

Carbon dioxide production as an indicator of *Aspergillus flavus* colonisation and aflatoxins/cyclopiazonic acid contamination in shelled peanuts stored under different interacting abiotic factors

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Abstract

Aspergillus flavus is the main xerophytic species in stored peanuts often resulting in contamination with aflatoxins (AFs) and cyclopiazonic acid (CPA). This study evaluated the relationship between storage of shelled peanuts under interacting abiotic conditions on (a) the temporal colonisation kinetics and cumulative respiration rates, (b) quantified the effect on dry matter losses (DMLs) and (c) quantify accumulation of aflatoxin B₁ (AFB₁) and CPA. Both naturally contaminated and inoculated peanuts with *A. flavus* conidia were stored for 7-days under different water activity (a_w ; 0.77-0.95) and temperature levels (20-35°C). Gas Chromatography (GC-TCD) was used to measure the temporal respiration rates (R) and the total accumulated CO₂ production. Mycotoxin contamination was quantified at the end of the storage period by LC-MS/MS. There was an increase in the temporal CO₂ production rates in wetter and warmer conditions in all treatments, with the highest respiration in the 0.95 a_w treatments of stored peanuts + *A. flavus* inoculum at 30°C (2474 mg CO₂ kg⁻¹ h⁻¹). The DMLs were modelled to produce contour maps of the environmental conditions resulting in maximum/minimum losses. Maximum mycotoxin contamination was always at 0.95 a_w although optimal temperatures were different for AFB₁ and CPA. Thus, 30-35°C was optimum for CPA and 25-30°C for AFs. A significant positive correlation (Spearman $p < 0.0001$) were found between Log₁₀AFB₁ and Log₁₀DML in both naturally contaminated stored peanuts ($r^2 = 0.7744$) and that inoculated with *A. flavus* ($r^2 = 0.8464$). Small dry matter losses (0.56%) resulted in AFB₁ contamination above the EU legislative limits. Real time CO₂ monitoring is a powerful tool to provide an early indication of biological activity, our results show a correlation with high CO₂ production fungal growth and mycotoxin accumulation. These results provide valuable information for the creation of a database for the development of a post-harvest decision support system for determining relative risks of contamination with these mycotoxins in stored shelled peanuts.

Highlights (3 to 5/ 85 character per bullet point including spaces)

- ✓ Higher optimum temperature for *A. flavus* growth (30-35°C) than for AFB₁ production (25-30°C)
- ✓ Optimum cyclopiazonic acid production was at (30-35°C), with none at 0.90 a_w
- ✓ Dry matter losses varied from 0.3-17% at 0.90-0.95 a_w in stored peanuts depending on the temperature.

- ✓ Dry matter losses can be used as a sensitive early indicator of initiation of fungal spoilage
- ✓ Dry matter losses due to fungal activity (0.56%) resulted in aflatoxin contamination exceeding EU limits

Keywords (6 keywords)

Groundnuts; temperature; water activity; mycotoxins; dry matter losses, storage, food safety

1. Introduction

Peanuts (*Arachis hypogaea* L.) also known as groundnuts, is a legume that originated in South America. Peanut plants are grown widely in China, India, Africa and the USA. Peanut world production in 2016 was approx. 43.9M tonnes (Food and Agriculture Organisation of the United Nations, 2018). Peanut crops are very susceptible to fungal diseases, especially by mycotoxigenic fungi during the pod filling phase at pre-harvest, particularly during drought stress episodes (Paterson and Lima, 2011). Subsequently, poor drying and storage can result in further mycotoxin contamination, as peanuts are hygroscopic and absorb moisture easily. *Fusarium*, *Penicillium*, and *Aspergillus* species are commonly isolated from peanuts during the whole production phase from growth to storage. *Aspergillus flavus* infection occurs during the pre-harvest stage but colonisation can also occur during drying and storage, when the most commonly isolated species have been reported (Atayde et al., 2012; Gonçalves et al., 2008; Sultan and Magan, 2010; Zorzete et al., 2011, 2013). This results in significant contamination with toxic secondary metabolites, especially aflatoxins (AFs) and cyclopiazonic acid (CPA). Such contamination has major impacts on the quality of the product and the potential for its export from producer countries resulting in significant economic impacts, especially in Lower Middle Income Countries (LMICs) (Atayde et al., 2012; de Souza et al., 2014; Zorzete et al., 2011, 2013).

Peanuts like other edible seeds respire at very low levels under safe storage and reduced water availability conditions (water activity, a_w ; <0.70 a_w = 8% moisture content (m.c.). Under these conditions, while fungal contaminants remain viable, they are unable to initiate spoilage or any additional toxin contamination (Fleurat-Lessard, 2017; Paterson and Lima, 2011). However, poor storage conditions or inadequate silo hygiene can lead to the introduction of water from outside, boosting the pest and disease activity. This biological activity can lead to pockets of spoilage resulting in toxin contamination. This also results in an increase in the respiration of the stored peanuts and of the associated mycobiota. Spoilage fungi are able to utilise the lipids present, leading to a deterioration in quality and associated dry matter losses (DMLs) (Seitz et al., 1982). Saul and Lind (1958) were the first to correlate the impact the respiration (CO_2 production) and DMLs due to fungal colonisation and mycotoxin production.

According to Seitz et al. (1982), mould colonisation increases the DML during storage at a rate dependent on the prevailing m.c., temperature, level of kernel damage and the fungal community present on the phyllosphere surfaces of the peanuts.

Recent studies have shown that changes in CO₂ production during storage of cereals, including maize, wheat, oats and rice can be used as an indicator of DML, as well as the relationship with mycotoxin contamination (Garcia-Cela et al., 2018; Garcia-Cela et al., 2019; Martín Castaño et al., 2017ab; Mylona et al., 2012 ab; Mylona and Magan, 2011). In these studies it was found that it is possible to use the progressive increase in the aerobic respiration rate under increasingly conducive interacting abiotic factors. This is related to mould growth, as their activity leads to an oxidation of carbohydrates/lipids and hence CO₂ production. Therefore, it can be linked to the quality losses as DML percentages. Respiration rates (R) using Gas Chromatography and the associated DMLs can be used to establish an “index of risk in storage” to predict overall quality changes and mycotoxin contamination in stored cereals and nuts (Magan et al., 2010).

