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Effect of storage temperature and duration on concentrations of 27 fungal secondary metabolites spiked into floor dust from an office building

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Abstract

Fungi are ubiquitous in environments and produce secondary metabolites that are usually low-molecular-weight organic compounds during growth processes. Dust samples containing these fungal secondary metabolites collected from study sites are often stored in certain temperature conditions for an extended period until laboratory analysis resources are available. However, there is little information on how stable fungal secondary metabolites are over time at different storage temperatures. We examined the stability of 27 fungal secondary metabolites spiked into floor dust samples collected from a moisture-damaged office building. Ninety-five dust aliquots were made from the spiked dust; five replicates were randomly assigned to a baseline (time=0) and each of the 18 combinations of three temperatures (room temperature, 4 °C, or −80 °C) and six time points (2, 12, 25, 56, 79, and 105 weeks). At the baseline and each subsequent time point, we extracted and analyzed the fungal secondary metabolites from the spiked dust using ultra-performance liquid chromatograph-tandem mass spectrometer. To estimate change in concentration over storage time at each temperature condition, we applied multiple linear regression models with interaction effect between storage temperature and duration. For 10 of the 27 fungal secondary metabolites, the effect of time was significantly (p-values <0.05) or marginally (p-values <0.1) modified by temperature, but not for the remaining 17 metabolites. Generally, for most fungal secondary metabolites, storage at room temperature was significantly (p-values <0.05) associated with a larger decline in concentration (up to 83% for 3-nitropropionic acid at about 11 months) than storing at 4 °C (up to 55% for emodin) or −80 °C (55% for asperglaucide). We did not observe significant differences between storage at 4 °C, or −80 °C. Storage temperature influenced degradation of fungal secondary metabolites more than storage time. Our study indicates that fungal secondary metabolites, including mycotoxins in floor dust, quickly degrade at room temperature. However, storing dust samples at 4 °C might be adequate given that storing them at −80 °C did not further reduce degradation of fungal secondary metabolites.

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Keywords

Degradation; dust storage; moisture damage; Mycotoxin; UPLC-MSMS

INTRODUCTION

Excess moisture in building materials promotes fungal growth, which could result in occupant exposure to fungal agents including secondary metabolites, and potentially lead to various respiratory and non-respiratory illnesses- *e.g.*, exacerbation of asthma, upper and lower respiratory symptoms, respiratory infections, and eczema (WHO guidelines for indoor air quality 2009; Mendell et al. 2011; Park and Cox-Ganser 2011). Fungal secondary metabolites (FSMs) are also produced during fungal growth in moisture-damaged environments (Calvo et al. 2002). A study by Kirjavainen et al. (2016) analyzed dust samples from 93 houses with or without water-damage for microbial metabolites, and detected 42 microbial secondary metabolites, including 3-nitropropionic acid, alternariol, alternariol monomethylether, emodin, ochratoxin A, roquefortine C, stachybotrylactam, and sterigmatocystin. The study reported that moisture-damaged homes with visible mold or mold odor tended to be associated with an increased number of metabolites. Täubel et al. (2011) detected 33 microbial secondary metabolites in 69 samples from homes, including alternariol, alternariol monomethylether, chaetoglobosin A, emodin, roquefortine C, stachybotrylactam, and sterigmatocystin. More recently, Park et al. (2018) reported 29 FSMs in 22 floor dust samples collected from a water-damaged office building, including 3-nitropropionic acid, asperglaucide, alternariol monomethylether, citreorosein, cyclo(L-pro-L-tyr), emodin, and neoechinulin A. These studies indicate that FSMs might be ubiquitous in indoor environments.

Although FSMs are likely to be present in indoor environments, their measured concentrations are generally lower than a few nanograms per milligram of dust (less than a nanogram for many FSMs) (Täubel et al. 2011; Kirjavainen et al. 2016; Park et al. 2018). In an earlier publication, we demonstrated that there were substantial matrix effects in an analytical method for quantification of FSMs in indoor dust using an ultra-performance liquid chromatograph-tandem mass spectrometer (UPLC-MSMS) (Jaderson and Park 2019). Thus, the matrix effects are likely to contribute to an underestimation of the true concentrations of those FSMs. Additionally, those FSMs may undergo chemical changes through biological degradation by fungi or bacteria (Ji et al. 2016), or chemical reactions with existing other chemicals in dust. Some FSMs may quickly degrade at room temperature environments once they are released, which may explain presence of FSMs with generally low concentration indoors. However, there are few studies examining the stability of FSMs in indoor environments and other storage conditions.

Dust samples collected at study sites are usually transported at room temperature or in a cooler to laboratories before they are processed, aliquoted, and analyzed. Researchers frequently need to store dust samples at 4 °C, −20 °C, or −80 °C for a few weeks until they are analyzed. In some cases, samples may need to be stored for a longer period until resources for the analysis are available. Therefore, room temperature or other temperature

conditions and storage time may influence the concentration of FSMs. In this study, we examined the stability of FSMs spiked into floor dust samples collected from a moisture-intruded office building; the samples had been stored at room temperature and two additional storage temperatures (4 °C and −80 °C) for up to approximately two years.

MATERIALS AND METHODS

Chemicals and standards

We selected 27 FSM standard materials. Of these, two thirds (18 of 27) were mycotoxins that have been demonstrated to be harmful to humans and animals (Table 1) (Miller and McMullin 2014) and the rest were FSMs that are frequently and commonly detected in indoor environments (Täubel et al. 2011; Kirjavainen et al. 2016; Park et al. 2018). Table 1 lists the FSMs and mycotoxin standards included in our experiment and their suppliers. Methanol (> 99.9%, LC-MS grade), acetic acid (≥ 99.7%, LC-MS grade), and ammonium acetate (≥ 99.0%, LC-MS grade) were purchased from Fisher Scientific (Waltham, MA, USA). Acetonitrile (≥ 99.5%, LC-MS grade) was purchased from EMD Millipore (Burlington, MA, USA) and Fisher Scientific. Ultrapure water was collected through an Advantage 10 ultra-filtration assembly (EMD Millipore) with resistivity of 18.2 MΩ•cm at 25 °C.

