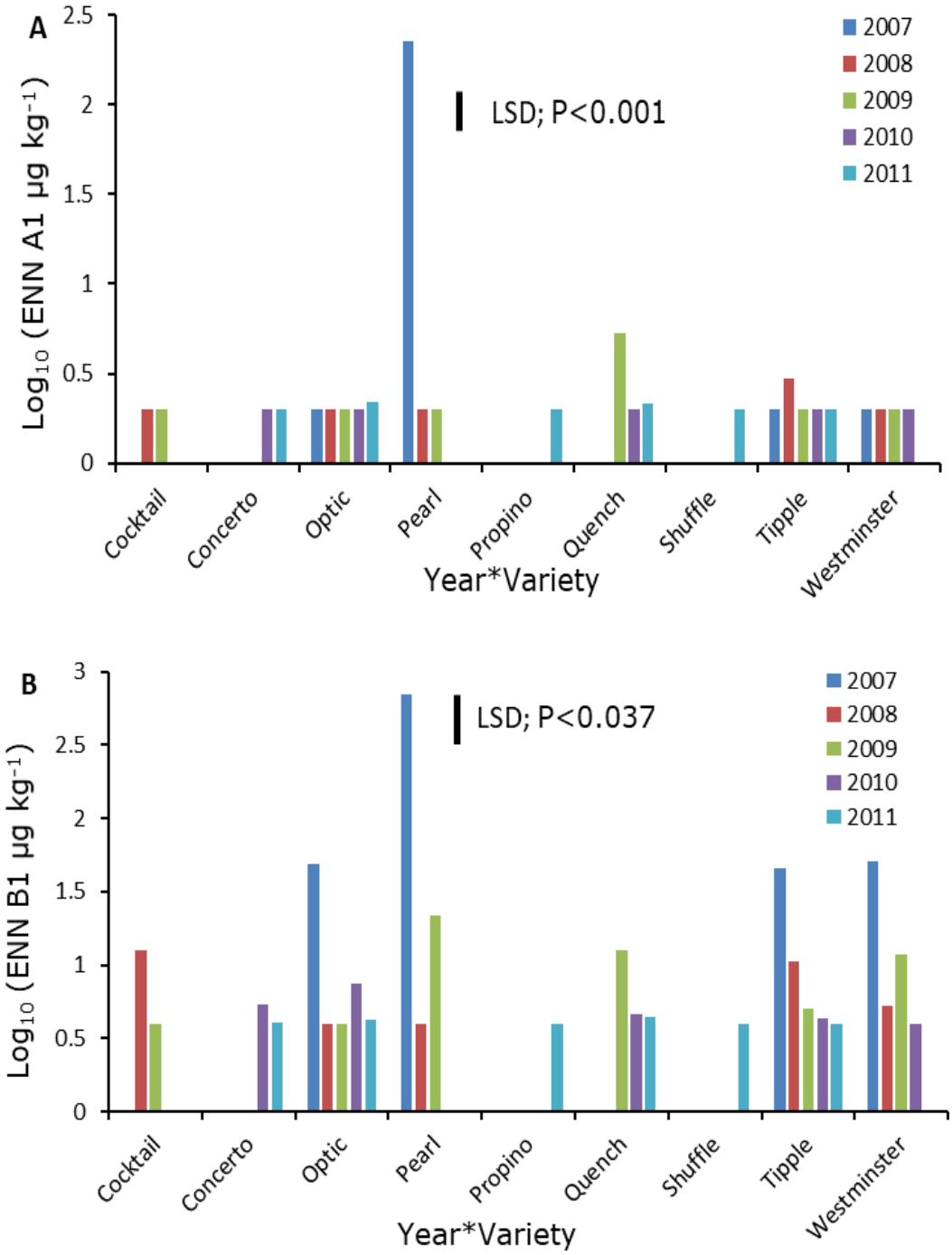

Figure (4): Seasonal variation and crop difference on enniatins concentration in previous crops of UK collected from 2007 to 2011, described as log10 of ENN A1, B and B1 concentrations (µg kg-1). A) highly significant interaction ($p<0.001$, n= 116) between year and crop in ENN A1 when previous crops are set aside and spring barley in 2007 to 2011. B) a significant interaction ($p=0.023$, n= 116) between year and crop in ENN B1 when previous crops were set-aside and spring barley from 2007 to 2011.