Previously, DML values as low as 0.04, 1 and 2% indicated impacts on seed germination and risk of mycotoxin contamination in the context of the EU legal maximum limits (Garcia-Cela et al., 2018; Garcia-Cela et al., 2019; Lacey et al., 1994; Martín Castaño et al., 2017ab; Mylona et al., 2012b; Mylona and Magan, 2011; White et al., 1982). However, very little data is available on nuts, especially peanuts, although studies by Mylona (2012a) examined *A. flavus* and CO₂ production in hazelnuts, [showing](#) that very small DMLs resulted in aflatoxin B₁ (AFB₁) contamination exceeding the EU legislative limits.

Indeed, subsequent studies have suggested that CO₂ production could be a very powerful tool for the early prediction of the initiation of spoilage mould activity and [therefore mycotoxin](#) contamination of wheat, maize and rice (Mylona et al., 2012b; Martín Castaño et al., 2017a,b). This approach could have benefits in the development of sensing systems for the early indication of spoilage initiation, based on real time temporal monitoring of CO₂.

Thus, the aims of our study were to (a) examine temporal respiration (R) and cumulative total CO₂ production by stored naturally contaminated shelled peanuts or those artificially inoculated with *A. flavus* conidia stored under different conditions of a_w (0.77-0.95) and temperatures (20-35°C) conditions; (b) relate R production during storage to relative DMLs; (c) quantify AFs and CPA in all stored conditions; and (d) investigate the relationship between %DMLs and AFB₁ contamination relative to the EU legislative limits for food and feed use. The potential outcomes for using such data sets and models as a predictive tool of the relative risk of AFB₁ contamination during storage of peanuts by monitoring of CO₂ production are discussed.

2. Material and Methods

2.1. Fungal isolate

An aflatoxigenic type strain of *A. flavus* (NRRL 3357; Northern Regional Research Laboratories (NRRL) of the US Department of Agriculture USDA, New Orleans) was used in this experiment. The strain was maintained in glycerol:water (70:30, v/v) at -20°C in the culture collection of the Applied Mycology Group, Cranfield University.

2.2. Peanuts samples

Shelled peanuts from China, were used as naturally contaminated peanuts for storage experiments. [Water activity](#) (a_w) of both batches was 0.77, and they were stored at 4°C in resealable zip-lock polyethene bags until their use.

2.3. Peanut water activity adjustment

Known volumes of sterile water (0-2 mL) were added to 5 g sub-samples of naturally contaminated peanuts in 40 mL glass Universal bottles. These were sealed, shaken vigorously and stored overnight at 4°C to allow water absorption and equilibration. Samples were then equilibrated at room temperature (T) before the measurement of a_w values of each sub-sample, using the dew point/water activity meter AQUALAB Model Series 4TE (Decagon Instruments; Washington, USA). Water adsorption curves were determined by plotting the amounts of added water (mL) against a_w values measured. These curves were used to determine the exact quantities of water needed to reach the target a_w levels for each set of experiments performed. After this, the m.c. of the samples was determined by drying them in an oven at 105°C for 16 h.

2.4. Inoculation and storage experiments carried out

The *A. flavus* strain was sub-cultured on malt extract agar medium (MEA) in two 9 cm Petri dishes and incubated at 25°C for 7 days to obtain actively sporulating cultures. A sterile loop was used to remove some conidia from the culture and mix this with 9 mL sterile water containing 0.005% Tween 80. The suspension was homogenised and adjusted with sterile water to obtain a final concentration of 1×10^4 spores mL⁻¹ using a Thoma counting chamber (Marienfield superior, Germany).

Natural shelled peanut samples (90 g) were modified with sterile water (-1mL for spore inoculum), moisture adsorption curve, to obtain the target treatment a_w levels (0.95, 0.90,

0.85), and stored at 4°C for 24 h. Control treatment was unmodified peanuts at 0.77 a_w . Four replicates (5 g) per treatment were placed in 40-mL clear glass Volatile Organic Analysis (VOA) vials with sealable PTFE caps containing a silicone septum (23188 Supelco) to allow for later gas sampling from the headspace. Peanut samples corresponding to the same a_w levels were incubated in 12-L polypropylene environmental chambers together with 2 x 500 mL beakers of glycerol/water solutions to maintain the target Equilibrium Relative Humidity (ERH) of the atmosphere relative to each a_w treatment value. Peanut samples were incubated for 7 days.

2.5. Respiration of peanuts stored under different water activity and temperature conditions

Treatments used in this study were temperatures of 20, 25, 30 and 35°C and a_w levels of 0.77-0.88-0.90 and 0.95. Carbon dioxide (CO₂) production was measured every 24 h. Sampling method used was that previously described by Mylona & Magan (2011). The percentages of CO₂ concentration were used to calculate (a) Respiration (R) rate in mg CO₂ (kg h⁻¹), (b) total cumulative production of CO₂ after 7 days storage and (c) the total DMLs (Mylona and Magan, 2011a).

2.6. Mycotoxin analysis

2.6.1. Sample preparation

At the end of the experiment peanuts from the different treatments were dried at 60°C for 48 h, milled and stored at 4°C until analysis. Between two and three grams of milled peanuts were used for mycotoxin extraction using 8 and 12 mL (peanuts powder/four times extract volume) of the extraction solvent (acetonitrile/water/acetic acid 79/20/1) followed by a 1+1 dilution using acetonitrile/water/acetic 79/20/1. Five µL of the diluted extract were directly injected into the sampling port for LC-MS/MS analysis. For concentrations exceeding 500 µg·kg⁻¹. The extract was further diluted 1+49 and reanalysed.

2.6.2. LC-MS/MS parameters

LC-MS/MS screening of targeted fungal mycotoxins was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurbolonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini C18-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 x 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). The chromatographic method as well as chromatographic and mass spectrometric parameters were previously described by Malachová et al. (2014).

