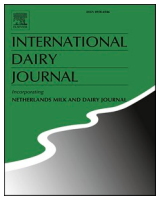
International Dairy Journal 160 (2025) 106103

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj



Research paper

The effect of variations in cold plasma conditions on the detoxification of Aflatoxin M1 and degradation products

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ARTICLE INFO

Keywords:
Dielectric barrier discharge
Plasma gas mix
Reactive species
Toxicity
Aspergillus sp.
Dairy

ABSTRACT

The aim of this study was to explore the chemical reactive species of different operating gases, and their effect on the degradation of aflatoxin M1 (AFM1) by cold plasma by measuring the reactive species concentration. Helium, at 80, 90 or 95%, was used mixed with oxygen, nitrogen and air. The efficacy of cold plasma on aflatoxin M1 (AFM1) reduction was improved when decreasing the ratio of helium in the gas mixture. The ratio of the gas mixtures changed the cold plasma chemistry believed to be due to the differences in the concentrations of the reactive species. The degradation products of AFM1 after cold plasma treatment using a helium/air gas mixture and the degradation pathway were identified by LCMS. AFM1 was oxidised by reactive species in the cold plasma to produce degradant products with, theoretically, lower toxicity than AFM1.

1. Introduction

Aflatoxin M1 (AFM1) in milk has been recognized as a toxin of concern for over 30 years and it is still a food safety issue worldwide. Cows consume feed contaminated with fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) that produce aflatoxin B1 (AFB1) which is converted into AFM1 in the cow and subsequently released into milk. The AFM1 concentration level in milk is lower than the maximum residue level in Italy, Morocco and Turkey (Alahlah, El Maadoudi, Bouchriti, Triqui, & Bougtaib, 2020; Armorini, Altafini, Zaghini, & Roncada, 2016; Özturk Yilmaz & Altinci, 2019). However, the highest level of AFM1 contamination in milk and milk products exceeded the MRL in some others countries such as Ethiopia, Nigeria, Brazil, Ecuador, Mexico, India, Indonesia and Iran (Agus, Nuryono, Purwanti, & Sumantri, 2019; Anthony et al., 2016; Gonçalves et al., 2017; Puga-Torres, Salazar, Cachiguango, Cisneros, & Gómez-Bravo, 2020; Quevedo-Garza, Amador-Espejo, Cantú-Martínez, & Trujillo-Mesa, 2018; Sharma, Jadhav, & Garg, 2020; Tadesse, Berhanu, & Woldegiorgis, 2020). This highlights the need for reducing AFM1 in milk as this compound, has been classified a group 1 carcinogen (IARC, 2002). Milk is one of the most common sources of nutrition for humans, especially for infants and elderly people, due to its nutritious components including protein, vitamins and minerals. Therefore, the occurrence of AFM1 in milk raises an important food safety issue leading to a risk to human health in the most susceptible in the human population. Consequently, the control of AFM1 in milk is necessary.

Many treatments have been used to decrease AFB1 from feedstuffs or AFM1 in milk to reduce the risk. These include yeast, lactic acid bacteria, enzymes, peroxide, ozone, UV light and plasma (Nguyen, Palmer, & Flint, 2020a). Plasma is the fourth state of matter following solid, liquid and gas. Plasma consists of a wide range of reactive species including ions, electrons, free radicals, stable conversion products and energetic photons (Bourke, Ziuzina, Han, Cullen, & Gilmore, 2017). These reactive species are responsible for controlling food contamination including microbial inactivation (Patil, Bourke, & Cullen, 2016). The production of these reactive species in plasma or the chemistry of plasma is determined by the operating gases and the set-up of plasma induction unit (Whitehead, 2016). Plasma is divided into nonequilibrium (non-thermal), which operates typically at room temperature and atmospheric pressure, and equilibrium (thermal) forms (Misra, Schlüter, & Cullen, 2016). This make it a potentially useful method for food treatment as it reduces the effect of temperature on nutritional components of food.

Cold plasma has been used to reduce AFB1 in corn; hazelnuts and

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https://doi.org/10.1016/j.idairyj.2024.106103
Received 17 April 2024; Received in revised form 25 September 2024; Accepted 30 September 2024
Available online 9 October 2024

AFM1 in skim and whole milk (Nguyen, Palmer, Phan, et al., 2022; Nikmaram & Keener, 2022; Shi, Cooper, Stroshine, Ileleji, & Keener, 2017; Siciliano et al., 2016). It is highly effective in degrading aflatoxins over a short time (several minutes), thus reducing the treatment cost and impact on product quality (Siciliano et al., 2016). Approximately 80–90 % of AFM1 was reduced after 3–5 min of cold plasma treatment without significantly changing milk quality including pH value, color, conductivity, total dissolved solids and nutrient composition in our previous study (Nguyen, Palmer, Phan, et al., 2022; Nikmaram & Keener, 2022). Cold plasma is a potential method for reducing AFM1 in milk to prevent food safety issues associated with AFM1. We hypothesise that cold plasma breaks down AFM 1 into non-toxic products. However, insight into the mechanism of degradation of AFM1 and the break down products using cold plasma are unknown. In this study, the chemical reactive species induced by different operating gases effecting the AFM1 reduction and the degradation products of AFM1 after cold plasma treatment were explored. Based on the structure of the AFM1 degradation products, the toxicity of AFM1 after treatment was predicted.

