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Letter to the Editor

"Analyses of Black *Aspergillus* Species of Peanut and Maize for Ochratoxins and Fumonisins," A Comment on: *J. Food Prot.* 77(5):805–813 (2014)

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In a recent article in *Journal of Food Protection,* Palencia and co-workers *(34)* published a survey on black *Aspergillus* species isolated from maize and peanuts reporting the production of some mycotoxins. An important claim in the article is their strains of *Aspergillus niger* produce fumonisins B_1 and B_3. However, for many reasons, we consider the production of these two fumonisin isomers unlikely.

Our respective groups *(8, 9, 14–16, 21–24, 27–30, 33, 38, 43, 47)* among others *(1, 2, 10, 35, 36, 42, 45, 46)* have screened more than 2,000 strains and never found any strain producing the B_1 and B_3 isomers. This includes more than 30 reference strains from the US *(15)* and also strains isolated from Californian raisins *(27).* Only one other study *(51)* claimed that these FB_1 and FB_3 isomers were produced by *A. niger* which was also questioned *(31),* and in later reports from this research group on *Aspergillus niger,* FB_1, and FB_3 were not detected *(52)* while all the expected B_2, B_6, and B_4 were. Many of the studies have used natural substrates *(9, 10, 27, 38, 52)* including peanuts *(26).*

We also note that Palencia et al. *(34)* have not identified the strains studied by appropriate methods, which currently is sequencing of β-tubulin and calmodulin *(15, 20, 39, 50).*

Further, there is no homologue of the FUM2 gene in the DNA sequences from the three genome sequenced isolates of *A. niger* strains (one originated from the USA). Since this gene encodes the enzyme used for the C-10 hydroxylation *(3, 12, 40)* and thus for the production of FB_1 and FB_3 *(37, 47),* current genome data suggest it is impossible for *A. niger* to produce FB_1 and FB_3.

Looking at the data of Palencia et al. *(34)* from a biosynthetic perspective, we find further evidence of an analytical problem. FBs are produced in a number of enzymatic steps to yield FB_4 as a single product. From this point on a P450-oxygynase (encoded by FUM2) will hydroxylate the C-10 and a dioxygenase (encoded by FUM3) hydroxylate the C-5. However the yields of these enzyme reactions are never 100% and thus FB_1, FB_2, FB_3, and FB_4 are always detected both in culture extracts and natural samples *(4–7, 11, 13, 17–19, 24, 40, 41, 48, 49, 53–56).* It is possible that FUM2 and/or FUM3 are defective or missing, but in this case, FB_1 and FB_3 will be missing or FB_1 and FB_2, respectively *(3, 12, 40).* Thus, samples such as RRC 458 where FB_3 is not detected while FB_1 and FB_2 are not, does not biosynthetically make sense.

Other problematic examples in the article include the data for *A. tubingensis* RRC 512. Currently, fully genome sequenced strains of this species do not contain the whole fumonisin gene cluster. Furthermore, strains of this species (when identified correctly by both β-tubulin and calmodulin sequences) *(15, 20, 39, 44, 50)* have not been shown to produce FBs.

There are also concerns with the description of the chromatography. These include an HPLC flow rate that appears is 4 to 5 times too low for the optimal flow rate for the column used. If this is correct, this would explain the poor separation illustrated in Figure 3. In our experience, it seems unlikely that FB_1 and FB_6 were separated. Since FB_6 elutes between FB_1 and FB_3, this may be a further source of problems. FB_1 and FB_6 cannot be differentiated by MS/MS without reference standard as they only differ by slight intensity differences due the water loss ability of FB_6 *(25).*

Finally we cannot read how the LC-MS^n data files were interpreted, was a positive identification done on a single extracted ion from full scan MS, e.g., $[M+H]^+$? Were qualifier ions used or were MS/MS spectra used each time as in Figure 3, and in that case how many ions and how were the MS/MS spectra interpreted? With the high numbers of metabolites produced by *A. niger* complex (e.g., Fig. 2 in Nielsen et al. 2009 *(32)*) several metabolites will give peaks near the retention time of fumonisins in the same *m/z* range as FB_1–FB_3, e.g., such as pestalamide A (=tensidol B) creating an intense signal at *m/z* 706, from the A+2 isotope of the $[2M+NH_4]^+$ ion.

We believe that the reporting of FB_1 and FB_3 by Palencia et al. *(34)* is not sufficiently well documented for publication and recommend that it be withdrawn until independently confirmed.

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Response

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In the published article of Palencia et al. *(13),* our objective was to provide evidence that isolates of the black *Aspergillus* species from maize and peanuts can produce the fumonisins, mycotoxins, and the ochratoxins that was indicated by analyses of isolates of this group from grapes and similar products here in the USA *(14).* The work was designed to provide a brief and certainly not a survey of hundreds of isolates from these two crops for this ability. We developed a rapid detection and identification procedure of the black species of *Aspergillus (12)* that was developed using a rep-PCR system with 20 morphotypes of black *Aspergillus* species, including several type species, obtained from Maren A. Klich *(6)* and identified with her assistance and directions. In this work we compared these morphospecies with data generated from ITS and partial calmodulin regions *(12).* This study served as the basis of our work on the species and strains used in Palencia et al. *(13).* We found no need to perform entire sequences as suggested. However, Nielsen et al. *(9)* in a Letter to the Editor criticized our identity of species within this group, and their ability to produce specific isomers, and finally concentrations of the fumonisins. Nielsen et al. *(9)* have issued other letters to the editor along these same lines of concern *(9, 11)*: valid determination of species and validity of concentrations.

To test the ability of these strains to produce the fumonisins and ochratoxins, we cultured them onto maize, a natural substrate from which some were isolated. Our data stands as published, and we are only one of two groups who used natural products to determine toxin production. In addition to our work, Magnoli et al. *(7, 8)* reported the production of ochratoxin on maize from black aspergilli strains isolated from maize and peanuts from Argentina. They did not examine, however, their strains for the fumonisins. While we examined our isolates for both toxins, we found the reverse: no strains producing ochratoxins while some were positive for isomers of the fumonisins. The point here is there are apparent differences in isolates, and these differences will reflect genetic differences. Isolates from grapes, dried vine fruits, and maize show differences in their ability to produce specific mycotoxins in the USA and other parts of the planet *(3, 14, 15),* not to mention further variation in structures of