Electrospray ionization (ESI-MS/MS) was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate

chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ± 27 and ± 48 seconds in positive and negative mode, respectively.

Quantification was performed by external calibration using serial dilutions of a multi-analyte stock solution made up of the following standards: AFB₁, aflatoxin B₂ (AFB₂) and CPA. The accuracy of the method has been verified on a continuous basis by regular participation in proficiency testing schemes (Malachova et al., 2015; Malachová et al., 2014).

2.7. Statistical Analysis

Statistical analysis was performed using package JMP® Pro 14 (SAS Institute Inc., 2016. Cary, NC, USA). Datasets were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene test, respectively. When data failed the normality test, variable transformation was performed to try to improve normality or homogenise the variances. Transformed data were still not normally distributed and therefore the Wilcoxon or Kruskal-Wallis test by ranks was used for the analysis of the data. Non-parametric comparisons for each pair using the Wilcoxon Method were used to find differences between groups.

Forward stepwise regression including all the linear, quadratic and the interaction terms (Equation 1) was used to obtain polynomial equations for DML with regard to the storage conditions (a_w and T) for both naturally and contaminated shelled peanuts. Non significant terms (p -value >0.05) were excluded. The assumptions of linearity and normally distributed residuals were assessed, producing normal plots of the residuals. Contour maps were built in JMP® Pro 14 using 5000 simulation data from predicted formula.

$$\text{DML} = b_0 + b_1 T + b_2 a_w + b_4 a_w^2 \times T^2 \quad \text{Equation 1}$$

3. Results and Discussion

3.1. Impact of a_w and temperature on the temporal and total accumulated CO₂ in naturally and contaminated shelled peanuts with *A. flavus* inoculum

Overall, the respiration rates of artificially inoculated shelled peanuts with *A. flavus* inoculum increased after two days storage, depending on the a_w and temperature treatment. Figure 1 shows the temporal (hourly) respiration rate (R) and the total accumulated CO₂ (cumulative R ; g CO₂ kg⁻¹ peanuts) at 30°C for the four different a_w levels. In general, respiration in the stored peanuts at 0.77-0.90 a_w was relatively low, regardless of the storage temperature. The highest respiration rates were at 0.95 a_w for the inoculated stored peanuts at 30°C (2474 mg CO₂ kg⁻¹ h⁻¹). Similar CO₂ production levels were found at 30 and 35°C in both naturally (2079 and 2281 mg CO₂ kg⁻¹ h⁻¹), and inoculated (2274 and 2096 mg CO₂ kg⁻¹ h⁻¹). In other cereal commodities, R for shelled peanuts was also optimum at 0.95 a_w in paddy rice, brown rice

and maize when inoculated with *A. flavus* at 30-35°C (Garcia-Cela et al., 2019; Martín Castaño et al., 2017b). The baseline data in the driest treatments were different for each cereal type because of the inherent cereal respiration and differences in the natural contamination with mycobiota and toxigenic fungi. For shelled peanuts, which have a high lipid content, the respiration rates due to the presence of surface contamination would be lower than for some other temperate cereals. Figure 2 shows that temporal and total accumulated CO₂ over the whole temperature range examined at a steady-state a_w of 0.95. The slowest respiration rates were observed at 20-25°C in the driest a_w treatment tested over the 7 day storage period.

Statistically, there were significant differences between both naturally contaminated and inoculated peanuts with *A. flavus* under the different temperature x a_w levels examined. The shelled peanuts inoculated with *A. flavus* had a higher respiration rates than the uninoculated naturally contaminated treatments. There were also statistically significant differences between the a_w treatments. This implied that a_w levels were more important in determining respiration rates and CO₂ production than the storage T.

3.2. Effect of storage conditions on dry matter losses in naturally contaminated and artificially inoculated shelled peanuts with *A. flavus*.

Figure 3 compares the effect of different temperature and a_w treatments on the relative DMLs of natural and contaminated peanuts. There was an increase in DMLs, as the storage a_w and temperature were increased. Statistical differences were found between both naturally contaminated shelled peanuts in relation to a_w treatments (Chi-square $p < 0.0001$). Previously, similar relationships were found for *Fusarium graminearum* colonisation of wheat and *A. flavus* colonisation of paddy and brown rice (Garcia-Cela et al., 2019; Martín Castaño et al., 2017b). In this study we have simulated natural contamination and high initial inoculum contaminated with *A. flavus* on shelled peanuts in order to evaluate the toxin production and elucidate the impacts related to the quality losses and mycotoxin contamination. This study has shown that DMLs were higher in the artificially inoculated treatments than the low naturally contaminated peanuts. The only exception was under relatively moist storage conditions at 35°C (14 vs 16% DML). Previously, optimum DMLs were found at 0.95 a_w and 30-35°C in maize of about 17% after 10 days storage (Garcia-Cela et al., 2019). For paddy and brown rice inoculated with *A. flavus* DMLs were 3.5% and 20% respectively (Martín Castaño et al., 2017b). In this case, the unprocessed paddy rice was protected by the outer grain layers, decreasing the potential fungal colonisation when compared with the processed brown rice. In the present study, the DMLs values were the lowest at $< 0.90 a_w$, even at 30-35°C. Polynomial equations generated for both naturally and artificially contaminated shelled peanuts with the % DMLs are presented

in Table 1. According to the model all terms except T^2 were significant ($p\text{-value}<0.05$), although the R^2 values were quite low.

Figure 4 shows the contour maps generated for the relationship between $a_w \times T$ and identifies the optimum and marginal conditions for DMLs in naturally and artificially contaminated peanuts. Higher DMLs were predicted in naturally contaminated peanuts due to *A. flavus* colonization between 20-30°C.