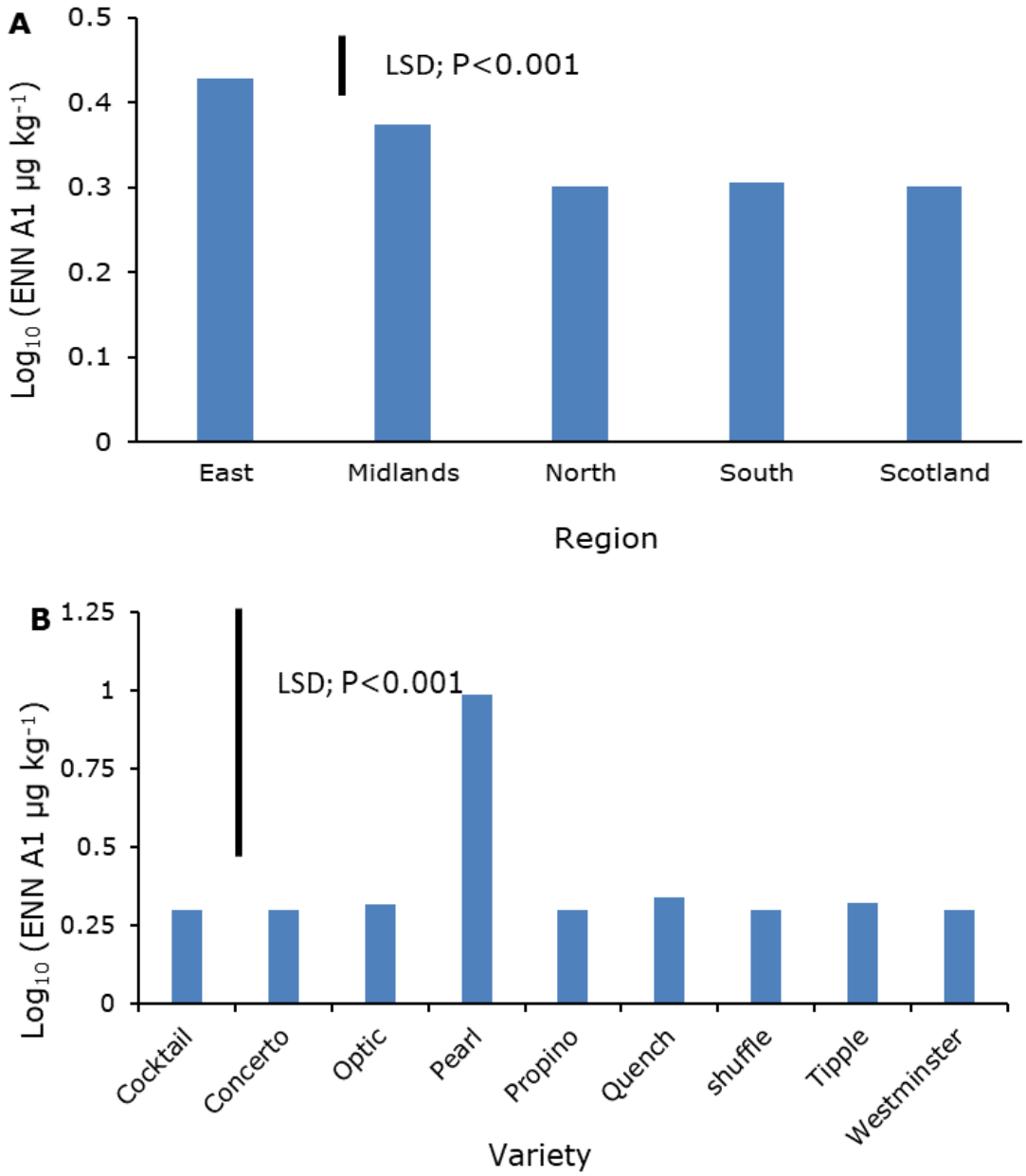

Figure (5): Regional variation and varietal difference of ENN A1 in varieties of UK barley, described as log10 of ENN A1 concentration (µg kg-1). A) highly significant difference ($p<0.001$) between regions in ENN A1; with higher concentration in the East of England compared to the rest of the region collected from 2007 to 2011. B) highly significant difference ($P=0.001$) between varieties in ENN A1 with higher concentration in Pearl compared to the rest of the varieties from 2007 to 2011.

