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Influence of Mycotoxin Producing Fungi (*Fusarium*, *Aspergillus*, *Penicillium*) on Gluten Proteins during Suboptimal Storage of Wheat after Harvest and Competitive Interactions between Field and Storage Fungi

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Cereals contaminated by *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. and their mycotoxins, for example, ochratoxin A (OTA) and deoxynivalenol (DON), are not only a risk to human and animal health but can also show poor technological properties and baking quality. The influence of these genera on the sulfur speciation of low molecular weight (LMW) subunits of glutenin was characterized by investigating suboptimally stored wheat samples in situ by X-ray absorption near edge structure (XANES) spectroscopy and baking tests. Field fungi of the genus *Fusarium* have hardly any influence on both the sulfur speciation of wheat gluten proteins and the baking properties, whereas storage fungi of the genera *Aspergillus* and *Penicillium* have a direct influence. An increased amount of sulfur in sulfonic acid state was found, which is not available for thiol/disulfide exchange reactions in the gluten network, and thus leads to a considerably reduced baking volume. From changes of the composition of the mould flora during suboptimal storage of wheat and from the mycotoxin contents, it can be concluded that microbial competitive interactions play an important role in the development of the mould flora and the mycotoxin concentrations during (suboptimal) storage of wheat.

KEYWORDS: LMW subunits of glutenin; XANES spectroscopy; quantitative analysis; wheat gluten; sulfur K-edge; suboptimal storage of wheat; moulds; competitive interactions; mycotoxins

INTRODUCTION

The sulfur containing gluten proteins largely determine the baking quality of wheat and functional properties of doughs. These proteins have been classified by their molecular weight and amino acid sequences into sulfur-rich, sulfur-poor, and high molecular weight (HMW) groups. The low molecular weight (LMW) subunits of glutenin (B- and C-types) and the α- and γ-gliadins form the sulfur-rich group, whereas the ω-gliadins and the LMW subunits of glutenin (D type) form the sulfur-poor group (*1*−*3*). In gluten proteins, intermolecular disulfide bonds within the glutenins are responsible for the elasticity by forming high molecular weight aggregates, whereas intramolecular disulfide bonds of the gliadins contribute to the viscosity. Especially the number of subunits belonging to the low molecular weight group is strongly correlated to the extensibility of doughs and therefore to the formation of the gluten network via intermolecular disulfide bonds (*4*). Whereas the total cysteine content of gluten proteins can be determined by amino acid analysis (*5*) and the amount of free S−H and S−S groups can be determined spectrophotometrically (*6*, *7*), the sulfur speciation can be characterized in situ on the basis of native samples using X-ray absorption near edge structure (XANES) spectroscopy. XANES spectroscopy at the sulfur K-edge using synchrotron radiation has been successfully used to determine the speciation of sulfur in a broad variety of scientific topics, which has been recently reviewed by Prange and Modrow (*8*). A more detailed insight into the application of XANES to investigate gluten proteins can be found in detail in Prange et al. (*9*−*11*).

Wheat and flour quality can be seriously diminished by the infection of moulds leading to contamination with mycotoxins and lowered baking quality. Moulds of the genera *Fusarium*, *Alternaria*, and *Cladosporium* belong to the typical field fungi infecting plants on the field. During storage of wheat, field fungi

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are increasingly displaced by the typical storage fungi *Aspergillus* spp. and *Penicillium* spp., which occur as conidia already on the field but invade and damage grains almost exclusively during storage (*12*). Storage conditions are considered as safe, if grain moisture is ≤14% at a temperature of 20 °C and an equilibrant moisture between grains and air of 65% (*13*). Improper storage accompanied by too high temperature and elevated moisture content in the grains favors mycotoxin production (*14*−*16*). The production of mycotoxins is influenced by various parameters (e.g., temperature, a_w, and pH), and it can be assumed that it is influenced by microbial interactions as well. However, only very little is known about interactions of different moulds on growth, mycotoxin production, and competitive influences. In years of abnormally prolonged rainy periods during harvest, crops may have to be harvested at higher moisture contents than desirable. For example, in Germany at the time of harvest, moisture contents of 20% and more can be encountered in rainy years, whereas in Scandinavian countries moisture contents of 20−30% are described as normal (*17*). In such cases, it is necessary to dry the grain immediately after harvest, but this can be a problem as smaller farms often lack appropriate drying and storage facilities; thus, it can happen that grain has to be stored suboptimally for some time before being dried (*18*).

Whereas the mycotoxin deoxynivalenol (DON) as well as other trichothecenes are normally produced during the vegetation period, it can be also produced during suboptimal storage on grains (*12*, *15*, *19*). DON is produced by *F. graminearum* and *F. culmorum* and is the *Fusarium* toxin detected most frequently and in highest concentrations (*19*, *20*) and can be thus regarded as a "leading toxin" (*21*). Unlike DON, the cancerogenic ochratoxin A (OTA), which is produced by different *Aspergillus* spp. and two *Penicillium* species (*22*), is rated as a storage toxin and does normally not occur before harvest (*23*). Its levels can drastically increase in wheat stored under suboptimal conditions (*24*). From a technological point of view and considering the impact of fungi on the baking quality, it can be assumed that the interactions of moulds and protein matrix within the grain might reduce the technological properties. Whereas it is generally accepted that infection of wheat plants with *Fusarium* spp. leads to loss in grain weight and shriveled grains (*25*), some reports indicate that infection of grains with moulds, especially *Fusarium* spp., results in loss of baking performance and a reduction of loaf heights in baking tests (*26*, *27*). Interestingly, we recently found that strong *Fusarium* spp. infection of wheat plants and grains accompanied by high DON contents did not reduce necessarily breadmaking properties and did not influence the sulfur speciation of LMW subunits of glutenin (*28*). In a single wheat sample that was heavily infected with storage fungi (*Aspergillus* spp./*Penicillium* spp.), significant amounts of higher oxidation states (sulfoxide and sulfonate state) were detected in LMW subunits of glutenin and a drastic reduction in baking quality (loaf volume) (*29*).