Studies on shelled peanuts colonised by an aflatoxigenic strain of *A. flavus* over the temperature $\times a_w$ range of 15-42°C and 0.85-0.99 a_w highlight that *A. flavus* colonisation was lower when temperature was $\leq 20^\circ\text{C}$ or $a_w \leq 0.85$ with optimum growth at 37°C/0.98 a_w (Liu et al., 2017). Previous research with *A. flavus* colonisation of peanuts have highlight an optimum growth temperature of 35°C and a minimum a_w of 0.78 (Hill et al., 1983). Other studies have highlighted that for peanuts, the optimum a_w range for *A. flavus* colonisation was 0.90-0.99 a_w (de Souza et al., 2014), while others have suggested that optima were at 0.90-0.95 a_w (Horn, 2017). Other studies by Nakai et al. (2008) and Zorzete et al. (2011) suggested a minimum a_w level for *A. flavus* growth in peanuts of 0.78-0.80 a_w .

Previous ecophysiological studies have provided valuable data to build boundary models based on real time temperature and relative humidity in silos. The aim was to predict the specific risk of fungal spoilage in a specific commodity (Garcia-Cela et al., 2019). However, Garcia-Cela et al. (2018) have suggested that CO_2 changes occur prior to temperature changes due to fungal activity and thus could provide a more sensitive earlier indication of risk.

On the other hand, according to our results (data no shown) and previous studies on stored oats have shown that higher populations of *F. langsethiae* ($\text{CFUs}\cdot\text{g}^{-1}$) were correlated with higher R rates (Mylona et al., 2011). It is noticeable that other indirect measurements like ergosterol or free fatty acid (FFA) could be used as an indicator of fungal contamination. However, the relationship between fungal populations and ergosterol is often unstable and related to mycelial biomass age (Dhingra et al., 2009). The relationship between FFA content and fungal contamination is controversial since some authors suggest that FFA changes in stored commodities such as soybean could partially be related to enzymatic activity of the soybeans instead of the fungal activity (Chapman and Robertson 1980). However, neither of these methods can be applied in real time. They require samples to be removed from a silo and laboratory time to obtain a result. In contrast, the CO_2 monitoring can be done in real time, which provides information on any changes in activity due to changes in moisture migration or

temperature. It may be also possible to link the CO₂ changes to the models for optimum and marginal conditions for toxin production.

3.3. Aflatoxin B₁, B₂ and cyclopiazonic acid production in shelled peanuts under different storage conditions

Figure 6 shows the effect of treatments on aflatoxins B₁ and B₂ and CPA. There were no significant differences in the mycotoxin contamination between naturally contaminated peanuts and that artificially inoculated with *A. flavus* (ChiSquare $p > 0.0001$). AFB₁ contamination represented >85% of the total AFs in the positive samples. A positive correlation ($r^2 = 0.9810$) between the two types of AFs analysed (Spearman $p < 0.0001$) was observed. Water activity significantly affected both AFB₁ and AFB₂ contamination (ChiSquare $p < 0.0001$) in all treatments assayed. Aflatoxins were not detected at 0.85-0.90 a_w and 20°C. Overall, the optimum temperature range for AFs production was between 25-30°C. Previous studies have tried to evaluate the effect of environmental conditions on AFB₁ accumulation by *A. flavus*. For example, Liu et al. (2017) found that the range of conditions for AFs production was 25-33°C and 0.92-0.96 a_w , with a maximum at 28°C/0.96 a_w . Also, Abdel-Hadi et al. (2010) observed higher AFB₁ production at 0.90-0.95 a_w . Regarding the minimum a_w for toxin production, Passone et al. (2010) found AFB₁ at 0.84-0.88 a_w but only after longer storage periods (>1 month). However, these previous studies were carried out with irradiated relatively sterile peanuts inoculated with *A. flavus*. Thus, interactions with and presence of other mycobiota were not considered. Few studies have examined the production of CPA in relation to different storage conditions in peanuts or indeed in cereals generally. Storage temperature x a_w significantly affected the production of CPA. However, CPA contamination was only detected in the wettest peanuts at 0.95 a_w with optimum temperature conditions between 30-35°C. Previously, Vaamonde et al. (2006) found that maximum accumulation of CPA was at 35°C / 0.94 a_w although some toxin was detected at 20°C and lower a_w levels. Zorzete et al. (2017) monitored fungal populations and mycotoxin contamination of stored peanuts for 6 months and reported that *A. flavus* was predominant, with co-occurrence of AFs and CPA in 14% of the samples. It is thus important to try and monitor both toxins during the storage of groundnuts.

3.4. Relationship between dry matter losses and AFB₁ accumulation on peanuts relative to the EU legislative limits for maximum contamination in food and feed

The AFB₁ data was plotted against DMLs for naturally contaminated shelled peanuts, and that inoculated with *A. flavus* in Figure 7. A significant positive correlation (Spearman $p < 0.0001$) were found between the Log₁₀AFB₁ and Log₁₀DML in both naturally and artificially contaminated stored peanuts ($r^2 = 0.0.7744$ and $r^2 = 0.8464$, respectively). Figure 7 includes

lines showing the EU legislative limits for AFB₁ in peanuts intended for human consumption (8 µg kg⁻¹) and for animal feed (20 µg kg⁻¹) (Commission, 2006, 2003). All treatments >0.90 a_w (>15% m.c.) exceeded the EU legal limits. Overall, small losses in dry matter of 0.56% [DMLlog₁₀ (-0.25)] resulted in AFB₁ contamination above the legislative limits.

There appears to be a low tolerance for quality losses due to fungal spoilage in stored shelled peanuts, especially if they are not dried effectively. Indeed, because peanuts are hygroscopic they will often reabsorb moisture from the environment when post-harvest management is not effectively controlled. In previous studies with cereals, mycotoxin contamination of about 1% resulted in type A and B trichothecenes, zearalenone (both in wheat) and fumonisins toxin levels (maize) exceeded the EU legislative limits (Mylona and Magan, 2011; Mylona et al., 2012b; Garcia-Cela et al., 2018; 2019). For fatty acid rich nuts such as hazlenuts, Mylona (2012a) found that only 0.4% DML resulted in AFB₁ contamination exceeding the EU limits. Thus, tolerances could be lower in nuts than for cereals. Unprocessed paddy rice offers some protection from fungal invasion and thus DMLs where mycotoxin contamination becomes a concern is higher than for brown rice where high DMLs occurred more rapidly in conjunction with higher AFB₁ and fumonisins contamination (Martin Castano et al., 2017a,b). From the CO₂ data sets it may be possible to develop three categories of low, intermediate, and high risks, relevant to human consumption or for animal feed use. Thus, this type of data could be utilised by silo Managers to help evaluate the temporal relative risk of mycotoxin contamination and take appropriate remedial action.