Study design and sample preparation

We collected 120 floor dust samples in a building with a history of water incursion located in the northeastern region of the United States in June 2007 (Park et al. 2017; Park et al. 2018). The sampling method involved vacuuming a 2 square meter (m^2) carpeted-floor area around each workstation or the floor along the edge of exterior walls for five min using a L'il Hummer backpack vacuum cleaner (100 CFM, 1.5 horse power, Pro-Team Inc., Boise, ID, U.S.A.) equipped with polyethylene filter socks. The dust was sieved and homogenized before being divided into aliquots. The detailed sampling method is described in earlier publications (Park et al. 2008; Cho et al. 2016; Park et al. 2017). We selected ten dust samples collected from the study and combined (pooled) them to secure enough dust (3.3 g dust) to make the appropriate number of aliquots for the experiment. The combined dust was homogenized again using a rotary homogenizer (model RKVSD, Appropriate Technical Resources, Laurel, Maryland, U.S.A.) for three hr to make a uniform medium within the pooled dust. Then we spiked the pooled dust with 2,195 μl of standard solution in acetonitrile having two different concentrations for the FSMs. Eleven FSMs were spiked at a concentration of 6.25 ng per mg dust (ng/mg) and the remaining 16 FSMs at a higher concentration of 25 ng/mg (Table 1). The spiking concentrations used were based on the different degree of loss (low versus high) by two groups of FSMs (Table 1) during extraction in the sample preparation (minor loss) and during analysis using the UPLC-MSMS due to dust matrix effect (major loss) (Jaderson and Park 2019). The spiked pooled dust in an open 10-ml polypropylene conical tube was placed in a chemical hood to allow for full evaporation of acetonitrile of the standard solution. The pooled dust was divided into 95 aliquots containing 30.0 to 31.2 mg dust measured with a balance (model AR0640, OHAUS, Pine Brook, New Jersey, U.S.A.). We randomly assigned five replicates of the dust aliquots to a baseline (time=0: without storage) and each of the 18 combinations between three

temperature conditions (room temperature, 4 °C, and −80 °C) and six different storage durations (2, 12, 25, 56, 79, and 105 weeks). The five dust aliquots that were randomly assigned to the baseline were extracted and analyzed immediately after we had spiked the dust aliquots with FSMs for comparison. The remaining dust aliquots were analyzed at each of the six different time points. The dust aliquots that were used for spiking FSMs were not autoclaved or sterilized.

We used a previously published extraction protocol for the FSMs (Jaderson and Park 2019). Briefly, we added 1,000 μl of extraction solution to each aliquot, shook the aliquots for 90 minutes using a rotary shaker (model 4625, LAB-LINE Industries, Inc., Melrose Park, Illinois, U.S.A.), centrifuged for three min at 1962.09×g (model Sorval Legend XT, Thermo Scientific, Waltham, Massachusetts, U.S.A.), and transferred 900 μl of the supernatant to an 8-ml borosilicate test tube. Then we evaporated the solution in each test tube under gentle nitrogen stream in a chemical hood, and reconstituted the dried extract with 150 μl of mobile phase solution (30% methanol/69% water/1% acetic acid by volume) for chromatography. Ten microliters of each extract were injected twice into the UPLC coupled with a tandem massspectrometer.

Analytical methods by UPLC-MSMS

We used an UPLC (Acquity H Class, Waters, Milford, MA, U.S.A.) equipped with an Acquity UPLC BEH C18 column for chromatographic separation of the FSMs. Programed gradient flow was used to control the flow of the mobile phase solvents. We used a tandem mass spectrometer (MSMS) (Acquity Xevo TqD Quadrupole Tandem Mass-Spectrometer, Waters, Milford, MA, U.S.A.) for quantification of the FSMs. Of the 27 FSMs, 23 were analyzed in positive ionization mode and the remaining four (alternariol, citreorosein, emodin, and zearalenone) in negative ionization mode (Table 2). The positive mode tune method included capillary voltage of 0.5 kV, a desolvation temperature of 350 °C, and a desolvation flow of 650 L/hr. MSMS parameters included RF lens 2.5 V, extractor 3 V, source temperature 150 °C, and cone gas flow 0 L/hr. The negative mode tune method had the same parameters as the positive mode except for a capillary voltage of 3.0 kV. Our previous publication[8] provides a detailed description of the UPLC-MSMS methodology. Table 2 lists the precursor ions, product ions, cone voltage, collision energy, and retention time for each of the analytes involved in the analyses using the UPLC-MSMS.

Statistical methods

For statistical analyses, we calculated the difference between the concentrations measured at each time point and the baseline. We first computed the amount of each FSM per mg of dust for each dust aliquot by dividing the total measured amount in 150 μl of the final reconstituted volume (ng/aliquot) by the total mass of dust. Then we subtracted the average concentration (ng/mg) of the five baseline replicates from the measured concentration at each of the timelines by temperature condition. Thus, positive or negative values in the difference indicate increase or decrease, respectively, in concentrations from the baseline. Using these difference data starting at Weeks 2 (the first assay after the baseline) to 105 (the last assay), we performed multiple linear regressions with interaction effects between temperature condition and storage time. The interaction models examined whether the effect

of the storage time for each FSM is modified by the storage temperature condition (Neter et al. 1996). In these models, we assumed that the change starting at Week 2 would be linear over the duration of the experiment for each temperature condition to examine general trend in changes over time. The interaction models included the changes from the baseline as a dependent variable, and the storage time (continuous as the number of weeks), the storage temperature condition (categorical), and interaction between the two as independent variables. For the FSMs with marginal ($0.05 < p < 0.1$) or significant ($p \leq 0.05$) interaction effects, we estimated the temperature-specific time effect for each FSM (slope: change/week) and calculated mean changes for each temperature at the midpoint (46.5 weeks: approximately 11 months) of the storage duration from the baseline. For the FSMs with no significant interaction effects ($p > 0.1$), we removed the interaction term from the interaction models, and then estimated the coefficient of the time variable and least square mean (LSM) changes (model-adjusted means) from the baseline for each temperature condition. We also calculated changes in concentration at the midpoint (46.5 weeks: approximately 11 months) of the storage duration from the baseline by multiplying the model coefficient of the storage time (slope: change/week) variable by 46.5. To compare the effects among the three storage temperature conditions, we performed multiple comparisons using the Tukey honestly significant difference (HSD) test (Tukey 1949).