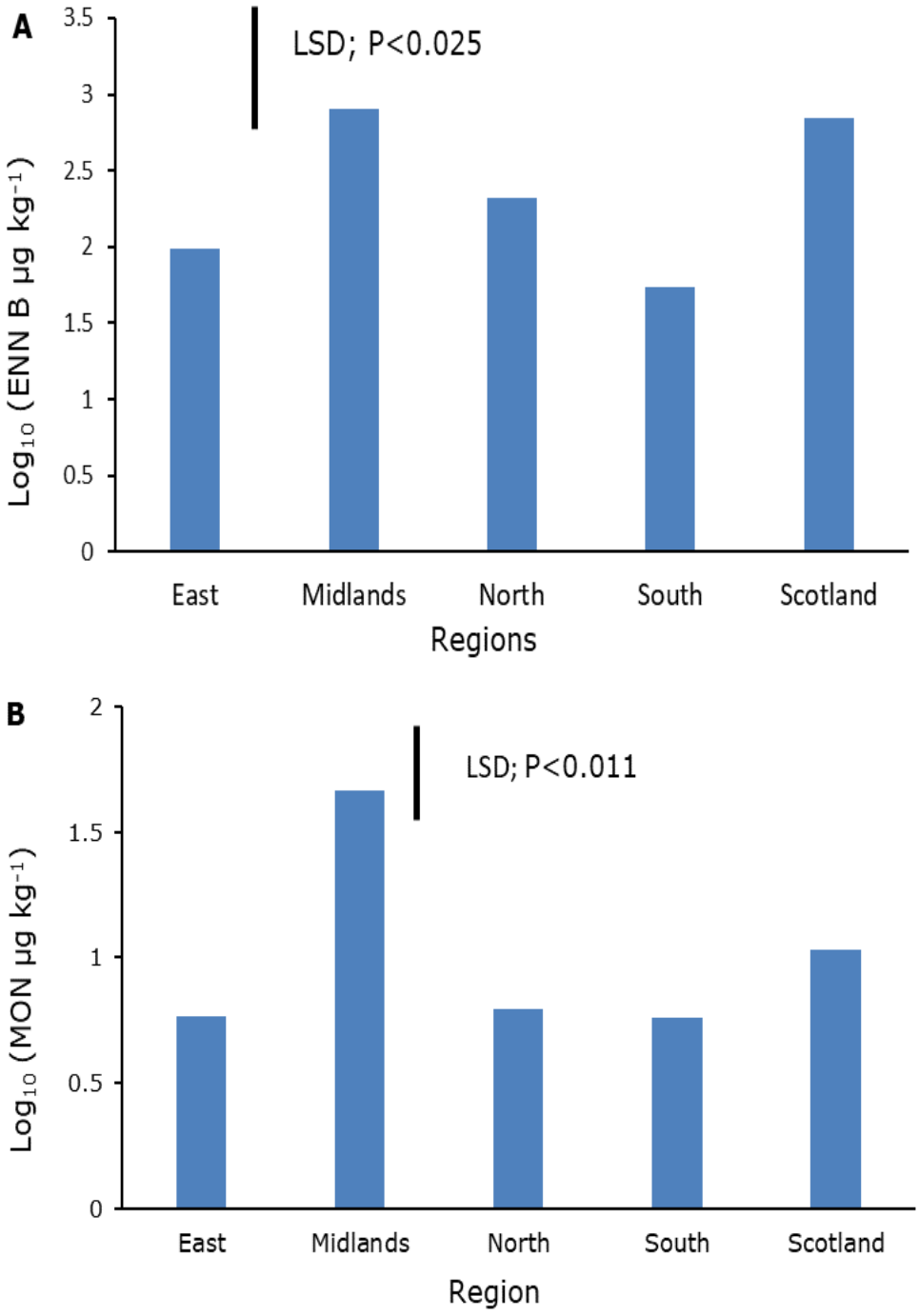

Figure (6): Regional variation of ENN B and MON described as log_{10} of ENN B concentration (µg kg^{-1}) from 2007 to 2011 and 2010 to 2011 of MON in UK. A) significant difference (p=0.025) between regions with higher concentration in the Scotland and Midlands of England compared to the rest of the region. B) significant difference (p=0.011) between regions with higher concentration in the Midlands of England compared to the rest of the region