4. Conclusions

This study showed that it is possible to use production of CO₂ as an early indicator of the onset of fungal colonisation or perhaps pest activity in stored peanuts. Small changes in DMLs resulted in AFB₁ levels exceeding the EU legislative limits in food and feed. Contamination with CPA occurred over a narrower range of conditions than that for AFB₁. Boundary conditions for colonisation and mycotoxin production could be effectively utilised to develop predictive models which can be used in conjunction with real-time CO₂ production to improve post-harvest management of peanuts as an effective tool for improving management of stored commodities post-harvest. This could improve sustainability and reduce post-harvest waste streams from such commodities.

List of Figures

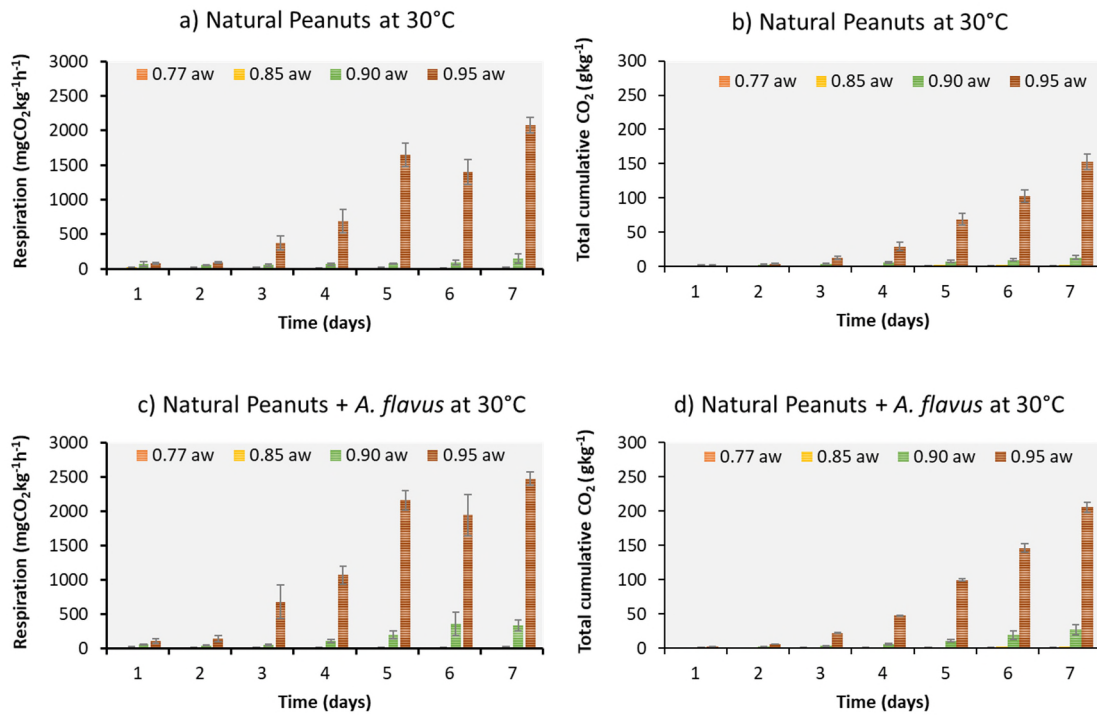


Figure 1. Temporal respiration (R) (a, c) and total accumulation (R cumulative) (b, d) of CO₂ obtained from GC measurements in naturally contaminated peanuts and those inoculated with *A. flavus* at 30°C and stored for up to 7 days at four different a_w levels. Vertical bars represent the standard error of the mean.

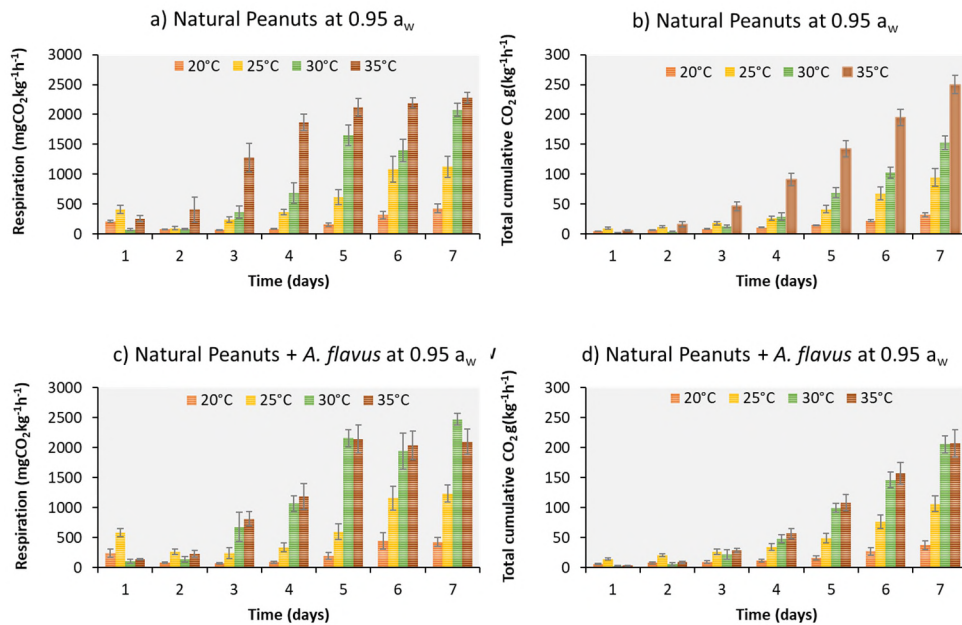


Figure 2. Temporal respiration (R) (a, c) and total accumulation (R cumulative) (b, d) of CO₂ obtained from GC measurements in naturally contaminated peanuts and those inoculated with *A. flavus* at 0.95 a_w level at four different temperatures over a 7 day storage period. Vertical bars represent the standard error of the mean.

