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Kinetic study of aflatoxins' degradation in the presence of ozone

S. Agriopoulou [a], A. Koliadima [b], G. Karaiskakis [b], J. Kapolos [a, *]

[a] Department of Food Technology, Technological and Educational Institution of Peloponnese, 24100 Kalamata, Greece
[b] Department of Chemistry, University of Patras, 26504 Patras, Greece

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ABSTRACT

The degradation of aflatoxins AFB1, AFB2, AFG1 and AFG2 after treatment of their solutions in triple distilled water with ozone was studied and the ability of ozone, even at low concentrations, to degrade aflatoxins was proved. Ozone concentrations of 8.5, 13.5, 20, 25 and 40 ppm were applied at different temperatures on aflatoxin solutions in triple distilled water of 10 ppb and 2 ppb and the complete and rapid elimination of AFB1 and AFG1 was observed while AFB2 and AFG2 remain more or less stable. The kinetic equations for the degradation procedure were calculated by applying ozone on neutral buffer solutions of aflatoxin at 298.15, 308.15, 318.15 and 328.15 K at ozone concentrations of 8.5, 13.5 and 20 ppm and the rate constants, were determined. The degradation of aflatoxins was described by a first order kinetic equation. Finally, the activation energies during degradation of aflatoxins were calculated from the Arrhenius equation.

1. Introduction

Aflatoxins (AFs) are widely distributed mycotoxins produced by strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Kurtzman, Horn, & Hesseltine, 1987). Additionally other species which produce aflatoxins are *Aspergillus pseudotamarii*, *Aspergillus ochraceoroseus*, *Aspergillus rambellii*, *Aspergillus toxicarius*, *Emericella astellata*, *Emericella olivicola* and *Emericella venezuelensis* (Reiter, Zentek, & Razzazi, 2009). Moreover other fungi of the genera *Aspergillus* (e.g., *A. ochraceus* and *A. carbonarius*) produce another important mycotoxin, ochratoxin A (OTA) (Sarigiannis, Kapolos, Koliadima, Tsegenidis, & Karaiskakis, 2014). Aflatoxins are extremely harmful to the health of humans and animals, as showing carcinogenic, mutagenic, teratogenic and immunosuppressive actions (Zinedine & Mañes, 2009). The known aflatoxins are about twenty, while the four main aflatoxins which are mainly found in tropical and subtropical climates (Inan, Pala, & Doymaz, 2007), are referred as aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2). The aflatoxins M1 (AFM1) and M2 (AFM2), which are hydroxylated metabolites of AFB1 and AFB2, can be found in all kinds of milk and dairy products from animals that have consumed contaminated feed with aflatoxins (Giray, Girgin, Engin, Aydın, & Sahin, 2007; Hussain & Anwar, 2008). Aflatoxins are frequently found in many human foodstuffs or animal feeds (Ali et al., 2005; Chiavaro et al., 2001; Nasir & Jolley, 2002; Park, 2002). From all the food-contaminating aflatoxins, AFB1 is usually the predominant mycotoxin (Jolly et al., 2006) and the International Agency for Research on Cancer (IARC) has classified aflatoxin AFB1 in the most toxic group (1 carcinogen), which primarily affects the liver (IARC, 1993). As food and health bodies have not established a tolerable daily intake (Tolerable Daily Intake, TDI) for humans, contamination in food, should be reduced to the lowest possible level (Pietri, Bertuzzi, Agosti, & Donadini, 2010).

Several factors have been reported as means of reducing the levels of aflatoxins. These are natural, such as mechanical separation of the clean product from the contaminated product (Karaca, 2010), heating at high temperatures, effects of radiation (Karaca, 2010; Reddy et al., 2009) and light, grinding, washing, and use of adsorbents (Karaca, 2010) or chemicals, such as ammoniation (Reddy et al., 2009), influence of acids and bases, influence of oxidizing agents or with various inorganic and organic chemicals (Karaca, 2010). Between the oxidizing agents used for the reduction or elimination of aflatoxins, a powerful oxidant is ozone. Ozone is a highly unstable trioatomic oxygen molecule (O_3) formed by the addition of an oxygen atom to a molecular diatomic oxygen (O_2) that can be generated readily and economically for application to several commodities (USA, 1997). It is one of the most potential oxidants that has several advantages, most prominently the absence of detectable residues on treated products. Ozone can be generated on-site, eliminating the need to store or dispose of

* Corresponding author.
E-mail address: jkapolos@teikal.gr (J. Kapolos).

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chemical containers. Ozone has been used to reduce various types of mycotoxins, including the aflatoxins (Chen et al., 2014; Inan et al., 2007; Luo et al., 2014; Zorlugenç, Kiroğlu Zorlugenç, Öztekin, & Evliya, 2008). Ozone reacts across the 8, 9 double bond of the terminal furan ring of aflatoxin through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids (Inan et al., 2007).

Although there have been many studies on ozone reactions with aflatoxins at many important agricultural products, very little kinetic data have been reported. In order to effectively treat aflatoxins with ozone and design appropriate treatment processes, reaction kinetics must be determined.