2. Methodology

To investigate the break down products of AFM1 treated with cold plasma, AFM1 standard in water was treated using cold plasma generated from different gases (see 2.1) in a di-electric barrier system (see 2.2) using 26 kv for different treatment times (see 2.4).

2.1. Chemicals and equipment

For the cold plasma treatment, the following gases were used: Helium (technical grade, compressed – BOC, Palmerston North, NZ). Nitrogen (industrial grade, oxygen free, compressed – BOC, NZ). Oxygen (industrial grade, compressed – BOC, NZ). Oxygen and ozone were detected using an oxygen analyser (Servomex – 570, England) and ozone detector (GT – 903, Korno, China) respectively.

The sample treatment was conducted in a plastic bag (heavy duty poly bags-polyethylene films 300 × 450mm, 70 μm, clear, OfficeMax).

Aflatoxin M1 standard 10 μg mL^{-1} in acetonitrile, in an ampule of 1 mL (Sigma - Aldrich) was used for cold plasma trials.

To detect some of the reactive species, Quantofix peroxide test sticks, 6 mm × 95 mm (pack of 100), ranged 1–100 mg L^{-1} (Germany); Quantofix® nitrite, 6 mm × 95 mm (pack of 100), ranged 1–80 mg L^{-1} and 0.1–0.3 g L^{-1}, 0.2 mm - thick plastic strips (Germany) were used with hydrogen peroxide (H_2O_2) 30% (LabServ - Thermo Fisher Scientific) and sodium nitrite ($NaNO_2$) (Merck, Germany) were used as controls.

For LCMS analysis, water (LCMS grade, ThermoFisher), methanol (LCMS grade, ThermoFisher), acetonitrile (LCMS grade, ThermoFisher), formic acid (LCMS grade, Sigma).

Gas flow meter, 1 and 10 L min^{-1} (Aalborg – USA) were used to mix the gases. 0.2 μm PTFE filters (Sartorius, Germany) were used to filter the samples before analysing by LCMS.

2.2. Dielectric barrier discharge system

To investigate the effect of different operating gases, various ratios of operating gases using cold plasma and the degradation products of AFM1 after treatment, a dielectric barrier discharge (DBD) cold plasma system was built at Massey University to generate High voltage atmospheric cold plasma (HVACP) (Nguyen, Palmer, Phan, et al., 2022) (Fig S1).

2.3. Effect of different gas ratios on the efficacy

The higher oxygen concentration in the gas mixture was proven to enhance the reduction of aflatoxins by cold plasma (Nguyen, Palmer, Phan, et al., 2022; Shi, Ileleji, Stroshine, Keener, & Jensen, 2017). In this study, three different concentrations of pure oxygen in helium (Table 1) were used to evaluate the effect of different operating gases on the reduction of AFM1 (10 μg L^{-1} in water/acetonitrile 85/15 v/v) after 3 min cold plasma treatment. Moreover, various levels of air and nitrogen in helium were also used as operating gases to explore the their effects on the reduction of AFM1 by cold plasma (Table 1). Gas flow meters were used to mix the gases.

The mixture of water/acetonitrile 85/15 v/v was used as a solvent to dissolve aflatoxins for extraction and analysis (Boonzaaijer, Osenbruggen, Kleinnijenhuis, & Dongen, 2008; Wang & Liu, 2007). In addition, there was no information regarding the effect of acetonitrile in the mixture on the reactive species or AFM1 degradation. In this study, this mixture was used as a solvent to dissolve AFM1.

2.4. Effect of different treatment times

AFM1 standard (10 μg L^{-1} in 85/15 water/acetonitrile) (2 mL) was held in a small Petri dish and exposed to the cold plasma for 3, 5, and 10 min. Air/helium (10/90) was used.

2.5. Measurement of hydrogen peroxide and nitrite produced during cold plasma treatment with test strips

Before testing the samples, the response of the strips was tested by using positive controls. For hydrogen peroxide testing, 83.33 μL H_2O_2 30% was pipetted to a 25 mL volumetric flask and filled with distilled water to make a 1000 ppm solution. The 10 and 100 ppm solutions were made with 10-fold, dilutions of the 100 ppm and 1000 ppm solutions. Distilled water was used as a negative control sample (zero ppm).

For nitrite testing, a positive control solution was made by weighing 0.2011 g of $NaNO_2$ and dissolving in 100 mL of distilled water (2011 ppm - μg L^{-1}). A set of different concentrations of 100, 10 and 1 ppm were diluted from the 2011 ppm solution.