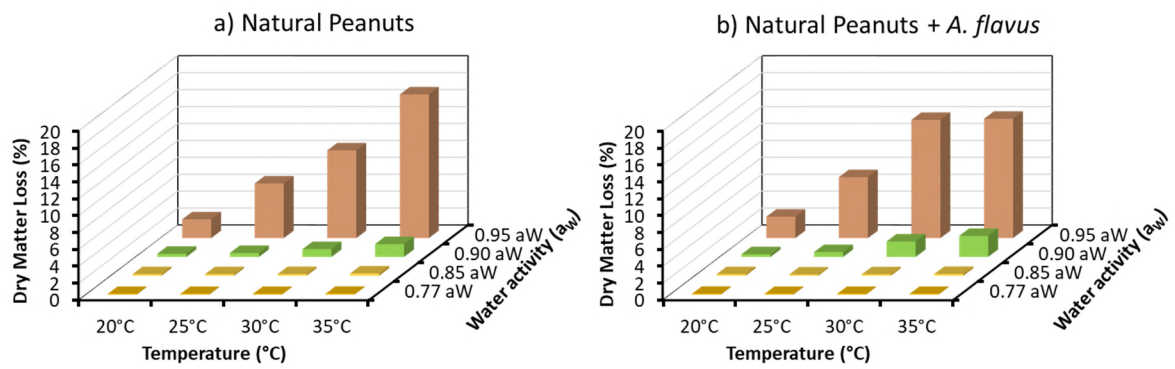


Figure 3. Percentage (%) Dry Matter Losses (DMLs) calculated from the total accumulated CO₂ production after 7 days in (a) naturally contaminated peanuts and (b) that inoculated with *A. flavus* spores and stored at different a_w levels and different temperatures.

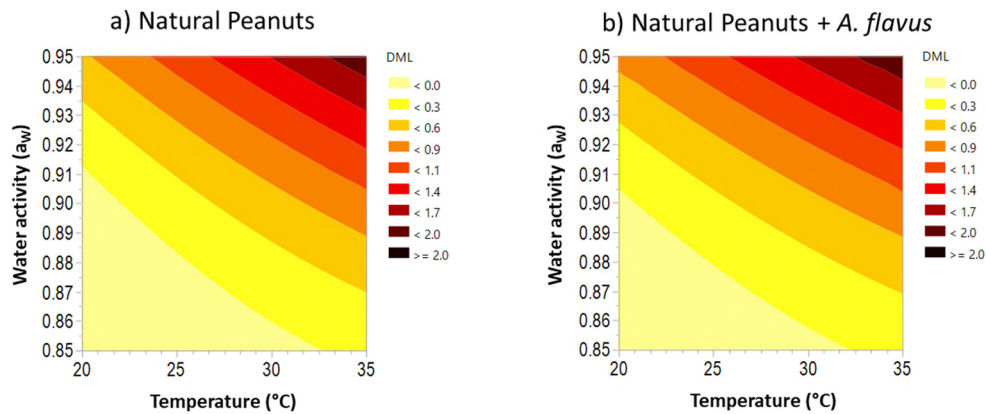


Figure 4. DMLs contour maps describing the relationship with different environmental parameters in (a) naturally contaminated and (b) that inoculated with *A. flavus* spores and stored under various combinations of environmental treatments.

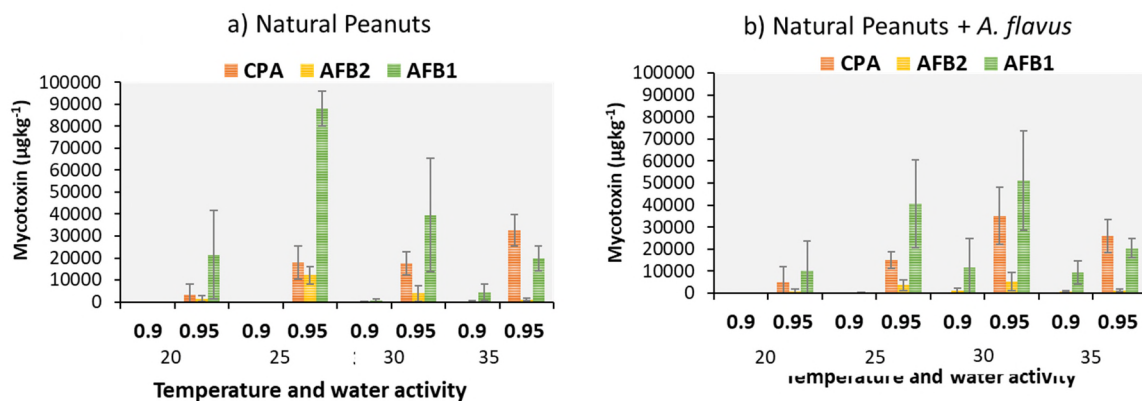


Figure 5. Mycotoxin contamination after 7 days in natural peanuts (a) and with *A. flavus* (b) and stored at different a_w levels and different temperatures. Vertical bars represent the standard deviation of the mean.