To clarify the exact influence of storage and field fungi on gluten proteins and their mycotoxin production under defined suboptimal conditions systematically, the first aim of this study was to examine the sulfur speciation of LMW subunits of glutenin isolated from (suboptimally) stored wheat samples of different storage trials by XANES spectroscopy. Besides XANES spectroscopy, micro-baking tests were performed to investigate the moulds' influence on baking quality. The second aim of the study was to investigate the influence of suboptimal storage conditions on the mould flora as well as competitive interactions of fungi during storage of wheat in relation to mycotoxin levels.

MATERIALS AND METHODS

Wheat Samples, Storage Trials, and Culture Conditions. Winter wheat (cv. Drifter) samples grown under integrated farming conditions in the mid-western part of Germany (Rhineland) were investigated. Samples had a weight of 5 kg, and each sample consisted of four equal subsamples of 1.25 kg weight. The natural *Fusarium* spp. infection rate of the wheat samples was 2%. After harvest, 1 kg was immediately frozen for DON analysis, and the remaining sample of 4 kg was stored at 4 °C until storage trials were started. Storage trials were conducted in a climatically controlled chamber (Rubarth, Laatzen, Germany) where temperature and humidity could be adjusted. A temperature of 20 °C and grain moisture of 20% were chosen as suboptimal storing parameters. Grains were harvested with comparable moisture contents of 14 ± 1%. Using a standard curve, each wheat sample was moistened with sterile water to attain water contents of 20% respectively. Samples (2 × 2 kg) for the storage trials were shaken (60 rpm) in a home-built overhead shaker for 24 h to allow the added water to soak thoroughly into the wheat samples. Samples were then transferred into sterile polyethylene bags, which incorporated filters allowing gas exchange, but preventing conidia from leaving the bags ($SacO_2$, Gent, Belgium). To ensure constant moisture of 20% in the grain, the humidity of the air had to be 90%. The moistures of the grain corresponded to a_w values of 0.86 and 0.91. Grain moisture and a_w values were checked weekly and remained constant. The storage period was 12 weeks. Every second week, 200 g samples of the grain were taken for further investigations. The moisture content of the grains was determined by drying at 105 °C until the weight did not change further. The a_w values were checked in a hygroscope (Rotronic, Esslingen, Germany) at a temperature of 20 °C.

The experimental design of the storage trials (storage period: 12 weeks) was as follows: storage trial (1) wheat moistened to a water content of 20 ±1%; storage trial (2) wheat mixture of untreated wheat and wheat inoculated with an isolate of DON- and NIV-producing *Fusarium culmorum* (isolate from wheat; culture collection of the Department of Food Microbiology and Hygiene, University of Bonn) (wheat mixture: ratio 75:25) moistened to a water content of 20 ± 1%; storage trial (3) untreated wheat, grain moisture 13.5 ± 1% (no water addition, control).

Determination of Fungal Species. Two hundred surface-sterilizated (3%-NaOCl, 3 min) kernels of grain were incubated on selective media to determine the rate of *Fusarium* infection (*30*) and on potato dextrose agar to determine the mould flora. Grains were surface-sterilized to focus on *Fusarium* spp., which are mostly localized under the outer pericarps of wheat. Genera were differentiated microscopically; further differentiation to species level was performed for *Fusarium* spp. according to Nelson et al. (*31*). Furthermore, a PCR assay was applied to detect the occurrence of *Fusarium* spp. quantitatively and to confirm the results obtained microscopically.

Semiquantitative Real-Time PCR Using SYBR Green. Genomic DNA was extracted from 10 g (pool samples, 4 × 2.5 g) wheat samples with the QIAGEN-DNeasy tissue kit using the method described previously (*32*). For (semi-)quantiative PCR detection to assess the total *Fusarium* infection and contamination, respectively, as well as changes during the storage period, the Tox5 PCR assay was performed with a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using the *tri5* specific PCR primers Tox5-1 (forward, 5′-GCTGCT-CATCACTTTGCTCAG-3′) and Tox5-2 (reverse, 5′-CTGATCTGGT-CACGCTCATC-3′) resulting in a 658 bp PCR product (*32*, *33*). DNA isolated from the wheat samples was diluted to 10 ng/μL and used as a template for real-time PCR analyses. The QuantiTect SYBR Green PCR kit (Qiagen, Germany) was used according to the manufacturers instructions. To amplify a 658 bp fragment of the *tri5* gene, a slightly modified protocol according to Schnerr et al. (*33*) was used. The real-time PCR conditions were: 95 °C for 15 min (activation of the polymerase), followed by 45 cycles of 95 °C for 5 s (denaturation), 62 °C for 10 s (annealing), and 72 °C for 20 s (elongation). This PCR quantification program was followed by a melting curve program

Table 1. Results of Fitting the Sulfur K-edge XANES Spectra of Different LMW Subunits of Glutenin (cf., **Figures 1–3**) to the Sum of Different Reference Spectra (cf., Materials and Methods and Prange et al. (*10*))

	percentage contribution of sulfur speciation					
LMW[a]	C–S–H (GSH[b])[c]	C–S–S–C (GSSG[b])[c]	C–S–C (methionine)[c]	C–SO–C (dimethyl sulfoxide)[c]	C–SO_3–H (cysteic acid)[c]	SO_4^{2-} (zinc sulfate)[c]
storage trial 1 (week 0)	40	15	38		5	(2)
storage trial 1 (week 4)	14	36	12	4	34	
storage trial 1 (week 8)	10	9	16		64	
storage trial 1 (week 12)	6	22	6	3	63	
storage trial 2 (week 0)	39	18	36		5	(2)
storage trial 2 (week 4)	40	9	43		7	
storage trial 2 (week 8)	39	12	33		14	
storage trial 2 (week 12)	20	48	22		5	(4)
storage trial 3 (week 0)	36	27	32		3	
storage trial 3 (week 8)	37	26	28		7	
storage trial 3 (week 12)	44	28	23		3	(3)

[a] LMW subunits of glutenin. [b] GSH, reduced glutathione; GSSG, oxidized glutathione. [c] Reference compounds for the different sulfur species are shown in parentheses. Error ≤ ±10%; blank cells, contribution <0.3%.

consisting of a melting and continuous measuring step at 0.1 °C/s up to 95 °C to detect the melting points of PCR products for each sample. Genomic DNA (0.02, 0.2, 2, 20, 200, and 2000 ng/μL) of *F. graminearum* DSMZ 4547 was isolated according to Lee and Taylor (*34*) and used under the same PCR conditions to obtain standard curves for calibration. Using the DNA standards, the LightCycler automatically quantified cDNA amounts of the samples of interest.