The strip indicators were completely dipped into 2 mL of sample and dried after 30 s. The colour of the strip was read after another 30 s. The change in the colour of the strip indicators for the positive control were tested before testing samples. The strip indicators turned blue – green when dipped into hydrogen peroxide solutions at 10 and 100 ppm while there was no colour change in water (negative control). The increase in the concentration of hydrogen peroxide increased the colour intensity of the strip indicators. Similarly, they turned pink – red when dipped into nitrite solutions while negative control strip indicators remained white. The colour intensity of the strip indicators increased gradually from 1 to 100 ppm of nitrite then turned red at the concentration of 2011 ppm.

2.6. Degradation products of AFM1 after cold plasma treatment

2.6.1. Treating AFM1 with cold plasma for identification of degradation products of AFM1 after cold plasma treatment

AFM1 standard (2 mL) in 15% acetonitrile in water (1 μg mL^{-1}) was held in a small glass Petri dish (ID 40 mm) placed in line with two electrodes in the reaction bag (Fig S1). The bag was filled with 10% air in helium, and the gas mixture was measured by an oxygen analyser (2% oxygen). The gap between the two aluminium electrodes was 20 mm. The plasma system was operated at 44 W, 20.5 kHz and 26 kV. The samples after plasma treatment were filtered through a 0.2 μm PTFE filter before LC - MS/MS injection.

Samples were initially analysed by LC – MS in full scan mode to detect all compounds present in the cold plasma treated samples (after 0, 3, 5 and 10 min treatment) and identify the ion masses of the compounds (chromatogram peaks). The peaks were integrated using the background subtracted spectrum. The areas of the peaks across replicates were assessed to check for consistency of the peak areas across replicates of the same sample. Only peaks that were consistent across the replicates, showed a change over the treatment time and were higher than 5% of the original AFM1 peak area were taken forward for structure

Table 1
The concentration of NO_2^-, H_2O_2 and ozone in cold plasma induced by different gas mixtures. Values with different letters (a, b, c) are significantly different ($P < 0.05$).

Gas ratio in helium	NO_2^- concentration (mg L^{-1})	H_2O_2 concentration (mg $^{-1}$L)	Ozone concentration (mg L^{-1})	Reduction of AFM1 (%)
100 % Helium	<1	3–10	–	0
5 % Air	5–10	≤30	$24.40^b \pm 3.10$	58.53 ± 2.99
10 % Air	10–20	30–100	$25.73^b \pm 3.82$	69.23 ± 13.26
20 % Air	≤20	≤100	$19.47^b \pm 8.43$	80.99 ± 11.59
5 % Oxygen	0–1	3–10	$560^c \pm 90$	53.35 ± 3.65
10 % Oxygen	1–5	10–30	$365^c \pm 152$	62.61 ± 10.16
20 % Oxygen	5–10	30–100	$410^c \pm 186$	88.85 ± 1.17
5 % Nitrogen	0–1	1–3	$4.43^a \pm 1.56$	47.92 ± 4.13
10 % Nitrogen	1–5	1–3	$5.57^a \pm 0.90$	48.01 ± 4.44
20 % Nitrogen	5–10	5–10	$4.43^a \pm 2.02$	47.20 ± 7.44

elucidation (peaks 1, 2, 3 and 4, Fig. 2) (smaller peaks had large variations between triplicates).

The METLIN database [https://metlin.scripps.edu/] was used to search for possible matching formula using the "find by formula" function. Molecular formula that matched the masses in the MS data within 5 ppm of the expected mass were selected for further investigation (Table S2). The formula with the highest score (closest to 100%) are selected.

2.6.2. HPLC-MSMS analysis

Samples were analysed on an Agilent UPLC - QTOF spectrometer equipped with an Agilent 1290 high speed binary pump, Agilent 1290 multisampler, Agilent 1290 multi-column thermostat, Agilent 1260 diode array detector, and Dual AJS ESI source using the following conditions (Nguyen, Palmer, Loo, et al., 2022).

- Column: Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 μm
- Gradient: 30–100% MeOH/water (+0.1% formic acid, FA)
- Flow rate: 0.3 mL/min
- Column temperature: 40 °C
- Run length: 17 min, 2 min post time
- MS polarity: positive
- Sample concentration: 1 μg mL^{-1}
- Sample injection volume: 1 μL

LC gradient details and the MS parameter are shown in Table S1.

2.7. Statistical analysis

All experiments were replicated three times. The significance analysis was carried out using ANOVA by running Minitab (Minitab reference manual, 2019).

3. Results and discussion

3.1. Effect of different operating gases

The reduction (%) of AFM1 by cold plasma with various operating gas mixtures is shown in Table 1. These results indicate that different gases and their ratios have influence AFM1 degradation by cold plasma. Helium was used to initiate the ionization of air/oxygen/nitrogen during treatment. When 100% helium was used as an operating gas, no reduction of AFM1 was observed. A relatively low concentration of hydrogen peroxide (3–10 ppm) and nitrite (approximately 1 ppm) were generated (Table 1) Approximately 4 ppm of ozone was produced by 100% helium after 3 min treatment. These low levels of reactive species (hydrogen peroxide, nitrite and ozone) produced in 100% helium plasma was likely caused by trace amounts of air dissolved in the solution. This was probably either insufficient to reduce AFM1 in the standard solution or no reactive species responsible for AFM1 reduction were generated in helium induced plasma.