We also calculated % Change in concentration at the midpoint of storage duration from the baseline for each FSM as follows:

$$\%Change = \frac{change\ at\ the\ midpoint\ \ from\ the\ baseline}{mean\ concentration\ at\ the\ baseline} \times 100 \quad (1)$$

To examine the repeatability of the instrument, we injected twice from each standard (neat) and sample (extract) vial and calculated percent coefficient of variation as follows (Matuszewski 2006):

$$\%CV = \frac{standard\ deviation\ of\ responses\ of\ duplicate\ injection}{mean\ of\ responses\ of\ duplicate\ injection} \times 100 \quad (2)$$

We considered $p \leq 0.05$ as statistically significant and $0.05 < p \leq 0.1$ as marginally significant. All analyses were performed using JMP 13.0.0 (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Changes in concentration for FSMs with interaction effect

Concentrations of most of the FSMs for the first assay at week two stayed similar to or declined from the baseline, except for citrinin, chaetoglobosin A, neoechinulin A, and roquefortine C, which increased in concentration (Figure 1). The linear regression models with the interaction effect indicated that for 10 of the 27 FSMs, the effect of the storage time was significantly or marginally modified by the temperature condition (Table 3 and Figure 2). For the majority of these FSMs, storage at room temperature had a significantly (Tukey HSD p-values<0.05) larger decline (from 36% for nivalenol to 83% for 3-nitropropionic acid) at the midpoint of the storage time than at 4 °C (11% for nivalenol to 47% for aflatoxin

G2) or at −80 °C (22% for nivalenol to 51% for aflatoxin G2) except for alternariol, citrinin, and citreorosein. For alternariol and citreorosein, although the interaction models identified marginal (for alternariol) or significant (for citreorosein) interaction effects, Tukey HSD multiple comparison did not find that they had different time effects by temperature. Concentration of citrinin declined substantially (55%) over time when stored at room temperature, compared with storage at 4 °C or −80 °C, for which there were no significant time effects. When stored at room temperature, citrinin, fumonisin B_1, 3-nitropropionic acid, and aflatoxins G_1 and G_2 were among the FSMs with the largest decline in concentration (55–83%) at the midpoint while deoxynivalenol, stachybotrylactam, and nivalenol concentrations declined less than 50%.

Changes in concentration for FSMs without interaction effect

The interaction models indicated that the storage time effect on concentration in dust for 17 of 27 FSMs was not modified by the temperature condition. For these FSMs, we performed regression analyses with no interaction and the results are presented in Table 4 and Figure 3. For four of the 17 FSMs (alternariol monomethylether, emodin, ochratoxin A, and zearalenone), there was no storage temperature effect on concentration while there was small (7–11% decline at 46.5 weeks from the baseline) but significant time effect. Concentration of T2-toxin was neither affected by storage temperature nor time. For two of the 17 FSMs [neosolaniol and cyclo(L-Pro-L-Tyr)], there was no storage time effect but a significant storage temperature effect. Tukey HSD multiple comparison among three temperature conditions for these two FSMs indicated that samples stored at room temperature had significantly lower concentrations (52–55% at the midpoint) than those stored at 4 °C (43–47% for both FSMs) or −80 °C (43% for neosolaniol only).

Concentrations of the remaining 10 among the 17 FSMs with no interaction were significantly but independently affected by both storage temperature and time. However, the effect of storage temperature was generally larger than the time effect except for neoechinulin A (Table 4 and Figure 2). Tukey HSD multiple comparison indicated that the decline of concentration in dust samples stored at room temperature (25 °C) was significantly larger than those at 4 °C or −80 °C, except for three FSMs - chaetoglobosin A, neoechinulin A, and roquefortine C. Concentration of chaetoglobosin A had substantially increased at the midpoint of the experiment time, and neoechinulin A and roquefortine C had only slightly increased at the midpoint (Figure 2). However, all three FSMs had a declining trend over time from week 2. Generally, degrees of decline from the baseline concentration for the other seven FSMs stored at 4 °C were not statistically different from those stored at −80 °C, while the FSMs in stored dust at room temperature had significantly larger decline in concentration than in the refrigerated or frozen samples.

Among those ten FSMs having independent temperature and time effects above, asperglaucide, aflatoxin B_1, and verrucarin A had a significant and the largest (68%, 55%, and 48%, respectively) decline at the midpoint when stored at room temperature compared with other FSMs. Sterigmatocystin, verrucarol, and aflatoxin B_2 also had moderate (38%, 36%, and 32%, respectively) decline from the baseline at room temperature. For those six FSMs, their decline when stored at room temperature was significantly greater than when

stored at 4 °C and −80 °C (6–44%). Yet, time effect for those six FSMs was substantially smaller (8–18%) than temperature effect, except for verrucarol and aflatoxin B_2 that had slightly increased concentrations over time. Percent coefficient of variation (%CV) of duplicate injections for each spiked dust sample ranged from 1.1% for stachybotrylactam to 8.2% for 3-nitropropionic acid, which indicated acceptable performance of the instrument for each analysis (Rosner 1995).

DISCUSSION

We found that FSMs (including mycotoxins) spiked into floor dust collected from water-intruded buildings were generally degraded over time in all three storage temperature conditions. Compared to room temperature, storage at 4 °C or −80 °C appeared to delay the degradation of FSMs. Nonetheless, aflatoxins B_1, G_1, and G_2, fumonisin B_1, 3-nitropropionic acid, asperglaucide, cyclo(L-pro-L-tyr), diacetoxyscirpenol, emodin, and verrucarin A still declined more than 30% at 4 °C or −80 °C at 11 months from the baseline. Although samples stored at −80 °C had slightly more degradation compared with those stored at 4 °C, we did not find a statistical difference between the two temperature conditions for all the FSMs. For the FSMs with independent effects of temperature and storage time (Table 4 and Figure 3) on concentration, we found that storage temperature condition was more important than storage time for most of the FSMs. The independent time effect at 11 months (the mid-point of the storage duration in our study) from the baseline for most of these FSMs was minimal (less than 20%) except for chaetoglobosin A, neoechinulin A, and roquefortine C that showed a substantial decline over time (higher than 45% decline). This finding indicated that dust samples containing FSMs should not be stored at room temperature condition for later analysis. However, storage at 4 °C may be acceptable and freezing dust for storage may not be necessary to delay degradation.