Determination of Mycotoxins. To determine B-trichothecenes (DON, NIV, 3-AcDON, 15-AcDON), GC-ECD analyses of the samples were performed commercially by IFA Tulln, Austria (www.ifa-tulln.ac.at), according to Weingaertner et al. (*35*). Detection limits by using GC-ECD were (±10%) 60 μg/kg (DON, NIV), 96 μg/kg (3-AcDON), and 109 μg/kg (15-AcDON). OTA was quantified by using HPLC analyses of the samples by CVUA, Stuttgart, Germany (www.cvuas.de), according to CVUA, Stuttgart and Hunt et al. (*36*, *37*). The detection limit by using HPLC was 0.02 μg/kg.

Isolation of LMW Subunits of Glutenin. Wheat samples were milled into flour with an ash content of 0.55% using a Brabender milling automat (FL 1/4, Brabender, Duisburg, Germany). LMW subunits of glutenin were extracted from wheat samples of the storage trials using a modified method according to Melas et al. (*38*). The residue (glutenins) was extracted stepwise with 50% (v/v) aqueous 2-propanol containing Tris-HCl (0.08 mol/L, pH 8) and dithiothreitol (1.0%) under nitrogen at 60 °C after removing albumins, globulins, and gliadins with 60% (v/v) aqueous ethanol. After centrifugation, HMW subunits of glutenin were precipitated by addition of acetone to a final concentration of 40% (v/v), while LMW subunits of glutenin remained in solution. The LMW fractions were dialyzed against nitrogen saturated acetic acid (0.01 mol/L) and freeze-dried. To prepare the samples for XANES spectroscopy measurements, 20 mg of freeze-dried LMW subunits of glutenin was pressed to a thin homogeneous film.

Determination of the Water Content and Water Absorption. The water content of the flour (1 g) of several samples (cf., Table 2) was determined by heating to 130 °C for 120 min according to Arbeitsgemeinschaft Getreideforschung e.V. (*39*). The mean water content of the flours was 10.4% ± 2.5% ($n = 6$). Farinograms were recorded by mixing flour (7 g), sodium chloride (0.14 g), and water in a micro-farinograph (Brabender, Duisburg, Germany) for 20 min at 60 rpm and 22 °C. The amount of water was adapted to obtain a maximum consistency of the dough of 550 Brabender Units (BU).

Microscale Baking Tests. The microscale baking test was adapted from the 10 g version (*40*) to a 7 g version. Flour (7 g), sodium chloride (0.14 g), baker's yeast (0.49 g), coconut fat (0.07 g), sucrose (0.07 g), ascorbic acid (0.14 mg), and water (4.70 mL) were mixed to a dough as described above, rested for 20 min at 30 °C in a water-saturated atmosphere, rounded to a spherical dough piece, and proofed for 35 min at 30 °C in a water-saturated atmosphere. The loaves were baked for 10 min at 230 °C. The oven was saturated with vapor of 50 mL of water prior to baking. The loaf volume was determined by measurement of the water displacement of the wax-coated breads (*41*).

Table 2. Results of Micro-baking Tests of the Different Wheat Samples of the Storage Trials (1–3)

flour sample	loaf volume [mL]	loaf height [mm]
storage trial 1 (week 0)	37.2	30
storage trial 1 (week 4)[a]	29.8	18
storage trial 1 (week 8)	23.2	18
storage trial 1 (week 12)[a]	25.2	15
storage trial 2 (week 0)	34.6	32
storage trial 2 (week 4)	33.3	31
storage trial 2 (week 8)	30.4	32
storage trial 2 (week 12)[a]	25.6	20
storage trial 3 (week 0)[a]	39.8	30
storage trial 3 (week 8)[a]	38.8	33
storage trial 3 (week 12)[a]	35.7	26

[a] Water content of these samples was determined. The mean water content of the flours was 10.4% ± 2.5% ($n = 6$). Maximum coefficient of variation ($n = 2$): 12% for loaf volume, 10% for loaf height.

XANES Spectroscopy − Experimental. XANES spectra were recorded at beamline BN3 using synchrotron radiation of the electron stretcher accelerator (ELSA) of the Institute of Physics, Bonn. The storage ring was operated at an energy of 2.3 GeV with electron currents between 70 and 20 mA. The synchrotron radiation was monochromatized by a modified Lemonnier type double crystal monochromator (*42*), which was equipped with InSb (111) crystals. The monochromatic flux rate per second was some 10^9 photons (at 50 mA). The measurements were performed in transmission mode with ionization chambers (filled with 60 mbar air). Further details of the experimental setup have been published previously (*43*). The spectra were scanned with step widths of 0.6 eV in the pre-edge region between 2440 and 2460 eV, 0.09 eV between 2460 and 2490 eV, and 0.2 eV between 2490 and 2510 eV, and with an integration time of 1 s per data point. A linear background determined in the pre-edge region was subtracted from the raw data to correct for the absorption from higher shells and from supporting materials. Spectra were normalized at 2510 eV where the variation of the absorption cross-section is small. For energy calibration of the spectra, the S K-edge XANES spectrum of zinc sulfate was used as a "secondary standard". The maximum of the first resonance was set to an energy of 2481.44 eV. According to the step width, this value is reproducible to ±0.09 eV (±1 step).