The nitrogen – helium mixture used in the cold plasma decreased the AFM1 concentration by about 50%. This indicates that the reactive nitrogen species (RNS) were able to react with the toxin, leading to its degradation. Reactive oxygen species (ROS) also contributed to AFM1 degradation by cold plasma induced by the pure oxygen in helium mixture which reduced approximately 50–90% of AFM1 (Table 1). These are likely to explain the reduction of AFM1 when air was used to mix with helium because RNS and ROS were both generated by cold plasma using air (78% nitrogen – 21% oxygen) as an operating gas.

A significant amount ($P < 0.05$) of AFM1 was degraded by air/helium and the pure oxygen/helium mixture induced plasmas. The degradation increased with an increase of 5–20% air or oxygen in

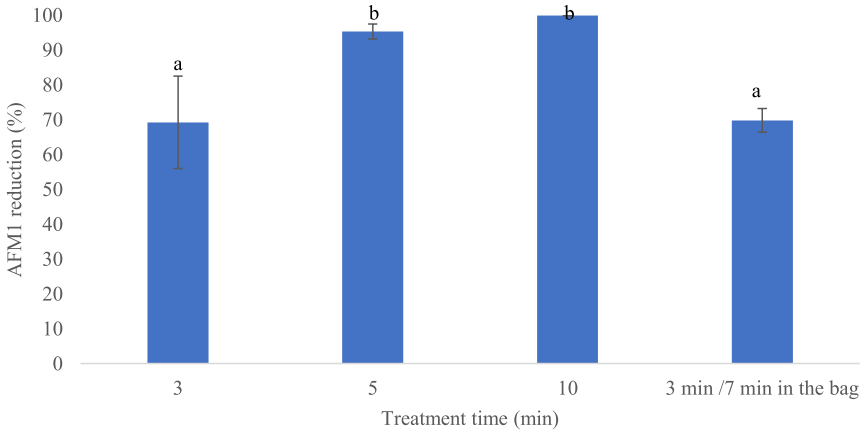

Fig. 1. The reduction of AFM1 (10 μg L^{-1}) by air/helium (10/90%) induced plasma at different treatment times (3, 5, 10 min and 3 min treatment with 7 min in the reaction bag). Values with different letters (a, b) are significantly different ($P < 0.05$).

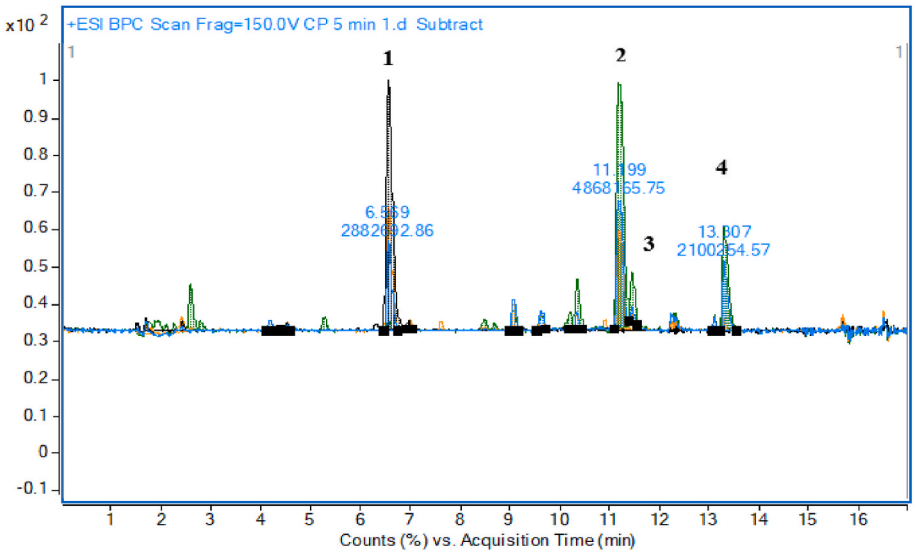

Fig. 2. Overlay of the base peak chromatograms (MS1) of the air/helium (10/90%) cold plasma treated samples (0 min – black, 3 min – green, 5 min – blue and 10 min – orange).

helium, (Table 1). The possible explanation is the increase in the ratio of air or pure oxygen (from 5 to 20%) in the gas mixture with helium that increased the concentration of reactive species leading to the improvement in AFM1 degradation. The concentrations of O_3, H_2O_2 and NO_2^- in the samples after cold plasma treatment using air and pure oxygen at different ratios indicates that the increase in the gas ratio in the mixture (air or pure oxygen from 5 to 20% in helium) generated high levels of these reactive species (Table 1).