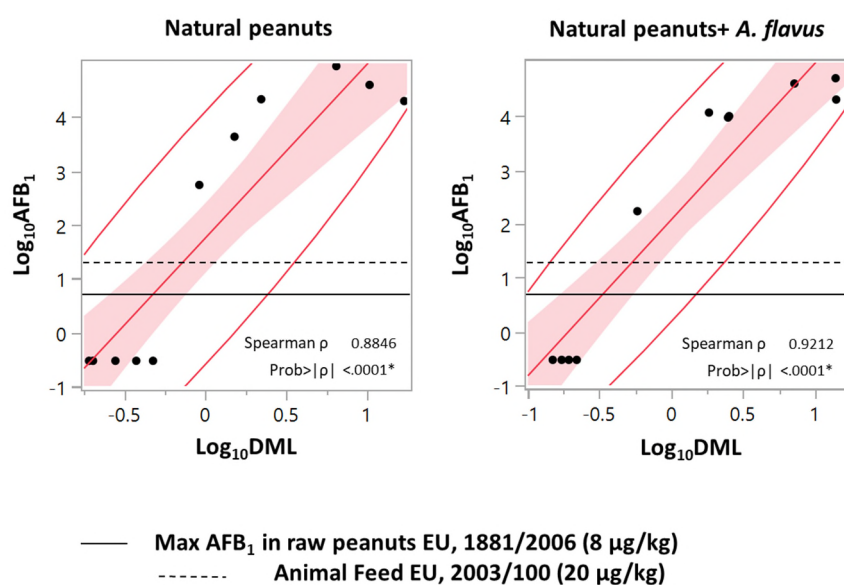


Figure 6: Scatter plot of DMLs and AFB_1 in stored peanuts after 7 days storage under all the environmental conditions examined in (a) naturally contaminated peanuts and (b) peanuts inoculated with *A. flavus*. Horizontal lines represent EU legislative limits. Non-parametric Spearman's correlation Ellipse $\alpha=0.95$ was used to analyse the data.

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Table 1. Estimated parameters from polynomial regression and maximum adjuster r^2 percentage (%) DMLs

	Natural Peanuts			Natural Inoculated Peanuts		
Parameter	Estimate	Std Error	p-value	Estimate	Std Error	p-value
intercept	-7.820	0.407	<0.0001	-8.13	0.446	<0.0001
T	0.037	0.005	<0.0001	0.035	0.005	<0.0001
a_w	7.779	0.429	<0.0001	8.258	0.470	<0.0001
$T \cdot a_w$	0.662	0.073	<0.0001	0.594	0.079	<0.0001
a_w^2	87.446	7.381	<0.0001	89.006	8.077	<0.0001
r^2		0.532			0.494	

Only significant parameters have been included in the table.

References

- Abdel-Hadi, A., Carter, D., Magan, N., 2010. Temporal monitoring of the nor-1 (afID) gene of *Aspergillus flavus* in relation to aflatoxin B1 production during storage of peanuts under different water activity levels. J. Appl. Microbiol. 109, 1914–1922.
<https://doi.org/10.1111/j.1365-2672.2010.04820.x>
- Atayde, D.D., Reis, T.A., Godoy, I.J., Zorzete, P., Reis, G.M., Corrêa, B., 2012. Mycobiota and aflatoxins in a peanut variety grown in different regions in the state of São Paulo, Brazil. Crop Prot. 33, 7–12. <https://doi.org/10.1016/j.cropro.2011.11.013>
- Chapman, G. W., Robertson, J. A. 1980. The effect of soybean moisture during storage on the lipid composition of extracted crude oil. J. Am. Oil Chem. Soc. 57(10), 339–342.
- Commission, E., 2006. Commission Regulation (EC) No. 1881/2006 of 19 December 2006, Setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union 364, 5–24.
- Commission, E., 2003. Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed (Text with EEA relevance). Off. J. L 285 , 01/11/2003 P. 0033 - 0037; L285, 0033–0037.
- de Souza, G., Mossini, S., Galerani, Arrotéia, C., Kemmelmeier, C., Machinski, M., 2014. Evaluation of the mycoflora and aflatoxins from the pre-harvest to storage of peanuts :a case study. Acta Sci. - Agron. 36, 27–33.
<https://doi.org/10.4025/actasciagron.v36i1.16972>
- Dhingra, O. D., Jham, G. N., Rodrigues, F. A., Silva Jr, G. J., Costa, M. L. N. 2009. Fumigation with essential oil of mustard retards fungal growth and accumulation of ergosterol and free fatty acid in stored shelled groundnuts. J. Stored Prod. Res. 45(1), 24–31.
- Fleurat-Lessard, F., 2017. Integrated management of the risks of stored grain spoilage by seedborne fungi and contamination by storage mould mycotoxins – An update. J. Stored Prod. Res. 71, 22–40. <https://doi.org/10.1016/j.jspr.2016.10.002>
- Food and Agriculture Organisation of the United Nations, 2018. Data-Production-Crops-World + total-Production Quantity-Groundnuts with shell-2016. [WWW Document]. FAOSTAT. URL <http://www.fao.org/faostat/en/#data/QC> (accessed 8.26.18).
- Garcia-Cela, E., Kiaitsi, E., Sulyok, M., Krska, R., Medina, A., Petit Damico, I., Magan, N., 2019. Influence of storage environment on maize grain: CO₂ production, dry matter losses and aflatoxins contamination. Food Addit. Contam. Part A 36, 175–185.
<https://doi.org/10.1080/19440049.2018.1556403>
- Garcia-Cela, E., Kiaitsi, E., Sulyok, M., Medina, A., Magan, N., 2018. *Fusarium graminearum* in stored wheat: Use of CO₂ production to quantify dry matter losses and relate this to relative risks of zearalenone contamination under interacting environmental conditions. Toxins (Basel). 10. <https://doi.org/10.3390/toxins10020086>
- Garcia-Cela, E.; Kiaitsi, E.; Medina, A.; Rubio-Lopez, F.; Magan, N., 2018. CO₂ production as early indicator of Pest and Fungal infestation: Effective for Post-harvest Decision Support System in stored Cereals. Poster 16 Sensors 2018. Innovative Chemical Sensing. London, United Kingdom. <http://www.rsc.org/events/detail/31365/sensors-2018-innovative-chemical-sensing>
- Gonçalves, E., Nogueira, J.H.C., Fonseca, H., Felicio, J.D., Pino, F.A., Corrêa, B., 2008. Mycobiota and mycotoxins in Brazilian peanut kernels from sowing to harvest. Int. J. Food Microbiol. 123, 184–190. <https://doi.org/10.1016/j.ijfoodmicro.2008.01.012>
- Hill, R., Blankenshi, Paul, Cole, R., Sanders, T., 1983. Effects of Soil Moisture and Temperature on Preharvest Invasion of Peanuts by the *Aspergillus flavus* Group and Subsequent Aflatoxin Development 45, 628–633.
- Horn, B., 2017. Colonization of wounded peanut seeds by soil fungi : selectivity for species from *Aspergillus* section *Flavi* Colonization of wounded peanut seeds by soil fungi: selectivity for species from *Aspergillus* section *Flavi*. Mycologia. 202–2017.
<https://doi.org/10.1080/15572536.2006.11832854>
- Lacey, J., Hamer, A., Magan, N., 1994. Respiration and dry matter losses in wheat grain