Garcia et al. reported aflatoxin G_1 was less stable than aflatoxin B_1 in standard solutions of ethyl acetate, methanol, and water, which is consistent with our finding of higher degradation of aflatoxins G_1 and G_2 than aflatoxins B_1 and B_2 in stored dust samples (Garcia et al. 1994). Widestrand and Pettersson examined stability of trichothecenes (T2-toxin, deoxynivalenol, and nivalenol) in thin film after the standard solution was evaporated under nitrogen stream (Widestrand and Pettersson 2001). They did not observe any changes in stability of T2-toxin over time in different temperature conditions (room temperature, 4 °C, or −80 °C), which is also consistent with our finding of no time and temperature effect on T2-toxin in stored dust. However, they observed significant degradation of deoxynivalenol (19% decline from the initial concentration) after 2 years and of nivalenol (22%) after nine months of storage at room temperature. They also found significant decomposition of deoxynivalenol after two years of storage at 4 °C (21%). In our dust samples stored at room temperature, we observed 46% and 36% degradation in 11 months for deoxynivalenol and nivalenol, respectively, while we found only 13% decline of deoxynivalenol in dust stored at 4 °C. Nonetheless, we are not aware of any published study evaluating the stability of FSMs in building dust stored in different temperature conditions over time. Our study indicated that most of the tested FSMs, including mycotoxins (except for T-2 toxin), in dust were degraded over time during storage with greater temperature effect than time effect.

When the FSMs remained at room temperature the decline was much more evident than at 4 °C or −80 °C. Many FSMs in samples aged 11 months at room temperature declined more than 50% from the initial concentration; these included aflatoxins B_1, G_1, and G_2, asperglaucide, cyclo(L-Pro-L-Tyr), emodin, neosolaniol, citrinin, fumonisin B_1, and 3-nitropropionic acid. Of these, 3-nitropropionic acid, aflatoxin G_2, and asperglaucide were the three FSMs that degraded the most. In general, most mycotoxins (FSMs demonstrated to be harmful to humans and animals) (Miller and McMullin 2014) were also easily degradable at room temperature, and the decline in the concentration in 11 months from the baseline was up to 66% for aflatoxin G_1 to 70% for aflatoxin G_2, except that the concentration of roquefortine C did increase slightly. Some of the most frequently found fungal species in damp environments are *Aspergillus versicolor* and *Aspergillus ochraceus*, which are known to produce sterigmatocystin (a precursor for aflatoxin) or ochratoxin A (Miller and McMullin 2014). These mycotoxins have been detected in dust from moisture-damaged building materials (Tuomi et al. 2000; Engelhart et al. 2002; Bloom et al. 2007; Bloom et al. 2009; Täubel et al. 2011; Kirjavainen et al. 2016); and their detected concentrations appeared to be extremely low (<5pg/mg of dust) or they were non-detectable for most of the floor dust samples. This infrequent low-level detection or non-detection might be explained by the following three conditions: 1) mycotoxins may not be frequently and sufficiently (i.e., enough to be detected) produced by relevant fungal species in water-damaged environments as indicated by Tuomi et al. (2000); 2) released mycotoxins may be degraded over time at room temperature as we found in our study; or 3) the dust matrix present during detection of mycotoxins with UPLC-MSMS may substantially suppress ionization, resulting in considerable underestimation of the true concentrations (Jaderson and Park 2019). In our study, we could not determine which of these conditions could generally play a dominant role in real environments, but it is reasonable to postulate that any combination of the three conditions could contribute to the low-level detection or non-detects of mycotoxins in floor dust samples.

There are some limitations in our study. During the study period (approximately two years), managing the shared UPLC-MSMS with other projects at the same level of sensitivity and accuracy was challenging. Thus, the variability in performance of the instrument over time might have contributed to the variable results over storage time. Unfortunately, we could not separate this variability from the results, which could be a limitation of our study. However, because the storage time effect was much smaller than the storage temperature effect, the effect of the variable performance of instrument on the results would be minimal. We observed increases in concentration for several FSMs (e.g., citrinin, deoxynivalenol, nivalenol, verrucarol, verrucarin A), particularly at weeks 25 and 56. Different level of performance of the instrument during the time period or growth of certain types of fungi producing those metabolites during the time might explain the increases although we were not able to confirm. To simplify the prediction of the amount of changes from the baseline and compare them among the different FSMs, we assumed that changes in concentration over time would be linear. However, the actual changes may not linearly occur over time and thus our linearity assumption could be another limitation of the study.

CONCLUSIONS

We found that FSMs were generally degraded over time in three storage temperature conditions (room temperature, 4 °C, and −80 °C) with larger temperature effect than time effect. Compared with storing the dust samples containing FSMs at 4 °C and −80 °C, storing at room temperature led to greater decline in concentration, indicating potential decomposition of FSMs including mycotoxins in indoor environments after they are released from fungi. Thus, it is recommended that dust samples to be analyzed for FSMs should not be stored at room temperature. The finding of no statistical difference in concentrations of FSMs between storage at 4 °C and −80 °C indicated that freezing dust samples to be analyzed for FSMs during storage is likely unnecessary.