The increase in the concentration of reactive species (hydrogen peroxide and nitrite) in the sample was demonstrated by increased darkness of the test strip colour. The concentration of hydrogen peroxide and nitrite in air induced plasma ranged from 30 to 100 mg L^{-1} and <5–20 mg L^{-1} (Table 1), respectively, with the air in helium increasing from 5 to 20%. Air is mainly made up of nitrogen and oxygen so the increase of the air ratio in the gas mixture with helium, which led to the increase of oxygen and nitrogen in the gas mixture that enhanced the production of reactive species because these elements are the main ingredients producing ROS and RNS, via the following steps (Surowsky, Bußler, & Schluter, 2016):

$$O_2 + e^- \rightarrow O + O$$

$$O + O_2 + O_2 \rightarrow O_3 \text{ and } O_2$$

$$N + O + M \rightarrow NO + M$$

$$O + NO + M \rightarrow NO_2 + M$$

$$O + H_2O \rightarrow 2OH^{\cdot} \quad (1)$$

$$2OH^{\cdot} \rightarrow H_2O_2$$

Similarly, the increase of oxygen from 5 to 20% in helium increased the concentration of hydrogen peroxide and nitrite from 10 to 100 mg L^{-1} and 1–10 mg L^{-1}, respectively (Table 1). Increasing the oxygen ratio in the gas mixture improved the efficacy of cold plasma treatment due to the increase in the amount of atomic oxygen. This in turn led to an increase in hydrogen peroxide induction, which was also observed in a previous study where jet plasma was used to control *Citrobacter freundii* in apple juice (Surowsky, Fröhling, Gottschalk, Schlüter, & Knorr, 2014). However, the amount of nitrite induced by the oxygen/helium mixture tended to be lower than in the air/helium mixture, probably due to the reduced nitrogen in the oxygen/helium gas mixture. In addition, at the same ration of gas mixture, the concentration of hydrogen peroxide induced by the mixture of air/helium tends to be slightly higher than the oxygen/helium mixture, this could be due to the humidity of the air, while pure oxygen was dry.

The concentration of hydrogen peroxide induced by the nitrogen/helium plasma was much lower than in the air/helium or oxygen/helium mixtures, ranging from 0 to 10 mg L^{-1} (Table 1). The decrease in these reactive species induced by nitrogen/helium is probably due to the lack of oxygen in the gas mixture. Therefore, the formation of hydrogen peroxide is likely based on either the trace of air in the sample or the reaction between hydroxyl radicals ($OH^{\cdot}$) which are produced by the dissociation of water from the electron collision (reaction **) instead of the combination of the reactions (*), (**) and (***).

$$e + H_2O \rightarrow H^{\cdot} + OH^{\cdot} + e \text{ (Whitehead, 2016)} \quad (2)$$

$$2OH^{\cdot} \rightarrow H_2O_2 \quad (3)$$

The concentration of nitrite rose from 1 to 10 mg L^{-1} responding to 5–20% nitrogen in helium gas. However, the generation of RNS in the nitrogen/helium was likely to be less than in air which is due to the absence of oxygen. Oxygen is a primary ingredient in the generation of nitrite together with nitrogen. The lower concentration of nitrite induced by oxygen/helium cold plasma is also due to the absence of nitrogen, a primary ingredient in the gas mixture.

The concentration of ozone produced by various gases during cold plasma treatment was different for the nitrogen/helium, air/helium and oxygen/helium mixtures (Table 1). The highest concentration of ozone was induced by the pure oxygen/helium mixture. Plasma induced by air/helium contained less ozone than pure oxygen/helium which was also due to the amount of oxygen as at the same ratio in the gas mixture, the percentage of oxygen in air was five time less than pure oxygen. For example, in 5% of the gas mixture, the oxygen in air was 1% while it was 5% in the pure oxygen gas mixture with helium. Therefore, based on the percentage of oxygen in the gas, the ozone concentration in the pure oxygen/helium induced plasma should be five times higher than in air but it was approximately twenty times more than in air (Table 1). This was probably due to the distribution of the input energy for ionization and dissociation of the operating gas molecules during cold plasma treatment. The first ionization energy of oxygen is 13.6181 eV and of nitrogen is 14.5341 eV, which indicates that the strengh of the bond between nitrogen molecules is stronger than oxygen. The ionization and dissociation of oxygen occurs at a lower level of energy compared to nitrogen (Whitehead, 2016). Therefore, most of the input energy tranferred oxygen into ozone in the pure oxygen-induced plasma, while it

consumed more energy to dissociate and ionize nitrogen in air induced plasma. That may lead to the ozone concentration generated in air being much smaller than in pure oxygen plasma.

The absence of oxygen could explain the low level of ozone generated by nitrogen/helium gas. However, insignificant ($P > 0.05$) differences in the amount of ozone were observed in all three gases (nitrogen/helium, air/helium and oxygen/helium). The possible explanation is that the small differences in the gas ratio were insufficient to make a significant change in the ozone amount generated.

