Degradation of aflatoxin M1 in skim milk using UVC or Cold Plasma

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Nguyen Thi Thu (Thu Nguyen)

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Abstract

Contamination of AFM1 in milk and milk products has been an issue for decades as it is a food safety risk, classified as a Group 1 carcinogen. Cows consuming feed contaminated with fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) that produce AFB1, convert AFB1 to AFM1 that is released into the milk. The best way of controlling AFM1 contamination in milk is to keep the feed dry to prevent the growth of fungi to avoid the production of AFB1. However, this is challenging in some tropical countries where the weather is hot and humid all year around. Treating milk contaminated with AFM1 is an alternative method of control. The aim of this study was to investigate two methods for milk treatment - UVC and cold plasma to reduce AFM1 in milk, investigate the factors influencing these treatments and identify the degradation products after treatment.

UVC (254 nm) reduced AFM1 in skim milk to below MRL (0.5 μg/L) from an initial level of 1 μg/L after 20 min treatment. Treatment time (min), depth of samples (mm) and the stirring of the milk sample during treatment were found to significantly ($P < 0.05$) enhance the reduction of AFM1 in milk. The contamination level (μg/L) and fat content in milk did not significantly ($P > 0.05$) effect the UVC efficacy. A change in milk colour was observed but the pH of the milk samples did not change. The degradant of AFM1 after UVC treatment was identified as an oxidation product which resulted in hydroxylation occurring at the double bond of the furan ring of AFM1 molecules.

High voltage atmospheric cold plasma (HVACP) was used to reduce AFM1 in skim milk and explore the effect of treatment times (5, 10 and 20 min), operating gases (air and MA65 - 65% O_2, 30% CO_2, 5% N_2), three voltages (60, 70 and 80 kV), using direct and indirect treatment, AFM1 contamination levels (0.1; 1 and 50 μg/L) and the volume of

the sample (10, 20 and 30 mL). A reduction of 64.99 and 78.86% of AFM1 in skim milk after 20 min HVACP treatment using air and MA65, respectively, was achieved with the initial level of 1 µg/L. HVACP did not change the milk colour after 20 min treatment but a slight change in pH was observed. Different treatment times, different operating gases and voltages, direct and indirect treatments were found to have the most effect on AFM1 reduction. While AFM1 contamination levels (0.1; 1 and 50 µg/L) had an insignificant ($P > 0.05$) effect on AFM1 reduction in milk.

A dielectric barrier discharge (DBD) cold plasma set up with small capacity high voltage generator was used to investigate the effects of other operating gases with different mixtures (5, 10 and 20% of air, pure oxygen and nitrogen in helium) and the effect of milk components (casein, lactose and whey protein) on AFM1 reduction. The degradation products of AFM1 after cold plasma treatment were determined. Although this small capability system reduced approximately 70-100% of AFM1 in water after 3 and 10 min treatment by using air/helium (10/90), the reduction of AFM1 in skim milk, whey and casein was much less, although 70% of AFM1 was reduced in lactose. The reduction of AFM1 in water was significantly ($P < 0.05$) improved by cold plasma with the increase in the concentration of air/pure oxygen in helium but it was unchanged regardless of the ratio of nitrogen in helium. The structure of three degradants of AFM1 after cold plasma treatment was elucidated with the confirmation of two of them resulting from damage to the furan ring of AFM1 molecules. The structure of the third one was proposed but another analysis technique is required to confirm.

Acknowledgements

“All knowledge is connected to all other knowledge. The fun is in making the connections.” — Arthur C. Aufderheide.

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Declaration

This thesis contains 6 chapters. Chapter 2 has been published, the results from chapters 4 and 5 have been structured as submitted manuscripts. Chapter 3 describes the methods used for analysis in chapters 4 and 5, however, the treatment methods are different in each chapter.

The DRC 16 form for the contributions of the authors in the publication has been attached in the Appendix D.