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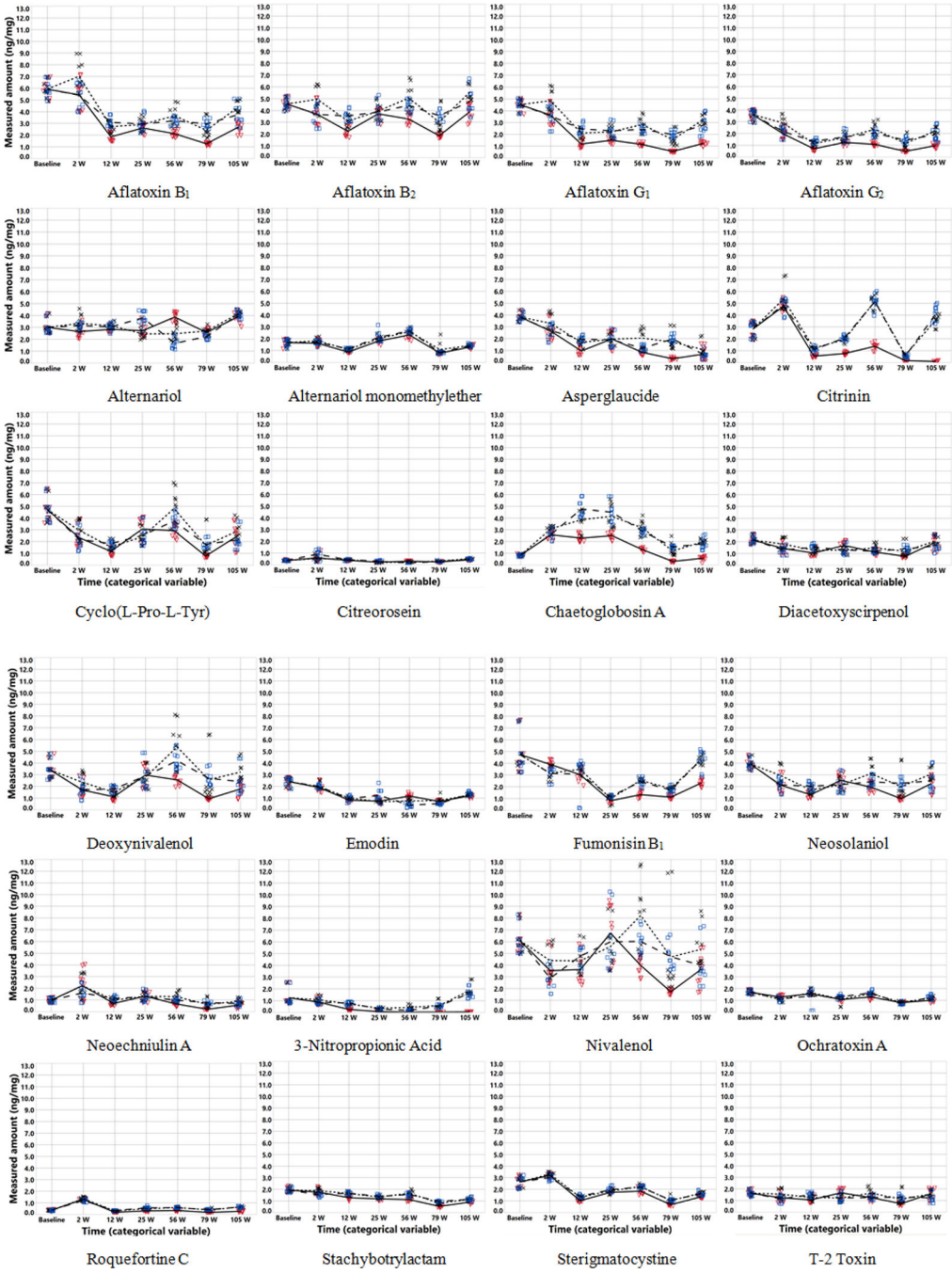

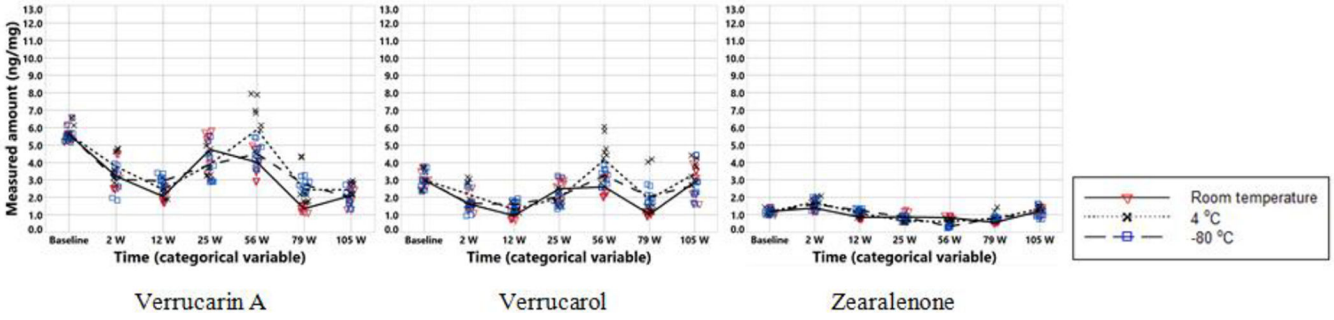

Figure 1.

Actual measured concentrations of fungal secondary metabolite for the entire period of storage from the baseline to weeks 105 (two years) by storage temperature condition.