Reference Compounds. Glutathione (oxidized and reduced), methionine, dimethyl sulfoxide, cysteic acid, and zinc sulfate were used as reference compounds. They were of reagent grade, purchased from Sigma (Deisenhofen, Germany), and used as received. Solid samples were ground to fine powder and put on a sulfur-free self-adhesive Kapton film (type 7010) purchased from CWC Klebetechnik (Frankenthal, Germany). Dimethyl sulfoxide was pipetted on a sulfur-free

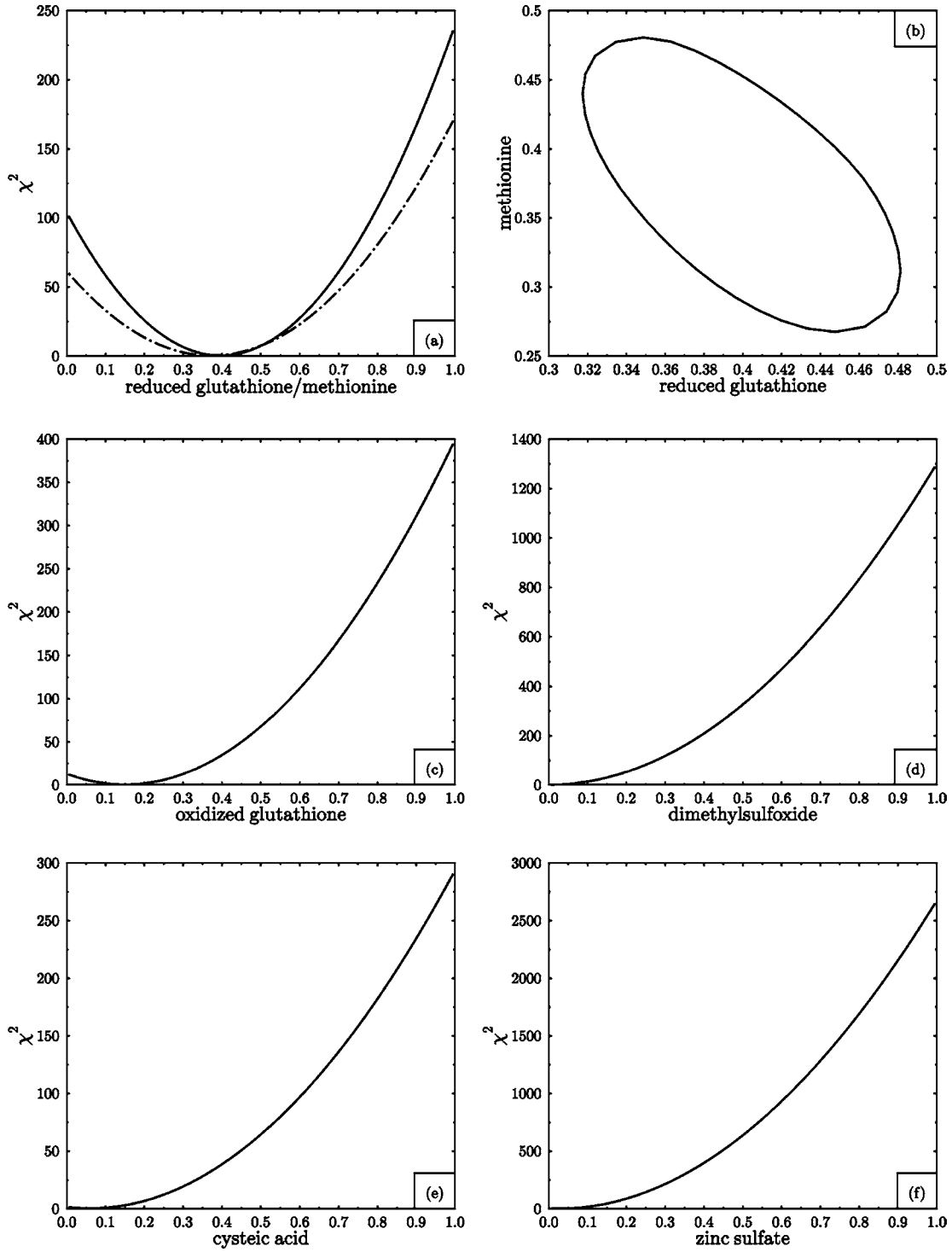

Figure 1. Statistical information on the fit of LMW subunits of glutenin (storage trial (1), wheat moistened to a water content of 20%, week 0; cf. **Figure 3a**): (**a**) χ^2-fit of reduced glutathione (solid) and methionine (dash and dot), (**b**) contour plot of these parameters as discussed in section "Quantitative analysis – MINUIT fitting of XANES spectra", (**c**) χ^2-fit of oxidized glutathione, (**d**) χ^2-fit of dimethyl sulfoxide, (**e**) χ^2-fit of cysteic acid, and (**f**) χ^2-fit of zinc sulfate.

filter paper for measurements. The thickness and homogeneity of the samples were optimized to avoid possible thickness as well as pinhole effects.

Quantitative Analysis – MINUIT Fitting of XANES Spectra. The interactive fitting and plotting package Mn-Fit 4.04/15 was used to analyze the XANES spectra quantitatively (available at: http://zina06.physik.uni-bonn.de/~brock/mn_fit.html). Mn-Fit 4.04/15 uses the function minimization tool "MINUIT" to fit histograms or data. MINUIT is a tool to find the minimum value of a multiparameter function. In this study, a set of six reference spectra (reduced and oxidized glutathione, methionine, dimethyl sulfoxide, cysteic acid, and zinc sulfate) was used. A χ^2 criterion was applied to find the linear combination of these spectra that reproduces the XANES spectrum of interest with the highest probability. A detailed description and explanation for the choice of this set of reference compounds to fit LMW subunits of glutenin as well as a figure with the corresponding reference spectra is given in Prange et al. (*10*). Further details concerning this approach to the quantitative analysis of XANES spectra, especially its verification, potential, and restrictions, have been published elsewhere (*10*, *45*). The errors of the percentage contributions of sulfur (**Table 1**) can be estimated to be smaller than ±10% (absolute value) (*10*, *44*, *45*). As an example, in **Figure 1**, χ^2-plots (i.e., the change of