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List of Publications and Presentations

Publication

1) Nguyen, T., Flint, S., & Palmer, J. (2020). Control of aflatoxin M1 in milk by novel methods: A review. *Food Chemistry*, 311, 125984. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/31855773. doi:10.1016/j.foodchem.2019.125984

Presentations

1) Thu Nguyen. Degrading aflatoxin M1 in milk. NZMS Conference, Dunedin, NZ, Poster (Nov. 2018)

2) Thu Nguyen. Inactivation of aflatoxin M1 in skim milk by UVC (254 nm). NZMS Conference, Palmerston North, NZ, Poster (Nov. 2019)

3) Thu Nguyen. Inactivation of aflatoxin M1 in skim milk by high voltage atmospheric cold plasma (HVACP). NZMS online Conference, Oral presentation (Nov. 2020) and Massey University Postgraduate Food Science Symposium. Oral presentation (Sept 2020).

4) Thu Nguyen. Degradation of Aflatoxin M1 by cold plasma. FAOBMB (NZMS) online Conference, Oral presentation (Nov. 2021)

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List of abbreviation

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
AFs	Aflatoxins
DBD	Dielectric barrier discharge
ELISA	Enzyme-linked immunosorbent assay
FDA	The United States Food and Drug Administration
HPLC	High pressure liquid chromatography
HVACP	High Voltage Atmospheric Cold Plasma
IAC	Immuno-affinity column
LC - MS/MS	Liquid chromatography mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MA65 gas	Modified atmosphere (65% O_2, 30 %CO_2, 5% N_2)
MRL	Maximum residue limit
PBS	Phosphate-buffered saline
RSD	Relative standard deviation
SD	Standard deviation
SPE	Solid phase extraction
TLC	Thin layer chromatography
UHT	Ultra - high temperature
UV/ UV LED	Ultraviolet/ Ultraviolet Light Emitting Diodes
UVC	Ultraviolet C
Ppm/ppb	Parts per million/ parts per billion

CHAPTER 1. INTRODUTION

1.1 Background and problem statement

Mycotoxins have become an increasing concern to public health and the food industry, particularly in Vietnam. According to the Thuy Chau Nguyen survey in 1996 in Nghe An, Hanoi and Dong Nai, 25% of maize samples were found contaminated with aflatoxin from 25-36 ppb (Nguyen, 1996). Aflatoxin contaminated 95% of 115 food samples from commercial animal feed samples tested at the Institute of Hygiene and Public Health (2002.vnexpress.net). In 2005, Le and his colleagues investigated the occurrence of aflatoxin in feed in Vietnam (Le, Nguyen & Du, 2005) (Table 1.1).

Table 1. 1 Aflatoxin contamination in animal feed in Vietnam

Feedstuff	Average contamination level (ppb)	Maximum contamination level (ppb)
Maize	205	600
Rice and broken rice	22	25
Soybean	50	50
Rice bran	29	55
Sesame oil meal	8	10
Copra meal	17	50
Soybean oil meal	12	50
Peanut oil meal	1200	5000
Dry cassava slice	40	40
Feed pallet	105	500

Aflatoxin contamination level in feed is seasonal (Table 1.2) (Le et al., 2005). The contamination levels in feed are higher in the rainy season and lower in dry season. This is due to the weather conditions in the rainy season including hot temperature and high humidity. All those favour fungus growing in feed.

Table 1. 2 Aflatoxin contamination in animal feed in rainy and dry seasons

Feedstuff	Sample (n)	Average contamination level (ppb)	Maximum contamination level (ppb)
Peanut oil meal (rainy season)	17	1520	5000
Maize (rainy season)	18	240	750
Peanut oil meal (dried season)	18	525	1160
Maize (dried season)	13	120	450

According to an investigation by the Preventive Medicine Center of Ho Chi Minh city, Vietnam, the level of aflatoxin contamination in peanuts was 263 times higher than the maximum limit (15 µg/kg) (8-1:2011/BYT, 2011). In 2009, the Institute of Vegetable Oils surveyed 11 oil samples for their aflatoxin levels, five samples were contaminated with 20 - 112 mg/kg of aflatoxin (2 to 11 times higher than the permitted level) (Aflatoxin. 2009). In another study, more than 50% of selected maize samples were positive for aflatoxin with the average concentrations of 740 µg/kg and 1757 µg/kg in the Southeastern provinces and Central Highlands provinces of Vietnam, respectively

(Huong, Tuyen, Tuan, Brimer, & Dalsgaard, 2016; Phuong, Thieu, Ogle, & Pettersson, 2015). Aflatoxin B1 was found in 85.7% of surveyed food samples in Lao Cai, Vietnam (Huong, Tuyen, Tuan, Brimer, & Dalsgaard, 2016).