The difference in AFM1 reduction by air and pure oxygen induced plasma was insignificant ($P > 0.05$) at all three concentrations (5, 10 and 20%) of the gases added to helium, although the components of the gases were different. The exact degradation mechanism remains unknown, but the explanation is likely to be the chemistry of the plasma, driven by the operating gases. For instance, at a ratio of 20% in the gas mixture, the amount of oxygen in pure oxygen mixture was five times higher than in air, leading to a higher level of ozone induced by the pure oxygen/helium plasma than air/helium. However, the concentrations of hydrogen peroxide and nitrite were lower in pure oxygen/helium induced plasma than in air/helium. It should be noted that there may be more reactive species induced by these operating gases at different concentrations, but the measurement for these species was not available. Ozone, hydrogen peroxide and nitrite all play an important role in AFM1 reduction. In addition, the reduction of AFM1 by nitrogen induced plasma was no different for the various gas ratios (Table 1). The reduction of AFM1 was approximately 50% at gas mixtures ranging from 5 to 20% nitrogen/helium. At 5% nitrogen/helium, plasma reduced AFM1 by 50%, same as air/helium and pure oxygen/helium. However, the long - life reactive species (ozone, hydrogen peroxide, nitrite) levels in nitrogen/helium were much lower than in air/helium and pure oxygen/helium. This possibly illustrated that the short – lived reactive species, which are produced during the treatment and disappear once plasma induction has stopped, also play a key role in degrading AFM1 by cold plasma together with the long – lived species including ozone, hydrogen peroxide and nitrite.

Although the efficacy of cold plasma treatment using air/helium and pure oxygen/helium on AFM1 reduction was relatively similar, the mixture of air/helium was used for further experiments due to the cost advantage of using air compared with pure oxygen.

3.2. Effect of treatment time on degradation of AFM1 by cold plasma

In the present trial, the increase in the concentration of reactive species (short- and long – lived ones) over time was the most likely explanation for the improvement in AFM1 reduction. The concentration of hydrogen peroxide and nitrite increased from 10 to >100 mg L^{-1} and 0.1–0.3 g L^{-1}, respectively, with treatment times increasing from 3 to 10 min (Table 2). However, the ozone concentration at different treatment times tended to be unchanged (Table 2). A similar result was found in another study where the concentration of ozone reached the peak at 10 min of plasma treatment and remained unchanged for 10–30 min (Shi, Cooper, et al., 2017). The ozonation process by plasma treatment is quick, so that ozone saturation is achieved after a short time and remains stable, regardless of treatment time.

Table 2
Reactive species (NO_2^-, H_2O_2 and ozone) induced by 10% air in helium at different treatment times, values with different letters are significantly different ($P < 0.05$).

Treatment time (min)	NO_2^- concentration (g L^{-1})	H_2O_2 concentration (mg L^{-1})	Ozone concentration (mg L^{-1})
0	0	0	$0.4^a \pm 0.1$
3	<0.1	10–30	$25.73^b \pm 3.82$
5	≈0.1	30–100	$23.40^b \pm 7.83$
10	≈0.3	>100	$20.43^b \pm 6.98$

The effect of treatment time on AFM1 reduction by 10/90% air/helium induced plasma was significant (Fig. 1). AFM1 was undetectable after 10 min treatment while approximately 70% was reduced after 3 min which indicated that the increase in treatment time enhanced the AFM1 degradation by cold plasma.

To evaluate the effect of short – lived and long – lived reactive species (particularly ozone - O_3), we carried out an experiment to compare AFM1 reduction in two samples with the same treatment time (3 min) but one of them was kept in the reaction bag for another 7 min (10 min in the reaction bag in total) to prevent the O_3 leakage. No significant ($P < 0.05$) difference in AFM1 degradation between these samples was found (Fig. 1). This indicated that reactive species other than O_3 also play a key role in the AFM1 reduction by cold plasma. It should be noted that the insignificant ($P < 0.05$) difference in AFM1 degradation by cold plasma between 3 min (with and without in-bag resting time) could also represent the decrease in the penetration of cold plasma into samples when the power was off. In addition, the reaction between the AFM1 molecule and reactive species is likely to be happening during the treatment, when the power is on which confirms the contribution of short – lived species in the degradation of AFM1 by cold plasma.

An improvement in the cold plasma efficacy due to the increase in treatment time was also found in previous studies. *Escherichia coli* and *Listeria monocytogenes* in pork loins, *E. coli* in milk, *Enterococcus faecalis*, *E. coli*, *L. monocytogenes*, and *S.* Typhimurium in milk were reduced by cold plasma (Chang & Chen, 2016; Gurol, Ekinci, Aslan, & Korachi, 2012; Kim, Yong, Park, Choe, & Jo, 2013; Kim et al., 2015). However, the efficacy varied, mainly due to different set - ups of the cold plasma generator, the sample substrate and the targeted subjects (microorganisms, toxin, etc.).

3.3. Identification of degradation products of AFM1 after cold plasma treatment and the toxicity prediction

Peaks 2, 3 and 4 were the degradation products of AFM1 (peak 1) by cold plasma and only appeared after the treatment started. Before the treatment at 0 min, the area of peaks 2, 3 and 4 were zero and the AFM1 peak area accounted for 100% of peak areas of importance. When the treatment time increased, the AFM1 peak reduced with an increase in the production of peaks 2, 3 and 4. The production of peaks 2, 3 and 4 varied in each of the replicates leading to the difference in their peak areas. The change in the peak areas of the degraded AFM1 over treatment time (Fig. S2). AFM1 reduced gradually over time; from approximately 50% after 3 min of treatment, to about 60% after 5 min of treatment, and almost 100% after 10 min of cold plasma treatment. Meanwhile, the formation of degradation products increased gradually.