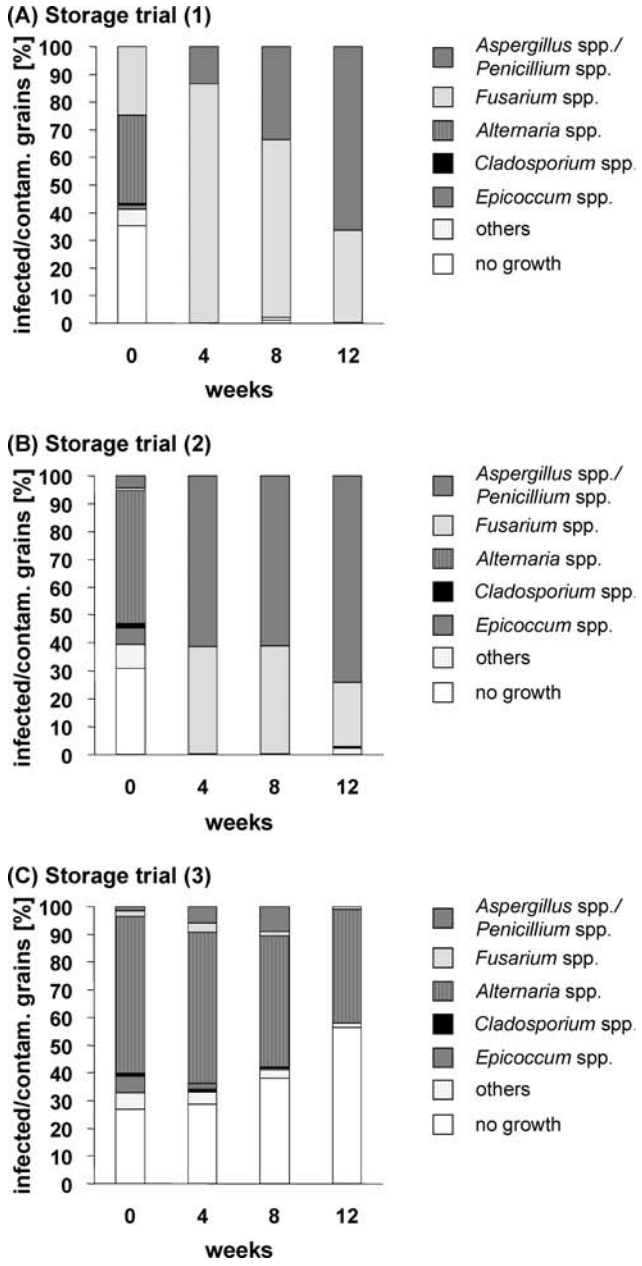

Figure 2. Mould floras of wheat stored at a temperature of 20 °C over a storage period of 12 weeks: (**A**) storage trial (1), wheat moistened to a water content of 20%; (**B**) storage trial (2), wheat mixture of untreated and *F. culmorum*-inoculated wheat (ratio 2:1) moistened to a water content of 20%; (**C**) storage trial (3), untreated wheat (no water addition, control).

the goodness-of-fit parameter with a change of the parameter value) and a contour plot for reduced glutathione and methionine (i.e., the characterization of the parameter space within which a change in the χ^2-value of 0.1 or less is induced by variation of this pair of parameters while allowing for reoptimization of the remaining variables) are provided as additional statistical information for the S K-edge XANES spectrum of LMW subunits of glutenin (storage trial (1): wheat moistened to a water content of 20%, week 0; cf., **Figure 3a**). This is a critical case, because potential linear dependence in the set of reference spectra affects mainly the references that play an important role in this fit. The presented, representative example clearly points out the quality and consistency (cf., Prange et al. (*10*)) of the fits and speciation results (**Table 1**).

RESULTS AND DISCUSSION

Following our earlier investigations (*9*−*11*), it was one aim of the present study to investigate the exact influence of storage and field fungi on sulfur speciation in gluten proteins systematically by performing S K-edge XANES measurements of LMW subunits of glutenin isolated from wheat samples of different storage trials and by analyzing the obtained spectra. It should be noted at this point that the reference compounds (cf., **Table 1**), which were used for the quantitative analysis of fitting the XANES spectra of the samples, are only representatives for a given class of an atomic environment of the sulfur atom. Although zinc sulfate crystals are obviously not present in the gluten proteins, this compound was used as a proxy for sulfate anions, which indicates the presence of sulfate in general.

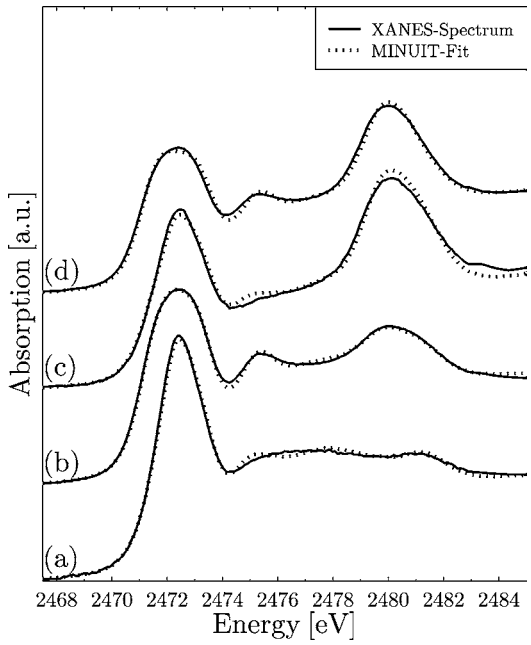

Figure 3. Sulfur K-edge XANES spectra of LMW subunits of glutenin and corresponding MINUIT fits: storage trial (1), wheat moistened to a water content of 20%, (**a**) week 0, (**b**) week 4, (**c**) week 8, and (**d**) week 12.

Influence of Storage Fungi (*Aspergillus* spp., *Penicillium* spp.) on Sulfur Speciation in LMW Subunits of Glutenin. At the beginning of storage trial (1), week 0, ∼60% of the grains were infected with moulds, mainly by *Alternaria* spp. (∼50%), *Aspergillus* spp./*Penicillium* spp. (∼5%), *Epicoccum* spp. (∼6%), and some few *Fusarium* spp., *Cladosporium* spp., and others (**Figure 2A**). Four weeks later, already, a strong infection with *Aspergillus* spp. and *Penicillium* spp. was visibly detectable by white and green mycelia on the grains accompanied by the typical musty smell of moulds. All grains were infected or contaminated by moulds, but the mould flora changed almost completely and only three genera were present: *Aspergillus* spp./*Penicillium* spp. (∼62%) and *Fusarium* spp. (∼36%). During the rest of the storage period, mainly these three genera were detected in similar amounts, and in week 12 the amount of the storage fungi *Aspergillus* spp./*Penicillium* spp. increased to ∼72%. At the beginning of the storage trial (week 0), a typical sulfur speciation for LMW subunits of glutenin (i.e., sulfur is mainly present in the thiol (C−S−H), the disulfide (C−S−S−C) or the thioether (C−S−C) states (*10*, *28*)) could be (**Figure 3**, **Table 1**). Sulfur present in the sulfonic acid state increases from ∼5% in week 0 to ∼63% in week 12 (**Table 1**). Furthermore, results of fitting the spectra showed minor percentages of sulfur in the sulfoxide state (∼3−4%, weeks 4 and 12). The increase of sulfur in the sulfonic acid state can be correlated with an increase of storage fungi (cf. below; discussion sulfur speciation of storage trial (2)). Thus, it can be