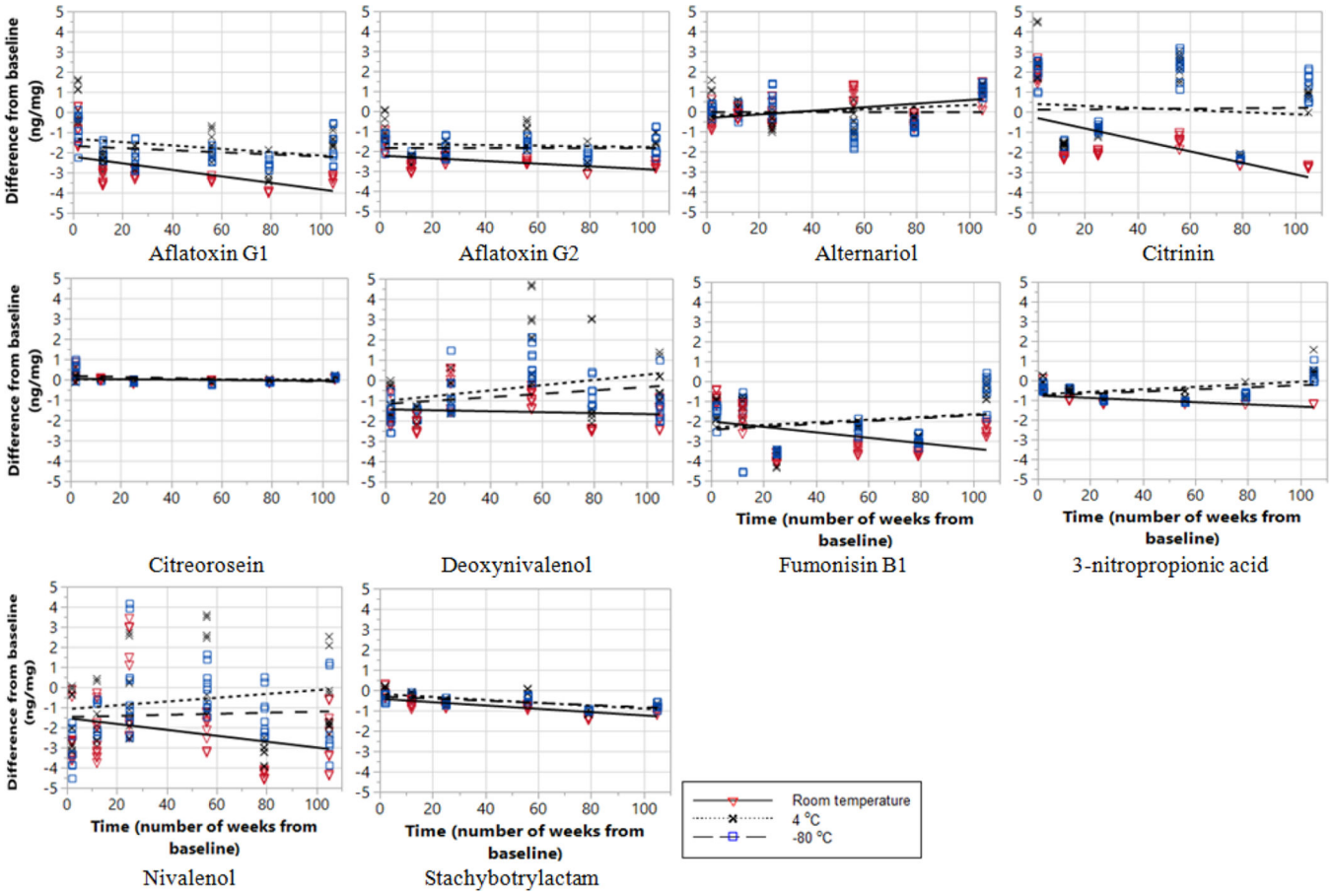

Figure 2.

Trend of the changes in fungal secondary metabolite concentration with linear regression line by temperature for the metabolites with interaction effects.

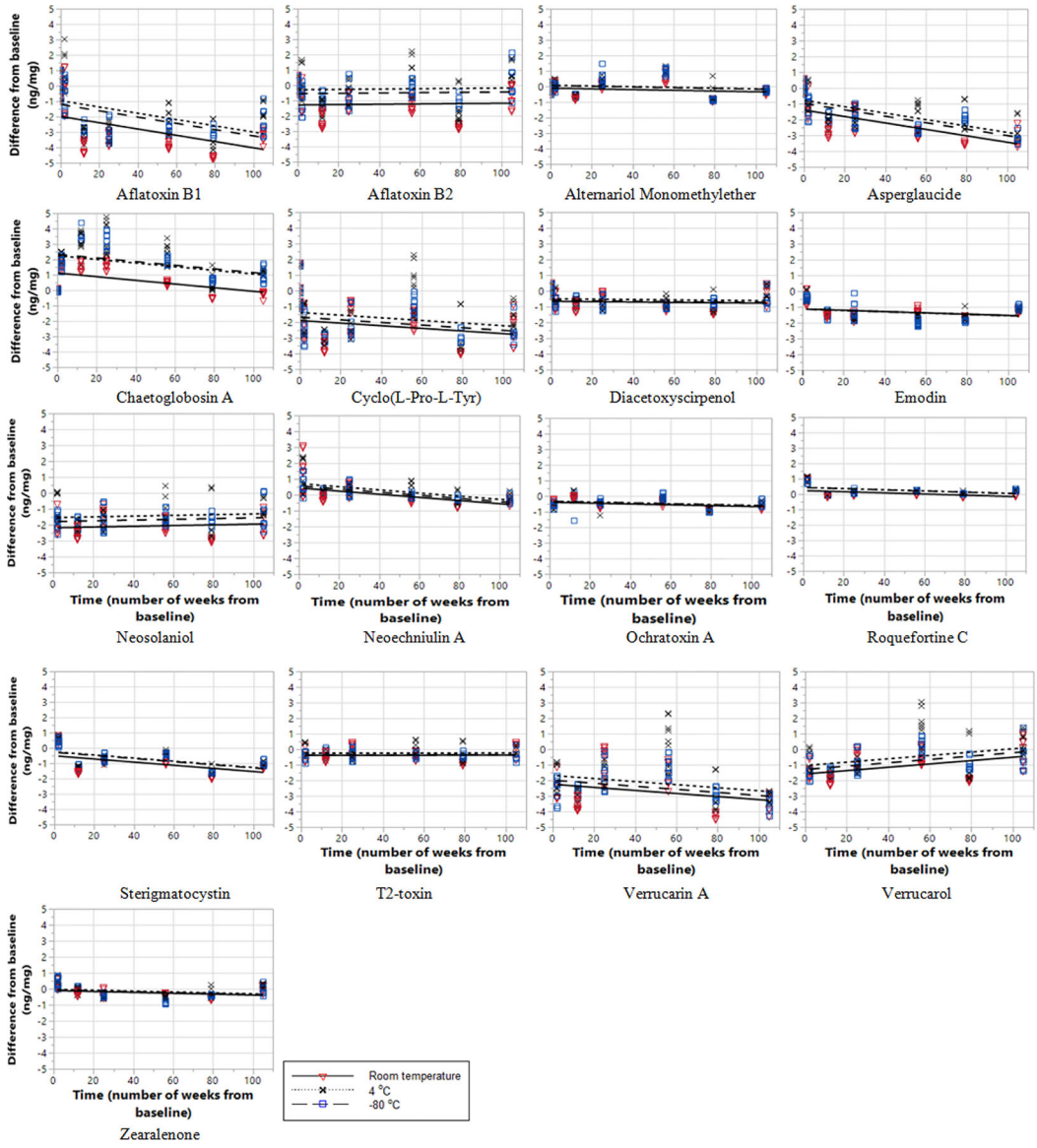

Figure 3.