4. Discussion

In this study, new separation conditions were developed and validated using a sensitive, specific and reproducible method for the simultaneous detection and quantification of BEA, ENNs and MON in UK barley samples using LC-MS/MS, although sensitivities vary due to different extraction methods and chromatography analyses, such as LC-DAD (Zinedine et al., 2011), LC-MS/MS (Habler and Rychlik., 2016), UPLC-MS (Bolechová et al., 2015) and LC-QTRAP -MS/MS (Tolosa et al., 2019) as well as UHPLC with the isotopically labeled $^{13}C_2$-MON standard (Von Bargen et al., 2012) and (Scarpino et al, 2013). To our knowledge, this is the first report on the presence of BEA and ENN mycotoxins in commercially grown malting barley in the United Kingdom. The results of our study indicate a high incidence of ENN B, followed by ENN B1 and A1, while BEA and ENN A were not detectable or were below the LOQ. These results are consistent with previous reviews conducted by Santini et al. (2012), who reported the highest levels of ENN B contamination in barley grain in Europe. Climate conditions are among the most notable factors influencing the occurrence and distribution of *Fusarium*. As a result of warm and moist conditions, especially during the period of anthesis, are considered critical factors for FHB disease development. Countries in the Mediterranean region, such as Morocco (Zinedine et al., 2011), Tunisia (Oueslati et al., 2011) and Spain (Meca et al., 2010), have high concentration levels of ENN A1 in barley. In Spain, mycotoxin contamination in barley was investigated by Meca et al. (2010), who found BEA and ENN A1, B and B1 at concentrations of 4870, 148200, 21400 and 46000 µg kg^{-1}, respectively, whereas ENN A was not detected. In Morocco, Zinedine et al. (2011) detected ENN A1, B and B1 in barley with mean concentrations of 84000, 18000 and 22000 µg kg^{-1}, respectively, but no ENN A was detected. In Tunisia, barley samples were contaminated with ENN A, A1, B and B1 at concentrations of 33600, 116400, 27500 and 31000 µg kg^{-1}, respectively (Oueslati et al., 2011). Concentrations of ENNs (ENN A1 > B > B1 > A) and concentrations in barley samples in these Mediterranean countries are much higher and different from the ENNs (ENN B > B1 > A1) reported in our results. In cooler regions, higher contents of ENN B have been reported compared with those reported in warmer zones. This could be explained by variations in climatic conditions, which may favor the proliferation of different *F. avenaceum* isolates or other *Fusarium* spp., which produce specific ENNs. Our study also indicated that the concentrations of MON in barley were much lower than those of ENNs in both 2010 and 2011. This agrees with the study conducted by Jestoi et al. (2004) in Finland, who found MON in barley with an incidence rate of 74% and maximum concentration of up to 290 µg kg^{-1}. Similarly, in Norway, MON in barley at the maximum concentration was 380 µg kg^{-1}, while the incidence was 30% in 2001 (Uhlig et al., 2004).

For the modeling of ENNs A1, B and B1 and MON concentrations of barley samples against year, region, previous crop and variety, regional and seasonal variation of ENN A1 and B1 across the United Kingdom showed higher ENN A1 and B1 towards the east of the United Kingdom in 2007, although the distribution of ENN B1 was very different across all regions because the level fluctuated inconsistently between