assumed that the storage fungi induce oxidation of thiol and disulfide groups mainly to the sulfonic acid state; that is, cysteine is oxidized to cysteic acid and can therefore no longer participate in thiol-interchange reactions of the gluten network. One could imagine that oxidation of cysteine−sulfur can be performed by the storage fungi itself. However, there is no experimental evidence for this hypothesis. It has to be kept in mind that the storage fungi normally invade and grow into the grains so that they directly degrade the gluten proteins and use it for their own metabolic activities (*46*), which, however, cannot explain the high percentage of sulfur in the sulfonic acid state. Probably, the oxidation to higher oxidation states might be a "pure" chemical process and not performed directly by the fungi. Storage fungi of the genera *Aspergillus* and *Penicillium*, in contrast to the field fungi *Fusarium* spp. and *Alternaria* spp., are well known as effective producers of acids, for example, citric acid production by *Aspergillus* spp. (*47*). Therefore, the production of acid in the moistened environment during suboptimal storage and lowered pH might lead to an oxidative process involving oxygen from the air or can be the starting point for the oxidation. This hypothesis is further supported be the observation that oxidation of LMW subunits of glutenin by oxidizing agents to higher oxidation states of sulfur (sulfoxide and sulfonate state) was much effective at low pH values (pH 2, pH 4) than at neutral or basic pH (*10*). To verify this hypothesis in future experiments, one can envision monitoring the effects of addition of, for example, citric acid to uninfected suboptimally stored grains in the presence of oxygen using the above-described experimental techniques. The results of the baking tests (**Table 2**) show that the volumes and the heights of the loaves continuously decrease from week 0 to week 12. This can be correlated to the occurrence and increase of sulfur in sulfonic acid state (see above; **Table 1**) as well as degradation of gluten proteins by the storage fungi leading to a drastic reduction of the baking quality. The storage fungi invade the grains (*46*) and degrade and depolymerize, in contrast to *Fusarium* spp. (see below), the gluten network. This observation is in good accordance with the investigation of gluten proteins isolated from wheat highly infected by storage fungi showing an extremely lowered amount of gluten proteins (*48*).

The same phenomenon, that is, the correlation between an increase of storage fungi and increases of sulfur present in the sulfonic acid state in the LMW subunits of glutenin, could be observed in storage trial (2). First, in the grains at week 0, ∼25% of the grains were infected by *Fusarium culmorum* (as expected because of the inoculated grains), and other typical field fungi (∼30% *Alternaria* spp., ∼5% *Cladosporium* spp.) could be detected (**Figure 2B**). In analogy to storage trial (1), ∼30% of the grains were not infected. From week 4 and during the rest of the storage trial, the mould flora changed completely. In week 4, ∼85% of the grains were infected only by *F. culmorum* (*Fusarium* spp. was differentiated microscopically, data not shown) and ∼15% by *Aspergillus* spp./*Penicillium* spp. Over the course of the storage trial, the amount of *F. culmorum*-infected grains decreased (week 8 ∼65%, week 12 ∼32%), whereas the amount of grains infected by storage fungi increased in a correlated way (week 8 ∼35%, week 12 ∼68%). Because of this interesting development of the mould flora, competitive interactions between *F. culmorum* and the storage fungi can be assumed. Furthermore, it can be observed that the sulfur speciation changes with the dominant occurrence and increase of storage fungi (**Table 1**). By quantitatively analyzing the corresponding S K-edge XANES spectra (**Figure 4**), sulfur present in the sulfonic acid state increases in the LMW subunits

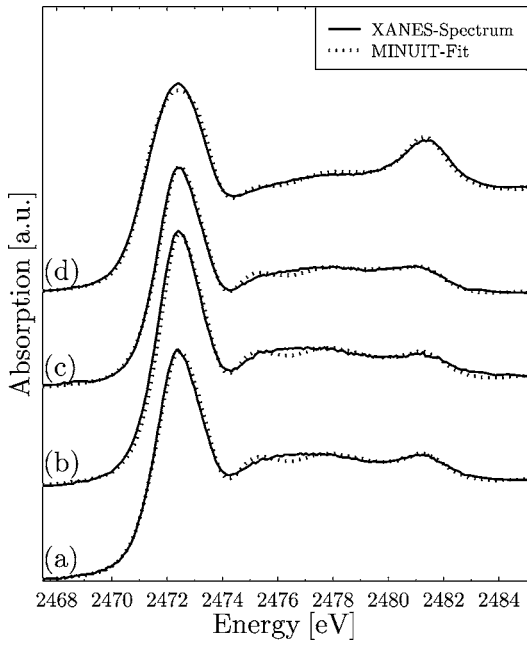

Figure 4. Sulfur K-edge XANES spectra of LMW subunits of glutenin and corresponding MINUIT fits: storage trial (2), wheat mixture of untreated and *F. culmorum*-inoculated wheat (ratio 2:1) moistened to a water content of 20%, (**a**) week 0, (**b**) week 4, (**c**) week 8, and (**d**) week 12.