Trend of the changes in fungal secondary metabolite concentration with linear regression line by temperature for the metabolites without interaction effect.

Table 1.

Fungal secondary metabolites used in the study and the amount spiked into dust.

No.	Metabolite	Supplier	CAS No.	Spiked amount (ng/mg)
1	3-Nitropropionic acid	Sigma-Aldrich	504-88-1	6.25
2	Aflatoxin B_1 *	Sigma-Aldrich	1162-65-8	6.25
3	Aflatoxin B_2 *	Fermentek	7220-81-7	6.25
4	Aflatoxin G_1 *	Sigma-Aldrich	1165-39-5	6.25
5	Aflatoxin G_2 *	Sigma-Aldrich	7241-98-7	6.25
6	Alternariol	Sigma-Aldrich	641-38-3	6.25
7	Alternariol monomethylether	Adipogen	26894-49-5	6.25
8	Asperglaucide	ChemFaces	56121-42-7	6.25
9	Chaetoglobosin A *	Adipogen	50335-03-0	25
10	Citreorosein	ChemFaces	481-73-2	25
11	Citrinin *	Sigma-Aldrich	518-75-2	6.25
12	Cyclo(L-Pro-L-Tyr)	Bioaustralis	4549-02-4	6.25
13	Deoxynivalenol *	Sigma-Aldrich	51481-10-8	6.25
14	Diacetoxyscirpenol *	CAYMAN	2270-40-8	25
15	Emodin	Sigma-Aldrich	518-82-1	6.25
16	Fumonisin B_1 *	Sigma-Aldrich	116355-83-0	25
17	Neoechinulin A	ChemFaces	51551-29-2	25
18	Neosolaniol *	Sigma-Aldrich	36519-25-2	6.25
19	Nivalenol *	Fermentek	23282-20-4	6.25
20	Ochratoxin A *	Sigma-Aldrich	303-47-9	25
21	Roquefortine C *	Santa Cruz	58735-64-1	25
22	Stachybotrylactam	Santa Cruz	163391-76-2	25
23	Sterigmatocystin *	Sigma-Aldrich	10048-13-2	6.25
24	T2-Toxin *	Fermentek	21259-20-1	25
25	Verrucarin A *	Sigma-Aldrich	3148-09-2	6.25
26	Verrucarol *	Sigma-Aldrich	2198-92-7	6.25
27	Zearalenone *	Sigma-Aldrich	17924-92-4	25

Sigma-Aldrich, St. Louis, MO, U.S.A.; Fermentek, Jerusalem, Israel; Adipogen, San Diego, CA, U.S.A.; ChemFaces, Hubei, China; Bioaustralis, Smithfield, NSW, Australia; CAYMAN, Ann Arbor, MI, U.S.A.; Santa Cruz, Dallas, TX, U.S.A.

* Mycotoxins that were known to be harmful to human beings.

Table 2.

UPLC-MSMS parameters for analyses of the fungal secondary metabolites.

Metabolite	Precursor ion (m/z)	Cone voltage (V)	Product ions (m/z) (1st, 2nd)	Collision energy (V) (1st, 2nd)	Retention time (min)
3-Nitropropionic acid *†	117.8 $[M-H]^-$	20	45.9	6	2.1
Aflatoxin B_1	313.1 $[M+H]^+$	62	241.1, 284.9	40, 24	5.4
Aflatoxin B_2	315.2 $[M+H]^+$	58	287.1, 259.1	28, 30	5.3
Aflatoxin G_1	329.1 $[M+H]^+$	58	243.0, 311.0	28, 22	5.2
Aflatoxin G_2	331.1 $[M+H]^+$	60	313.0, 245.0	26, 32	5.0
Alternariol *	257.0 $[M-H]^-$	56	212.3, 189.0	24, 24	5.9
Alternariol monomethylether	273.2 $[M+NH4]^+$	54	127.9, 115.0	50, 54	6.8
Asperglaucide	445.5 $[M+H]^+$	50	367.2, 349.2	18, 18	5.9
Citrinin	251.2 $[M+H]^+$	28	233.0, 191.0	18, 26	5.6
Citreorosein *	285.1 $[M-H]^-$	62	224.0, 241.0	34, 26	6.2
Chaetoglobosin A	529.5 $[M+H]^+$	32	130.0, 511.3	38, 10	6.6
Cyclo(L-Pro-L-Tyr)	261.2 $[M+H]^+$	36	136.0, 154.1	18, 28	4.3
Diacetoxyscirpenol	384.3 $[M+NH4]^+$	24	307. 2, 105.0	12, 32	5.6
Deoxynivalenol	281.2 $[M+H]^+$	26	109, 233.1	22, 10	4.7
Emodin *	269.0 $[M-H]^-$	66	225.0, 240.9	28, 28	7.5
Fumonisin B_1	722.6 $[M+H]^+$	62	352.3, 704.4	38, 32	5.8
Neosolaniol	400.3 $[M+NH4]^+$	20	305.1, 215.1	12, 20	4.7
Neoechinulin A	324.3 $[M+H]^+$	28	156. 9, 130.0	42, 52	5.8
Nivalenol	313.2 $[M+H]^+$	26	205.1, 125.0	12, 12	4.0
Ochratoxin A	404.3 $[M+H]^+$	32	239.0, 358.1	22, 14	6.3
Roquefortine C	390.2 $[M+H]^+$	16	193.0, 322.1	30, 22	6.3
Stachybotrylactam	386.4 $[M+H]^+$	70	178.1, 150.1	38, 46	7.0
Sterigmatocystin	325.2 $[M+H]^+$	50	310.1, 281.0	22, 54	6.8
T2-Toxin	484.4 $[M+NH4]^+$	26	305.1, 185.1	14, 20	6.3
Verrucarin A	520.4 $[M+NH4]^+$	24	249.1, 457.2	18, 14	6.2
Verrucarol	267.1 $[M+H]^+$	16	249.1, 231.1	6, 12	4.9
Zearalenone *	317.1 $[M-H]^-$	52	130.9, 174.9	30, 26	6.6

*Metabolites which were analyzed in negative ionization mode.