seasons and regions, a phenomenon that can be attributed to cooler temperatures. This was also probably due to fluctuations in weather between years and regions. The relative degree of mycotoxin contamination between cereals will vary from year to year and among regions, depending on climatic conditions, when each host species is at a susceptible growth stage (Edwards, 2017). Moreover, the higher ENN A1 and B1 identified in the East could be attributable to the higher intensity of cereals in rotation. Moreover, this is also probably due to fluctuations in weather between regions, particularly differences in rainfall distribution across the regions in the UK, which could be higher in the East before harvesting during June or July. Here, the rationale is that greater rainfall in June or July may result in the barley harvest extending longer into August. Crop debris is an important parameter of Fusarium ear blight epidemiology, in terms of the type and amount of crop debris (Edwards, 2007). There were significant interactions between years and varieties of barley that were included in our studies concerning to the levels of ENNs A1 and B1 and MON present. Modeling of ENN B concentration of barley samples showed significant seasonal variation from 2007 to 2011, where the highest levels of ENN B were found in 2007 and 2008 in spring barley, which gradually decreased from 2009 to 2011; this can be attributed to different climate conditions between years. The seasonal differences in the mycotoxin composition of the *Fusarium* spp. can potentially be explained by the differences in their environmental requirements for growth and infection and climatic differences in the different regions. This would indicate that the ENN-producing *Fusarium* spp. probably have different environmental requirements; for instance, *F. avenaceum*—the most potent ENN producer—is known to have an optimum temperature for sporulation and growth of approximately 20 °C (Popovski et al., 2013), whereas infection occurs between 10 °C and 25 °C (Xu et al., 2007). Moreover, this is probably due to the development of using agricultural practices and recommended techniques to prevent diseases of crops throughout the years. The findings suggest that contaminated malting barley samples may have been produced from previously infected cereal crops or variable environmental conditions that favour the colonisation process. More importantly, the climate conditions during critical phases of barley plant growth could lead to noticeable differences in mycotoxin diversity (Piacentini et al., 2019). Hence, with environmental conditions in years of high rainfall and *F. avenaceum* infection, it is likely that ENNs or MON will be found in barley, and the relative degree of mycotoxin contamination between barley crops will vary between years and regions depending on the climatic conditions when each host species is in flower. This confirms that geographical factors, including climate, are of superior importance for the occurrence of FHB and for the pattern of infestation by various *Fusarium* spp. As Polišenská et al. (2020) found, there was an association of a higher occurrence of ENNs with the harvest year characterised by the highest rainfall and lower temperature, where ENNs were more abundant in the colder and wetter areas. By the same token, Gautier et al. (2020) found that the producing species, mainly *F. avenaceum* and *F. tricinctum* in European areas, are characterised by a broad specificity of plant hosts, which certainly results from a high potential to adapt to diverse environments. Extensive studies published in the past decade have shown that

mechanisms involved in the adaptation of fungi to their environment also govern the biosynthesis of mycotoxins. However, about the regulation of ENNs in *Fusarium*, there is still much to do to confirm the existence of these regulatory pathways evidenced for other toxigenic species and to highlight their interconnection with the regulation of mycotoxin biosynthesis. Thus, research efforts should be pursued to fill these knowledge gaps and provide tools to improve strategies aimed at controlling the levels of this class of mycotoxins in food and feed products. With crop rotation, different types of crops will succeed each other in the field to limit the recontamination of crops. For example, sowing *Fusarium*-prone crops after each other increases the chance of recontamination from the soil (Janssen et al., 2018). Although the frequency of fungal species in residues varied with plant species, similar *Fusarium* spp. were commonly isolated from all residue types (Fernandez et al., 2008). According to increasing evidence of the worldwide and frequent occurrence of crop contamination with ENNs, together with the potential risk of health hazards associated with chronic exposure and the likelihood of interacting toxic effects when ENNs co-occur with other EU-regulated mycotoxins, ENNs may pose a major threat to human and animal health (Gautier et al., 2020). No studies could be found on the effect of agronomic factors on ENN levels or how to minimise their contamination in cereal products. Further studies are necessary to determine the best agronomic practices to minimize the contamination of emerging *Fusarium* mycotoxins in cereal crops.

5. Conclusion

In the present study, we developed a fast, highly sensitive and accurate method with new conditions for the separation and simultaneous detection of BEA, ENNs and MON. This study is the first to report natural ENN A1, B and B1 contamination in malting barley between 2007 and 2011 and in MON between 2010 and 2011, during growing seasons from several UK regions with different climate conditions. Our results suggest that the risk of ENNs contamination could increase in cropping areas for growing seasons with higher rainfall and low temperatures during the cropping season. The presence of ENNs with other mycotoxins (DON, NIV, ZEA, T-2 and HT-2) can be mutually positively correlated, which can imply potential for the combined risk leading to simultaneous toxicological effects after consumption. Because Emerging mycotoxins are not currently regulated, they are not regularly monitored in cereals intended for food production. It will be of great value for the EFSA in terms of assessing the risks posed by these mycotoxins to human and animal health.