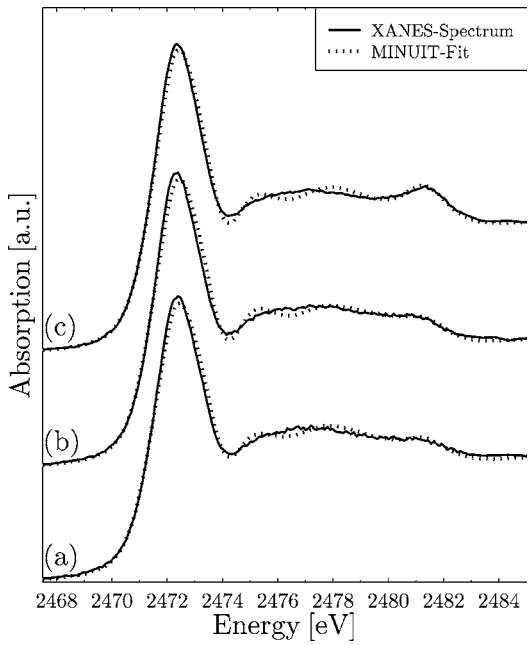

Figure 5. Sulfur K-edge XANES spectra of LMW subunits of glutenin and corresponding MINUIT fits: storage trial (3), untreated wheat (no water addition, control), (**a**) week 0, (**b**) week 8, and (**c**) week 12.

of glutenin in week 8 (∼14%) and week 12 (∼9%). The results of the corresponding baking tests correlate with the occurrence and increase of storage fungi. Furthermore, a significant reduction of loaf volume and loaf height from week 0 to week 8 could not be observed in the baking tests using flours from this storage trial. Only after 12 weeks of storage did the volume and height of the bread decrease significantly (**Table 2**).

Storage trial (3) was performed as a control experiment. The loss of moisture led to a complete other mould flora over the storage period (**Figure 2C**): *Alternaria* spp. dominated the mould flora during the storage period, whereas the storage fungi and *Fusarium* spp. were present but only detectable in a

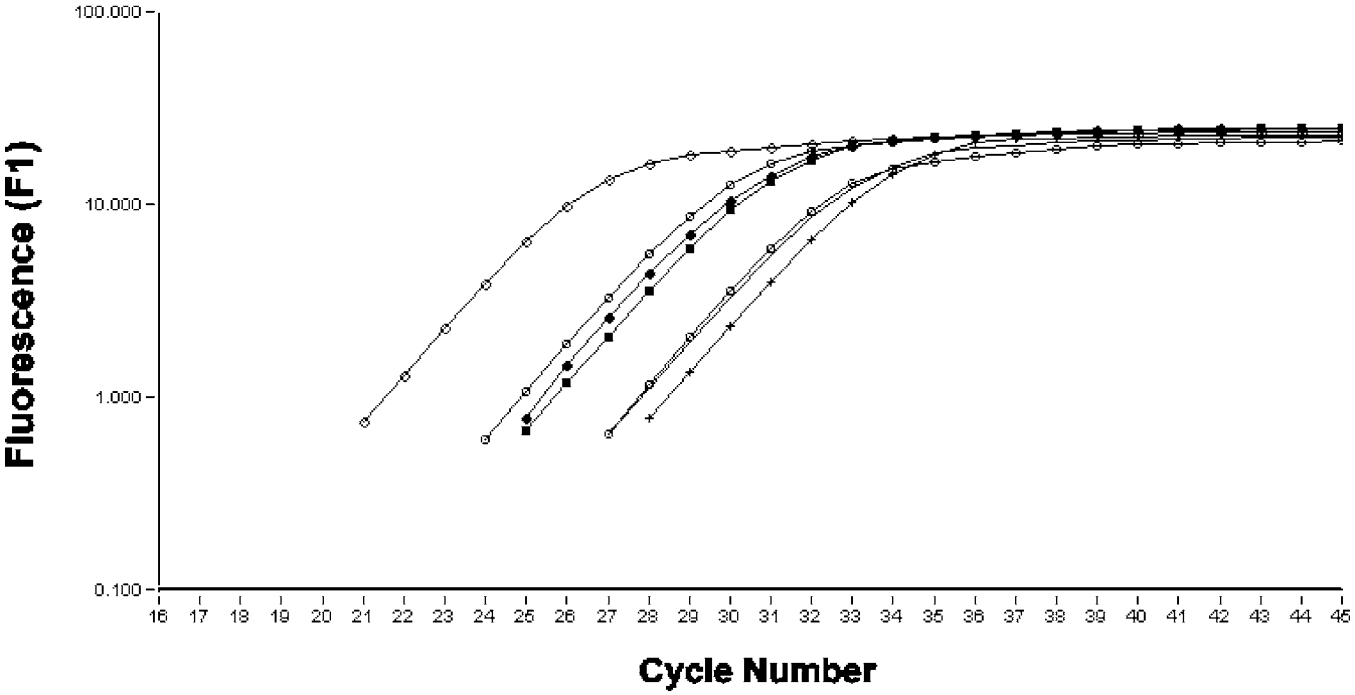

Figure 6. Light Cycler results for the detection of the *tri5*-PCR products: First, amplification curves of genomic *F. graminearum* DNA standards used for quantification and calibration with concentrations of 20, 2, and 0.2 ng/μL (–○–, from left to right). The Light Cycler software calculated the corresponding standard curve with an error of linear regression of 0.05 (curve not shown). Second, amplification curves of *Fusarium* spp. DNA samples isolated from samples of storage trial (2): week 0 (–×–), week 4 (–●–), week 8 (–■–), and week 12 (–).

Table 3. Mycotoxin Content of the Different Wheat Samples of the Storage Trials (1–3)

	mycotoxin content of wheat samples from the storage trials [μg/kg dry weight]				
	DON (GC-EDS)	NIV (GC-EDS)	3-AcDON (GC-EDS)	15-AcDON (GC-EDS)	OTA (HPLC)
storage trial 1 (week 0)	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]
storage trial 1 (week 4)	nd[a]	nd[a]	nd[a]	nd[a]	0.24
storage trial 1 (week 8)	nd[a]	nd[a]	nd[a]	nd[a]	23.55
storage trial 1 (week 12)	216	nd[a]	nd[a]	nd[a]	122
storage trial 2 (week 0)	2745	982	nd[a]	nd[a]	nd[a]
storage trial 2 (week 4)	1827	488	nd[a]	nd[a]	0.03
storage trial 2 (week 8)	1351	622	nd[a]	nd[a]	1.17
storage trial 2 (week 12)	2670	1020	nd[a]	nd[a]	0.88
storage trial 3 (week 0)	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]
storage trial 3 (week 8)	nd[a]	nd[a]	nd[a]	nd[a]	0.07
storage trial 3 (week 12)	nd[a]	nd[a]	nd[a]	nd[a]	0.02

[a] nd: not detectable (cf., detection limits "Determination of Mycotoxins", Materials and Methods).

maximum of ~10% of the grains. The sulfur speciation of the LMW subunits of glutenin did not significantly change during the storage trial (**Figure 5**); higher oxidation states of sulfur were not found (sulfoxide state) or detected only as minor contributions (sulfonic acid state, ~3−7%) (**Table 1**). The corresponding baking tests showed as well no significant changes in loaf heights and volumes (**Table 2**), which indicates that the performed storage did not influence the baking properties as expected.