In the present study the influence of ozone on the degradation of aflatoxins AFB1, AFB2, AFG1 and AFG2 in triple distilled water was investigated, as well as the appropriate kinetic equations for the above procedure in neutral buffer solutions were determined. Determination of activation energy was also performed from the Arrhenius equation, for the degradation of aflatoxins AFB1 and AFG1, in the presence of ozone, in neutral buffer solutions. As far as we know rate constants and activation energies for the degradation of aflatoxins in the presence of ozone are calculated for the first time.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH) (all of HPLC grade) were purchased from Merck (Darmstadt, Germany). Standards solutions of AFB1, AFB2, AFG1, AFG2, were obtained from the company R-Biopharm (Rhône Diagnostic Technologies Ltd., Glasgow, UK). All solutions were prepared with triple distilled water. Triple distilled water was prepared in a special ultra-clean water system (Purelab Ultra, Elga Labwater, Marlow, UK) for producing ASTM Type I. The freshly prepared mobile phase was degassed for 20 min before use with a Branson 2510 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. Phosphate buffer solution was prepared with potassium dihydrogen phosphate (34.02 g) and disodium hydrogen phosphate (35.40 g) (all purchased form Merck) in 1 L triple distilled water. The final pH of the solution was adjusting to 7 by adding a few drops of 0.5 M NaOH. The solution was then stored at 4 °C in a sealed flask until use.

2.2. Aflatoxin standards in water

In order to study the degradation of aflatoxins in triple distilled water in the presence of different concentrations of ozone, aflatoxin standards in water were prepared. The stock solution of the four aflatoxins AFB1, AFB2, AFG1 and AFG2, was reconstituted by dissolving the content of standard in 10 mL of pure acetonitrile, obtaining 5 μg/mL of total aflatoxins (2 μg/mL of each aflatoxin AFB1 and AFG1 and 0.5 μg/mL of each aflatoxin AFB2 and AFG2) and stored at 4 °C. The preparation of standard solutions of aflatoxins included in a first stage the preparation of a stock solution, concentration of 200 ng/mL in acetonitrile. From this solution, were prepared by appropriate dilutions in water, working solutions with concentration of 1, 1.5, 2.5, 5, 7.5 and 10 ng/mL and stored at 4 °C until use in order to construct the four calibration curves.

Six-point calibration curves, with triplicate injections, covering the range of 1–10 ng/mL for each aflatoxin, were constructed. Calibration curves were drawn using the average response for each standard concentration employed.

2.3. Ozone production

Ozone was produced by using an ozone generator (TG-10 Ozone Generator, Ozone Solutions, IA, USA) for the transformation of O_2 molecules to O atoms and then forming O_3 by using high voltage current. Ozone was canalized to a constant conditions environmental chamber (KBF 115, Binder, Tuttlingen, Germany) in order to control temperature and relative humidity and after that, through a two port valve, was lead to an ozone analyzer (Ozone analyzer Model UV-100, ECO SENSORS, INC.). When a constant concentration of ozone was measured, the gas stream was guided throw the two port valve to a jacked beaker which was maintained at a constant temperature by mean of a heating circulator (F12-ED, Julabo Seelbach, Germany), (cf. Fig. 1).

For the ozonolysis experiments an appropriate volume of a stock solution, containing all the aflatoxins in CH_3CN, was added in 50 mL volumetric flask. Two different aflatoxin solutions in triply distilled water were prepared with final concentrations of 10 ppb for AFB1 and AFG1 and 2.5 ppb for AFB2 and AFG2 (first solution) and 2 ppb for AFB1 and AFG1 and 0.5 ppb for AFB2 and AFG2 (second solution). After that, each solution was added in the jacked beaker which was maintained at a constant temperature (298.15 K, 308.15 K, 318.15 K, 328.15 K and 338.15 K). Each aflatoxin solution in triple distilled water was exposed for 20 min on gaseous stream containing ozone at five different concentrations of 8.5, 13.5, 20, 25 and 40 ppm.

In order to obtain kinetic results for the degradation procedure, instead of triply distilled water, buffer solution at pH 7 was used for preparing the aflatoxin solutions of the concentration of 10 and 2 ppb. In contrast to the use of five ozone concentrations that were used for the ozonolysis, for the kinetic study the first three concentrations were used, because the two largest gave no results due to the rapid rate of the phenomenon of degradation. In order to study the influence of ozone on aflatoxin concentration as well as to determine the kinetic equations, every two minutes, a sample of 500 μL, was collected and analyzed. For each experiment fresh aflatoxin solutions were prepared. The quantification of aflatoxins was calculated by the peak area according to the calibration curve.

2.4. HPLC analysis of aflatoxins

The aflatoxin concentrations were determined by using an LC-20A Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a spectrofluorometric detector (RF-20A/20Axs), a system controller (CBM-20A/20Alite), a column oven (CTO-20A/20AC), an autosampler (SIL-20AHT/20ACHT) and an on-line degasser (DGU-20A3/20A5). The spectrofluorometric detector was operated at an excitation wavelength of $\lambda_{ex} = 365$ nm and an emission wavelength of $\lambda_{em} = 430$ nm. Water-methanol-acetronitrile (56:22:22 v/v/v) at a flow rate of 1 mL/min under isocratic conditions was used as mobile phase, the pressure was 113–115 bar and the total run time was 17 min. Separation was completed by Mycotox™ reversed-phase column (C18, 4.6 × 250 mm, 5 μm) (Pickering Laboratories Inc., California, USA). The column temperature was set to 42 °C and sample volumes of 10 μL were injected in triplicate. The samples were analyzed after post-column derivatization with a Pinnacle PCX instrument (Pickering Laboratories Inc., California, USA). The reagent used was iodine (100 mg/L of I_2 in water), the volume of the reactor was 1.4 mL, the flow rate was 0.3 mL/min and the reactor temperature was 90 °C. The solution of iodine was degassed using a nitrogen gas stream for 15 min. Aflatoxin AFG2 was eluted first followed by AFG1, AFB2 and AFB1 with retention times of approximately 7.5, 8.6, 9.3 and 10.8 min, respectively. The chromatograms were analyzed by LC solution LC-Assist software (Shimadzu). Aflatoxin concentration was expressed in μg of aflatoxin