†We found only one transition for 3-Nitropropionic acid.

Table 3.

The effect of storage duration by temperature condition for the fungal secondary metabolites with interaction effects.

Metabolite	Change, ng/mg (% change)*			P-value for interaction
	Room temperature	4 °C	−80 °C	
Aflatoxin G_1	-2.97^{B} (−66)	-1.71^{A} (−38)	-1.92^{A} (−43)	**0.05**
Aflatoxin G_2	-2.53^{B} (−70)	-1.70^{A} (−47)	-1.84^{A} (−51)	0.06
Alternariol	0.09^{A} (3)	0.04^{A} (1.2)	-0.01^{A} (−0.3)	0.06
Citrinin	-1.54^{B} (−55)	0.16^{A} (6)	0.14^{A} (5)	**<0.01**
Citreorosein	-0.01^{A} (−2)	0.01^{A} (2)	0.06^{A} (16)	**0.03**
Deoxynivalenol	-1.55^{B} (−46)	-0.43^{A} (−13)	-0.79^{A} (−23)	**0.05**
Fumonisin B_1	-2.65^{B} (−56)	-2.02^{A} (−43)	-2.10^{A} (−44)	**<0.01**
3-Nitropropionic acid	-1.03^{B} (−83)	-0.41^{A} (−33)	-0.53^{A} (−43)	**<0.01**
Nivalenol	-2.21^{B} (−36)	-0.65^{AB} (−11)	-1.36^{A} (−22)	0.07
Stachybotrylactam	-0.80^{B} (−40)	-0.51^{A} (−27)	-0.55^{A} (−28)	0.07

Results of Tukey HSD (honestly significant difference) multiple comparisons among three temperature conditions are indicated by superscripted letters. The levels that are not sharing the same superscripted letter indicate significant difference at α=0.05.

*The change (% change) was estimated at 46.5 weeks from the baseline (approximately the midpoint of the storage duration). Significant p-values are bolded.

Table 4.

The effect of storage duration and temperature for fungal secondary metabolites without interaction effects.

Metabolite	LSM* change in ng/mg (% change) in each temperature condition				Change in 46.5 weeks (% change)**	
	Room temperature	4 °C	−80 °C	P-value	Midpoint time	P-value
Aflatoxin B_1	-3.26^{B} (−55)	-2.07^{A} (−35)	-2.36^{A} (−40)	**< 0.01**	−0.58 (−10)	**< 0.01**
Aflatoxin B_2	-1.44^{B} (−32)	-0.28^{A} (−6)	-0.58^{A} (−13)	**< 0.01**	0.23 (5)	**0.02**
Alternariol MME†	-0.23^{A} (−14)	-0.01^{A} (−0.5)	-0.03^{A} (−2)	0.10	−0.11 (−7)	**0.05**
Asperglaucide	-2.58^{B} (−68)	-1.85^{A} (−49)	-2.08^{A} (−55)	**< 0.01**	−0.67 (−18)	**< 0.01**
Chaetoglobosin A	0.75^{B} (90)	2.06^{A} (246)	2.17^{A} (259)	**< 0.01**	−1.05 (−126)	**< 0.01**
Cyclo(L-Pro-L-Tyr)	-2.60^{B} (−55)	-2.01^{A} (−43)	-2.35^{AB} (−50)	**0.02**	0.05 (1)	0.68
Diacetoxyscirpenol	-0.82^{A} (−38)	-0.63^{A} (−30)	-0.76^{A} (−35)	0. 10	0.1 (5)	**0.03**
Emodin	-1.31^{A} (−55)	-1.32^{A} (−55)	-1.32^{A} (−55)	0.99	−0.19 (−8)	**< 0.01**
Neosolaniol	-2.08^{B} (−53)	-1.44^{A} (−37)	-1.68^{A} (−43)	**< 0.01**	0.11 (3)	0.14
Neoechinulin A	-0.03^{B} (−3)	0.25^{A} (25)	0.1^{AB} (11)	**0.03**	−0.46 (−47)	**< 0.01**
Ochratoxin A	-0.52^{A} (−31)	-0.45^{A} (−27)	-0.45^{A} (−27)	0.43	−0.12 (−7)	**< 0.01**
Roquefortine C	0.06^{B} (16)	0.26^{A} (70)	0.25^{A} (68)	**< 0.01**	−0.18 (−47)	**< 0.01**
Sterigmatocystin	-0.99^{A} (−38)	-0.72^{A} (−28)	-0.76^{A} (−29)	**0.05**	−0.47 (−18)	**< 0.01**
T2-toxin	-0.38^{A} (−23)	-0.24^{A} (−15)	-0.36^{A} (−22)	0.12	0.01 (0.4)	0.85
Verrucarin A	-2.71^{B} (−48)	-2.14^{A} (−38)	-2.44^{AB} (−44)	**0.05**	−0.46 (−8)	**< 0.01**
Verrucarol	-1.08^{B} (−36)	-0.54^{A} (−18)	-0.79^{A} (−26)	**< 0.01**	0.5 (17)	**< 0.01**
Zearalenone	-0.23^{A} (−20)	-0.16^{A} (−13)	-0.19^{A} (−16)	0.56	−0.13 (−11)	**< 0.01**

Results of Tukey HSD (honestly significant difference) multiple comparisons among three temperature conditions are indicated by superscripted letters. The levels that are not sharing the same superscripted letter indicate significant difference at α=0.05. Negative indicates decline from the baseline.

*LSM: least square mean.

**The change (% change) was estimated at 46.5 weeks from the baseline (approximately the midpoint of the storage duration).

†MME: monomethyl ether. Significant p-values are bolded.