Influence of Field Fungi (*Fusarium* spp.) on Sulfur Speciation in LMW Subunits of Glutenin. *Fusarium* spp., although typical field fungi, were detected in the stored moistened wheat (**Figure 2A**,**B**), which points out their probable occurrence and growth during suboptimal storage and confirmed earlier results (*15*). This presence of trichothecene producing *Fusarium* spp. was investigated and determined for the storage trials (1) and (2), additionally to the microscopic determination, by performing real-time detection of the *tri5* gene in *Fusarium* species by LightCycler-PCR showing that the DNA amounts detected correlate considerably with the percentages of *Fusarium* spp. infected grains (cf., **Figure 2**). As example, the results of the quantitative real time PCR results of storage trial (2) are shown in **Figure 6**. The corresponding DNA concentrations, as a measure *Fusarium* spp. biomass, calculated by the Light Cycler software (week 0, 0.11 ng/μL; week 4, 1.44 ng/μL; week 8, 1.31 ng/μL; week 12, 0.18 ng/μL) correlate with the development of *Fusarium* spp. during the storage trial investigated by cultural determination (cf., **Figure 2B**).

Microscopic differentiation of Fusaria showed that the *Fusarium* spectrum during the storage period of storage trial (1) consists of *F. culmorum* and *F. graminaearum*, who dominated the *Fusarium* spectrum (~40%), and *F. poae*, *F. avenaceum*, *F. equiseti*, *F. sporotrichoides*, and *F. tricinctum*, whereas in storage trial (2) the *Fusarium* spectrum consists of *F. culmorum* almost exclusively (data not shown). Although grains were highly infected by *Fusarium* spp., the results of fitting the XANES spectra of the LMW subunits of glutenin (**Table 1**) and the results of the corresponding baking tests

(**Table 2**) clearly indicate that *Fusarium* spp. did not have a direct influence on sulfur speciation of gluten proteins. This can be seen especially in the results for storage trial (2): From week 4 to week 12, *Fusarium* spp. Decreased, whereas the amount of infected grains by storage fungi increased continuously (**Figure 2B**). As discussed above, only the appearance of storage fungi leads to drastic changes in sulfur speciation and baking properties. Conversely, this implies that the exclusive or the dominant occurrence of *Fusarium* spp. (weeks 1 and 2, **Figure 2B**) has no significant influence on sulfur speciation of LMW subunits of glutenin and baking properties. These results confirm the finding that high *Fusarium* infection levels of wheat plants did not deteriorate baking quality of the grains (*28*).

Competitive Interactions between Field and Storage Fungi in Relation to Mycotoxin Contents during Suboptimal Storage of Wheat. It can be assumed that mycotoxins can be a competitive advantage during interactions of fungi, which is indicated by changes in the mould floras during the storage trials (see above) and changes in mycotoxin contents (**Table 3**). Although 65% of the grains (week 12) of storage trial (2) were infected by storage fungi *Aspergillus* spp./*Penicillium* spp., a visible contamination and infection with storage fungi was hardly recognizable, which may probably be explained by a direct inhibitory influence of *F. culmorum*. In storage trial (1), DON (exception: week 12, 216 μg/kg) and NIV as well as the acetylated DONs were not detected, although a significant amount (~25−35%) of *Fusarium* spp. (including the trichothecene producing species, data not shown) infected/contaminated grains were present in weeks 4−12, indicating that these "field fungi" can be regarded as "storage fungi" as well if storage conditions are suboptimal. In contrast, OTA was detected in significant and increasing amounts (0.24−122 μg/kg) in weeks 4−12, which correlates with the presence of storage fungi and with mouldy grains under suboptimal conditions (**Table 3**, **Figure 2A**). Interestingly, the situation in storage trial (2) is completely different. Extremely high amounts of DON (1351−2745 μg/ kg) and NIV (488−1020 μg/kg) over the whole storage period could be detected (**Table 3**), which were produced by the inoculated *F. culmorum*. During this time, however, the amount of *F. culmorum* infected grains decreased, whereas the number of contaminated grains with storage fungi increased inversely (up to 65%) (**Figure 2B**; see above discussion on mould flora). OTA, however, was hardly detected in the samples, although the base material was identical and thus contaminated with the same storage fungi. It has to be kept in mind that these results entail, as a consequence for consumer protection, that suboptimal storage of wheat (practical relevance: cf., Introduction), although not moulded visibly, can be heavily contaminated by, for example, DON and NIV. In the control experiment (storage trial (3)), production of mycotoxins during the storage period could not be observed. Regarding both storage trials (1) and (2), strong competitive interactions between field and storage fungi in relation to mycotoxin production during suboptimal storage of wheat can be assumed in these in vivo systems. If a strong DON and NIV producer is present and moisture and temperature conditions are suboptimal (cf., storage trial (2)), storage fungi of the genera *Aspergillus* and *Penicillium* can grow, but OTA production seems to be suppressed. In contrast, high OTA production during suboptimal storage of wheat seems to prevent the production of trichothecenes, although potentially trichothecene-producing Fusaria are present in significant amounts. This interesting result has to be elucidated in further interaction experiments in pure, mixed cultures of different mycotoxin producing moulds in vitro as well as in sterilized and then exactly defined inoculated grains in storage trials.

ABBREVIATIONS USED

LMW, low molecular weight; HMW, high molecular weight; XANES, X-ray absorption near edge structure; DON, deoxynivalenol; OTA, ochratoxin A; NIV, nivalenol; 3-AcDON, 3-acetylated deoxynivalenol; 15-AcDON, 15-acetylated deoxynivalenol.

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