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الحدوث الطبيعي لسموم الفيوزاريوم الناشئة في حبوب الشعير

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المستخلص

سموم الفيوزاريوم الناشئة هي أحد أنواع السموم الفطرية التي إنتشرت مؤخرآ في الحبوب حول العالم. بيوفيرسين (BEA) Beauvericin، الأنياتينات (ENA,A1, B B1) Enniatins، ومونيلفورمين(MON) ، هي نواتج ثانوية يفرزها بشكل رئيسي بواسطة *F. avenaceum*. الذي يتبع جنس الفيوزاريوم (*Fusarium* spp)، وهي تلوث محاصيل الحبوب الغذائية (القمح، الشعير) ومنتجاتها في جميع أنحاء العالم. ركزا الباحثون مؤخرا على بيوفيرسين، الأنياتينات، ومونيلفورمين، ومخاطرها المحتملة على صحة الإنسان والحيوان. لذلك، هذه البحث أرتكز على عينات جمعت لدراستين استقصائية ما بين (2007 – 2011) لأصناف حبوب الشعير المزروعة في المملكة المتحدة، حيث كان العدد الإجمالي إلى (223) عينة. وبالتالي، للكشف والتقدير الكمي لسموم الفيوزاريوم الناشئة، تم تجهيز العينات (الإستخلاص والتنقية)، ثم استخدم جهاز التحليل الكروماتوجرافي السائل مزود بالكاشف الطيف الكتلة المزدوج (LC–MS/MS). أوضحت نتائج التحقق من الطريقة بإستخدام LC–MS/MS، نسبة إسترداد يتراوح بين 87%–99% مع انحراف معياري نسبي Relative standard deviation (RSD) بنسبة 10% للبيوفيرسين والأنياتينات، بينما مونيلفورمين كان في مدى 83 – 89%، مع انحراف معياري نسبي (RSD 7%). حدود الكشف كانت بين 0.1 –0.9 ميكروجرام/كيلوجرام لـ ENN A و MON. أظهرت نتائج أن ENB كان من أكثر سموم فيوزاريوم الناشئة إنتشارآ في عينات حبوب الشعير بنسبة 100%، وبمتوسط تركيزات من 3072.9 إلى 3498.0 ميكروجرام/ كجم في سنة 2007 و2009. في حين، 2020 و2011 كانت متوسط تركيز لـ ENB 1940.5 و1977.9 ميكروجرام/ كجم، تليها ENN B1 و A1. تم اكتشاف MON فقط في عامي 2010 و2011، بمعدلات حدوث 10.1% و15.5% ومتوسط تركيز 5.1 إلى 45.3 ميكروجرام/كجم. في المقابل، فأن تركيزات لكل BEA وENA كانت أقل من حدود الكشف في العينات (دون حد الكشف للجهاز) في هذه الدراسة. علاوة على

ذلك، أظهر تحليل التوزيع الموسمي والإقليمي من 2007 إلى 2011، تفاعلات كبيرة بين السنة والمنطقة، والسنة والمحصول، والسنة والتنوع، في مستوي تركيزات ENN A1 تركيزات و B1 في عينات الشعير المجمعة من خمس مناطق في المملكة المتحدة (اسكتلندا، الجنوب، الشمال، ميدلاندز وشرق إنجلترا). وكانت هناك فروق دات دلالة إحصائية بين السنوات في مستوى ENN B والتي كانت أعلى مستوى في عام 2007 في شرق إنجلترا. تم تحليل MON في عامي 2010 و2011، وكشف عن تفاعلات كبيرة بين المنطقة والمحاصيل، وبين المحاصيل والتنوع والتي كانت أعلى في منطقة ميدلاندز. هذه النتائج، تدعم ما أوصت به الهيئة الأوروبية لسلامة الأغذية(EFSA) ، للمساهمة في لوضع تشريعات دولية بشأن هذه النوع من السموم الفطرية الموجودة في الحبوب ومنتجاتها، من أجل الإنسان والحيوانات من التعرض للأثار الضارة المحتملة المرتبطة بها.

الكلمات المفتاحية: سموم الفيوزاريوم الناشئة – تلوث الشعير – التحليل الكروماتوجرافي السائل – فطريات الفيوزاريوم

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