The structures of peaks 2 and 3 were proposed by analysing the fragmentation patterns in the associated MS/MS spectra. The results for peak 2 follow in Fig. S3.

Similarly, peak 3 structure was proposed by MS/MS fragmentation (Fig. S4).

The proposed structure for peak 4 was based in the ions observed in the MS MS^{-1} spectra (Fig. S5) and the literature. This was confirmed by the fragmentation of the compound in MS MS^{-1} (Fig. S6).

Proposed structures that matched the selected formulas are shown in Fig. 3.

The structures of the possible compounds were proposed based on the findings from the literature for aflatoxins degradants using various treatments. Due to the limited research on AFM1 and cold plasma, other aflatoxins and treatments were included in the search. The main degradants for aflatoxins that exhibit similar structures to AFM1 tend to be oxidation/epoxidation/hydrogenation across C8 = C9, reducing the ketone to an alcohol, and furan cleavage (Shi, Cooper, et al., 2017). The attack of reactive species [OH] induced by cold plasma at C8 = C9 led to oxidation producing an isomer. In addition, the reduction caused by the attack of reactive species [H] to ketone groups at C1 and C11 produced alcohol groups (Shi, Ileleji, et al., 2017) leading to the formation of

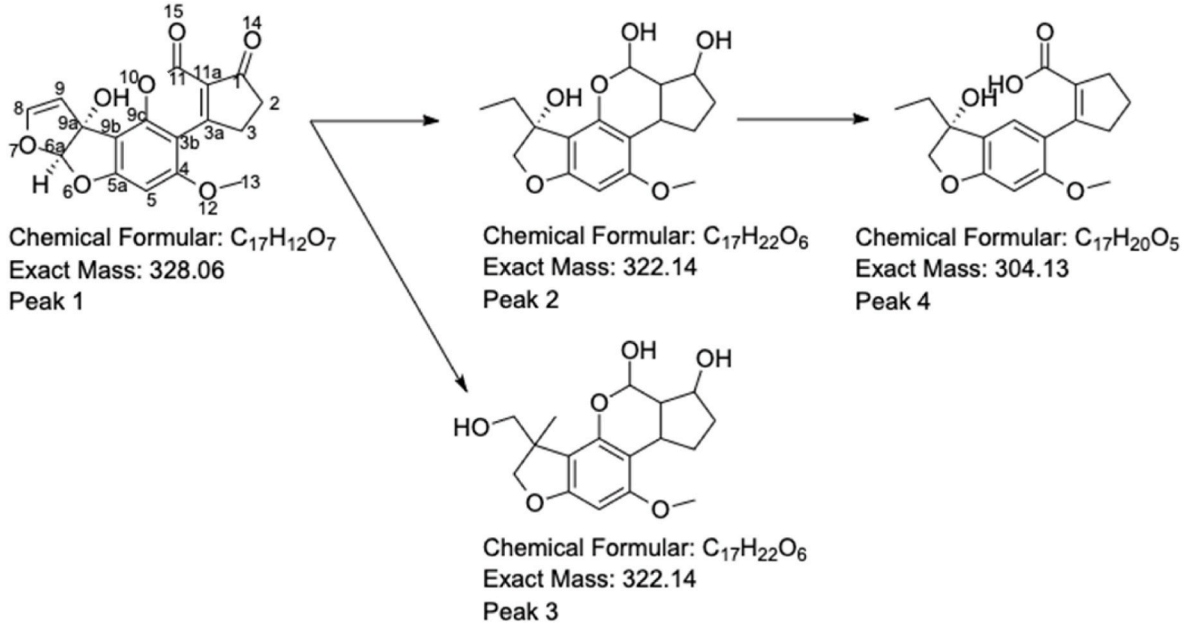

Fig. 3. Proposed AFM1 degradation pathway under cold plasma treatment conditions.

peaks 2 and 3. The reactive species ([O], [H]) attack opened the lactone ring of the AFM1 molecule, causing the oxidation of the ketone group at C11 to alcohol and the reduction water at C1. These are likely to explain the degradation of AFM1 by reactive species [OH], [H] and [O] and explain the increase in the concentration of these reactive species resulting in the degradation of AFM1. Similarly, the oxidation of the AFM1 molecule under UV treatment occurs at C8 = C9 (Nguyen, Palmer, Loo, et al., 2022; Stanley, Patras, Pendyala, Vergne, & Bansode, 2020), leading to two positional isomers, previously identified, and characterised as degradation pathway compounds for AFM1 under ultraviolet-C (UVC) treatment. Under the same conditions, this is followed by mild oxidation leading to epoxidation across the two diols from C1 and C11 under UVC treatment (Nguyen, Palmer, Loo, et al., 2022). However, cold plasma conditions are inducing a stronger oxidation than UVC, and the epoxy form rapidly oxidises to carboxylic acid. The proposed degradation pathway in cold plasma treatment is shown in Fig. 3.