- under different environmental factors, in: Highley, E., Wright, E.J., Banks, H.J., Champ, B.R. (Eds.), In *Stored Product Protection*. pp. 1007–1013.
- Liu, X., Guan, X., Xing, F., Lv, C., Dai, X., Liu, Y., 2017. Effect of water activity and temperature on the growth of *Aspergillus flavus*, the expression of aflatoxin biosynthetic genes and aflatoxin production in shelled peanuts. *Food Control* 82, 325–332. <https://doi.org/10.1016/j.foodcont.2017.07.012>
- Magan, N., Aldred, D., Mylona, K. Lambert, R.J.M., 2010. Limiting mycotoxins in wheat. *Food Addit Contam.* 27, 644-650
- Malachova, A., Sulyok, M., Beltran, E., Berthiller, F., Krska, R., 2015. Multi-Toxin Determination in Food – The Power of ‘Dilute and Shoot’ Approaches in LC–MS–MS.
- Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., Krska, R., 2014. Optimization and validation of a quantitative liquid chromatography–tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. *J. Chromatogr. A* 1362, 145–156. <https://doi.org/10.1016/j.chroma.2014.08.037>
- Martín Castaño, S., Medina, A., Magan, N., 2017a. Impact of storage environment on respiration, dry matter losses and fumonisin B1 contamination of stored paddy and brown rice. *World Mycotoxin J.* 10, 319–326. <https://doi.org/10.3920/WMJ2017.2237>
- Martín Castaño, S., Medina, A., Magan, N., 2017b. Comparison of dry matter losses and aflatoxin B1 contamination of paddy and brown rice stored naturally or after inoculation with *Aspergillus flavus* at different environmental conditions. *J. Stored Prod. Res.* 73, 47–53. <https://doi.org/10.1016/j.jspr.2017.06.004>
- Mylona, K., Magan, N., 2011. *Fusarium langsethiae*: Storage environment influences dry matter losses and T-2 and HT-2 toxin contamination of oats. *J. Stored Prod. Res.* 47, 321–327. <https://doi.org/10.1016/j.jspr.2011.05.002>
- Mylona, K. 2012a. *Fusarium* species in grains: dry matter losses, mycotoxin contamination and control strategies using ozone and chemical compounds. PhD Thesis. Cranfield University, Cranfield, Beds. MK43 0AL, U.K.
- Mylona, K., Sulyok, M., Magan, N., 2012b. Exposure & Risk Assessment Relationship between environmental factors , dry matter loss and mycotoxin levels in stored wheat and maize infected with *Fusarium* species. *Food Additives & Contaminants : Part A : Chemistry, Analysis, Control*, 37–41.
- Nakai, V.K., de Oliveira Rocha, L., Gonçalves, E., Fonseca, H., Ortega, E.M.M., Corrêa, B., 2008. Distribution of fungi and aflatoxins in a stored peanut variety. *Food Chem.* 106, 285–290. <https://doi.org/10.1016/j.foodchem.2007.05.087>
- Passone, M.A., Rosso, L.C., Ciancio, A., Etcheverry, M., 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of nor-1 gene, and effects of storage conditions on aflatoxin production. *Int. J. Food Microbiol.* 138, 276–281. <https://doi.org/10.1016/J.IJFOODMICRO.2010.01.003>
- Paterson, R., Lima, N., 2011. Further mycotoxin effects from climate change. *Food Res. Int.* 44, 2555–2566. <https://doi.org/10.1016/j.foodres.2011.05.038>
- Saul, R.A., Lind, E.F., 1958. Maximum Time for Safe Drying of Grain With Unheated Air. *Trans. ASAE* 1, 0029–0033. <https://doi.org/10.13031/2013.41203>
- Seitz, L., Sauer, D., Mohr, H., Aldis, D., 1982. Fungal Growth and Dry Matter Loss During Bin Storage of High-Moisture Corn. *Cereal Chem.*
- Sultan, Y., Magan, N., 2010. Mycotoxigenic fungi in peanuts from different geographic regions of Egypt. *Mycotoxin Res.* 26, 133–140. <https://doi.org/10.1007/s12550-010-0048-5>
- Vaamonde, G., Patriarca, A., Pinto, V.E.F., 2006. Effect of water activity and temperature on production of aflatoxin and cyclopiazonic acid by *Aspergillus flavus* in peanuts, in: *Advances in Experimental Medicine and Biology*. pp. 225–235. https://doi.org/10.1007/0-387-28391-9_15
- White, N.D.G., Sinha, R.N., Muir, W.E., 1982. Intergranular carbon dioxide as an indicator of biological activity associated with the spoilage of stored wheat. 24, 35–42.

- Zorzete, P., Baquião, A., Atayde, D., Reis, T., Gonçalves, E., Corrêa, B., 2013. Mycobiota, aflatoxins and cyclopiazonic acid in stored peanut cultivars. *Food Res. Int.* 52, 380–386. <https://doi.org/10.1016/j.foodres.2013.03.029>
- Zorzete, P., Reis, T.A., Felício, J.D., Baquião, A.C., Makimoto, P., Corrêa, B., 2011. Fungi, mycotoxins and phytoalexin in peanut varieties, during plant growth in the field. *Food Chem.* 129, 957–964. <https://doi.org/10.1016/j.foodchem.2011.05.053>
- Zorzete, P., Baquião, A.C., Atayde, D.D., Reis, T.A., Gonçalves, E., & Corrêa, B., 2013. Mycobiota, aflatoxins and cyclopiazonic acid in stored peanut cultivars. *Food Res Int*, 52(1), 380-386.

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