Dairy cows consuming animal feed containing mycotoxins leads to the presence of mycotoxins in the milk (Veldman, Meijs, Borggreve, & Heeres-van der Tol, 1992). In 2016, 15% of 20 milk samples in Ho Chi Minh city, Vietnam were found positive for AFM1 with levels ranging from 0.034-0.064 µg/L. The presence of AFM1 in the milk supply has been reported in many countries. The range of AFM1 contamination found in milk in four continents is shown in Table 1.3 (Iqbal, Jinap, Pirouz, & Ahmad Faizal, 2015). Generally, the level of AFM1 in milk is potentially higher in developing countries and where the weather is hot and humid that favors the growth of the fungi and aflatoxin production (Medina, Rodriguez, & Magan, 2014; Tajkarimi, Aliabadi-Sh, Salah Nejad, Poursoltani, Motallebi, & Mahdavi, 2008). The contamination levels are likely to exceed the MRL of 0.5 µg/L in those countries.

Table 1. 3 The range of amounts of AFM1 in milk from different countries across world.

Countries	Positive samples/samples	Range (µg/L)	Reference
Brazil	82/83	0.008 - 0.760	(Iha et al., 2013)
China	109/179	0.006 -.0154	(Zheng et al., 2013)
Egypt	50/50	0.018 - 0.250	(Motawee et al., 2009)
Greece	91/196	0.005 - 0.01	(Tsakiris et al., 2013)
India	30/45	0.1 - 3.8	(Siddappa et al., 2012)
Indonesia	113/113	0.006 - 0.015	Nuryono et al., 2009)
Iran	151/225 90/90 196/196	0.0058 - 0.528 0.0029 to 0.085 0.019 - 0.1261	(Fallah, 2010a) (Nemati, Mehran, Hamed, & Masoud, 2010) (Sani, Nikpooyan, & Moshiri, 2010)
Korea	48/100	0.002 - 0.08	(Lee, Kwak, Ahn & Jeon, 2009)
Kuwait	176/321	0.0049 - 0.0687	(Dashti et al., 2009)
Morocco	13/48	0.01 - 0.1	(Marnissi et al., 2012)
Pakistan	44/169 177/232 76/107	0.002 to 0.014 0.002 - 1.9 0.004 - 0.845	(Hussain,et al,. 2010) (Sadia et al., 2012) (Iqbal & Asi, 2013)
Serbia	148/150	0.01 - 1.2	(Kos et al., 2014)
Sudan	42/44	0.22 - 6.90	(Elzupir & Elhussein, 2010)
Syria	101/126	0.020 - 0.765	(Ghanem & Orfi, 2009)
Thailand	150/150	LOD - 0.114	(Ruangwises & Ruangwises, 2009)
Turkey	67/100	0.010 - 0.630	(Tekinsen & Eken, 2008)

Milk is one of the most common sources of nutrition for human, especially for infants and elderly people, due to its various nutritious components including protein, vitamins and minerals. Therefore, the occurrence of AFM1 that is carcinogenic (IARC, 2002) in milk raises an important food safety issue leading to a risk to human health. Consequently, the control of AFM1 in milk is necessary. The best way of preventing aflatoxin (AFs) contamination in milk is to keep the cattle feed dry to avoid the growth of toxin producing fungi as well as improve feed storage (Bovo, Corassin, Rosim, & de Oliveira, 2013). However, in Vietnam and some other countries, controlling the quality of animal feed is difficult because of the weather conditions and traditional feeds used for dairy cattle. Therefore, alternative methods of control are required.

1.2 Research questions

- Can the efficacy of UV light to degrade AFM1 be improved using a narrow wavelength of UVC light?

- Can high voltage atmospheric cold plasma (HVACP) reduce AFM1 in milk and if so under what conditions and what are degradation products?

1.3 Hypothesis

AFM1 in milk can be eliminated or reduced to safe levels by using UV light or HVACP.

1.4 Objectives

- To investigate how much AFM1 can be degraded in milk by UVC (254 nm) and evaluate the effects of the influencing factors.

- To identify the degradation products of AFM1 post UVC treatment.

- To build a cold plasma treatment unit.