Our proposed structures align with the findings of Nikmaram and her colleagues (Nikmaram, Brückner, Cramer, Hans-Ulrich, & Keener, 2023) who suggested that the degradation reaction between cold plasma and AFM1 is caused by the hydration/epoxidation of the C8-C9 double bond of AFM1 molecules. However, one key difference between our study and Nikmaram's is the choice of operating gas. While air mix gases were used in our experiments, Nikmaram utilized MA65. This variation in the operating gas is likely the main reason for the difference in the degradants observed. The decision to use air in our study was based on its availability and economic advantages. It is worth noting that the chemistry of reactive species is influenced by the choice of operating gases, as highlighted by (Whitehead, 2016). Therefore, the discrepancy in the concentration and types of reactive species produced by cold plasma generated from air and MA65 is likely responsible for the differing AFM1 degradants observed. Moreover, by considering the impact of operating gases on reactive species chemistry, the parameters to operate the cold plasma system including voltages, treatment time and medium to dissolve AFM1, we can better understand and explain the variations in the degradation reactions observed in our study compared to Nikmaram's.

The toxicity and mutagenicity of aflatoxins are believed to be due to the furan ring and lactone ring structures as they are the reactive sites of the AFM1 molecule (Siciliano et al., 2016; Wogan, Edwards, & Newberne, 1971). These sites of the aflatoxins molecules are easily converted to other substances that change their biochemical functions. Therefore, the treatments that destroy these furan and lactone ring structures of the toxin molecules, and prevent their binding are likely reduce the aflatoxins toxicity (Samarajeewa, Sen, Cohen, & Wei, 1990). The cleavage of the lactone ring of AFB1 was shown to have 450 times less mutagenic and 18 time less toxicity than the original AFB1 (Lee, Dunn, DeLucca, & Ciegler, 1981; Mendez-Albores, Nicolas Vazquez, Miranda-Ruvalcaba, & Mo- reno-Mart, 2008). Similarly, the toxicity of AFB1 decreased after treatment with UV and plant extracts due to alteration of the AFB1 molecules at the reactive sites (Iram, Anjum, Iqbal, Ghaffar, & Abbas, 2015; Iram et al., 2016; Mao et al., 2016). The molecular structures of AFB1 and AFM1 are similar in that they both have a double bond at C8 = C9 and a lactone ring that once destroyed reduces the toxicity. In this study, the reactive species induced by cold plasma reacted with the furan ring and the lactone ring leading to the degradation of AFM1 so that the toxicity of degradants is likely to be reduced. The reduction in toxicity of AFM1 degradants was presented in our previous study where HVACP was used to reduce AFM1 in skim milk (Nguyen, Palmer, Phan, et al., 2022).

4. Conclusion

A small scale DBD (37 kV) cold plasma system was built to explore the effect of different operating gases, various ratios of operating gases on AFM1 reduction, understand the production of the reactive species and identify the degradation products. Cold plasma treatment reduced AFM1 (10 μg/L) by approximately 50–90% after 3 min treatment with mixtures of 5, 10 and 20% of air, pure oxygen and nitrogen in helium. The measurements of ozone, hydrogen peroxide and nitrite indicated that the increase in the amounts of nitrogen and oxygen in the gas mixtures enhanced the production of the reactive species associated with the efficacy of cold plasma on AFM1 reduction. The efficacy of cold plasma on AFM1 reduction is affected by operating gases, ratio of the gas mixtures and treatment time which change the cold plasma chemistry considered to be due to the differences in the concentrations of the reactive species. The higher concentrations of the reactive species caused by the increase in the treatment time was associated with significant increases in the AFM1 reduction with 70 and almost 100% reduction after 3 and 10 min treatment, respectively. This proves that

cold plasma can successfully be used as an AFM1 reduction and can potentially be used to treat milk. The structure of the three different degradants of AFM1 after cold plasma treatment has been proposed for the first time. The degradation products were formed by the reaction between the reactive species in cold plasma with AFM1 at the oxidation sites of the toxin molecules that will in theory destroy the toxicity of the toxin.

The use of acetonitrile necessary to dissolve the AFM1 for analysis in this study, does differ from actual use of cold plasma in treating milk. This will have some effect on the reactive species produced during plasma treatment and must be considered as a limitation in these trials which aimed to provide insight into the degradation products produced by cold plasma treatment of AFM1.

CRediT authorship contribution statement

Thu Nguyen: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jon Palmer:** Writing – review & editing, Supervision, Conceptualization. **John Pedley:** Resources, Methodology. **Miruna Petcu:** Writing – review & editing, Software, Formal analysis. **Harriet L. Newson:** Software, Methodology, Formal analysis, Data curation. **Kevin Keener:** Supervision, Resources, Methodology, Investigation. **Steve Flint:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Funding

This work was supported in part by the New Zealand Ministry of Foreign Affairs and Trade (PLC-0000002886), and in part by the NZ Food Safety Science & Research Centre (33507).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank research team at Micro Lab, John Edwards at School at Food and Advanced Technology, Trevor Loo at School of Fundamental Sciences, Massey University for technical assistance and providing facility for sample extraction.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2024.106103.

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