- To investigate how much AFM1 can be degraded in milk by cold plasma and evaluate the factors influencing the degradation.

- To identify the degradation products of AFM1 following cold plasma treatment.

1.5 Significance of the research

This study will provide some guidelines for the control of AFM1 in milk and reduce an important food safety risk

1.6 The outline of the thesis

This thesis is made of six chapters. The current chapter is an introduction to the topic and problem statement. Hypothesis, objectives of the research and thesis outline are also included in this chapter. Chapter two is a comprehensive literature review introducing various methods to reduce AFM1 in milk. This review also compares the efficacy, influencing factors, (possible) mechanisms of activity, advantages, limitations, and potential future trends of these methods and provides some recommendations for the treatment of milk to reduce the risk of AFM1 contamination. The next chapter mainly describes methodologies used to determine AFM1 in milk before/after treatment by HPLC - MS.

Chapter four addresses the first two objectives including exploring the efficacy of UVC (254 nm) on AFM1 in milk reduction and degradation products of AFM1 under UVC (254 nm) treatment. Chapter five covers the three remaining objectives of the research, building of the cold plasma, the investigation of AFM1 degradation by cold plasma and the identification of the degradation products after cold plasma treatment. The last chapter summarizes the key findings of the research and suggests future work.

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CHAPTER 2. A REVIEW OF CONTROL OF AFLATOXIN M1 IN MILK BY NOVEL METHODS

Abstract

Aflatoxin M1 (AFM1) in milk and milk products has been recognised as an issue for over 30 years. Controlling AFM1 in milk is important to protect human health and trade. Preventing contamination by avoiding fungal contamination of cattle feed is the best method of control, however this is hard to avoid in some countries. Treating milk containing AFM1 is an alternative control measure, however, there is no single approved method. The challenge is to select a treatment method that is effective but does not affect the organoleptic quality of milk. This chapter reviews the strategies for degrading AFM1 in milk including yeast, lactic acid bacteria, enzyme, peroxide, ozone, UV light and cold plasma. The chapter also compares the efficacy, influencing factors, (possible) mechanisms of activity, advantages, limitations and potential future trends of these methods and provides some recommendations for the treatment of milk to reduce the risk of AFM1 contamination.

2.1 Introduction

Aflatoxins are mainly produced by two toxigenic species, *Aspergillus flavus* and *Aspergillus parasiticus.* There are more than 20 types of aflatoxins (Table 2.1), however, the most common aflatoxins are aflatoxin B1, B2, G1, G2, M1 and M2 (AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2). AFM1 and AFM2 are formed from the metabolism of AFB1 and AFB2, respectively (Veldman, Borggreve, & Heeres-van der Tol, 1992). Warm temperatures (25-30 ^{o}C), high humidity (80-90%), plant injuries in the field conditions during processing and storage promote fungal growth leading to aflatoxin production (Foster & Rosche, 2001). Aflatoxins appear naturally in many kinds of food and feedstuffs such as cereal, corn, nuts, cottonseeds, forages, cassava meal and straw. The contamination level depends on geographical regions (Cheeke, 1995). Cattle consuming feed contaminated with AFB1 results in the excretion of AFM1 via milk, urine and feces, with the majority found in milk and urine (Allcroft, Roberts, & Lloyd, 1968). AFM1 is formed by hydroxylation of AFB1 in the liver catalyzed by cytochrome P450 (CYP) (Koser, Faletto, Maccubbin, & Gurtoo, 1988), with 0.3-6.2% of AFB1 consumed (Britzi et al., 2013; Giovati, Magliani, Ciociola, Santinoli, Conti, & Polonelli, 2015; Veldman et al., 1992). Kuiman et al. (2000) described biosynthetic pathway of AFB1 from acetate with approximately 15 intermediates and many genes involved (Fig 2.1). The maximum AFM1 excretion level is detected in milk within the first 48 h of consuming feed contaminated with AFB1 reaching an undetectable level after 96 h (Allcroft et al., 1968). The transformation of AFB1 to AFM1 depends on the type of diet, ingestion rate, digestion rate, animal health, hepatic biotransformation capacity and milk yield (Duarte, Almeida, Teixeira, Pereira, Falcão, & Lino, 2013; Fink-Gremmels, 2008).

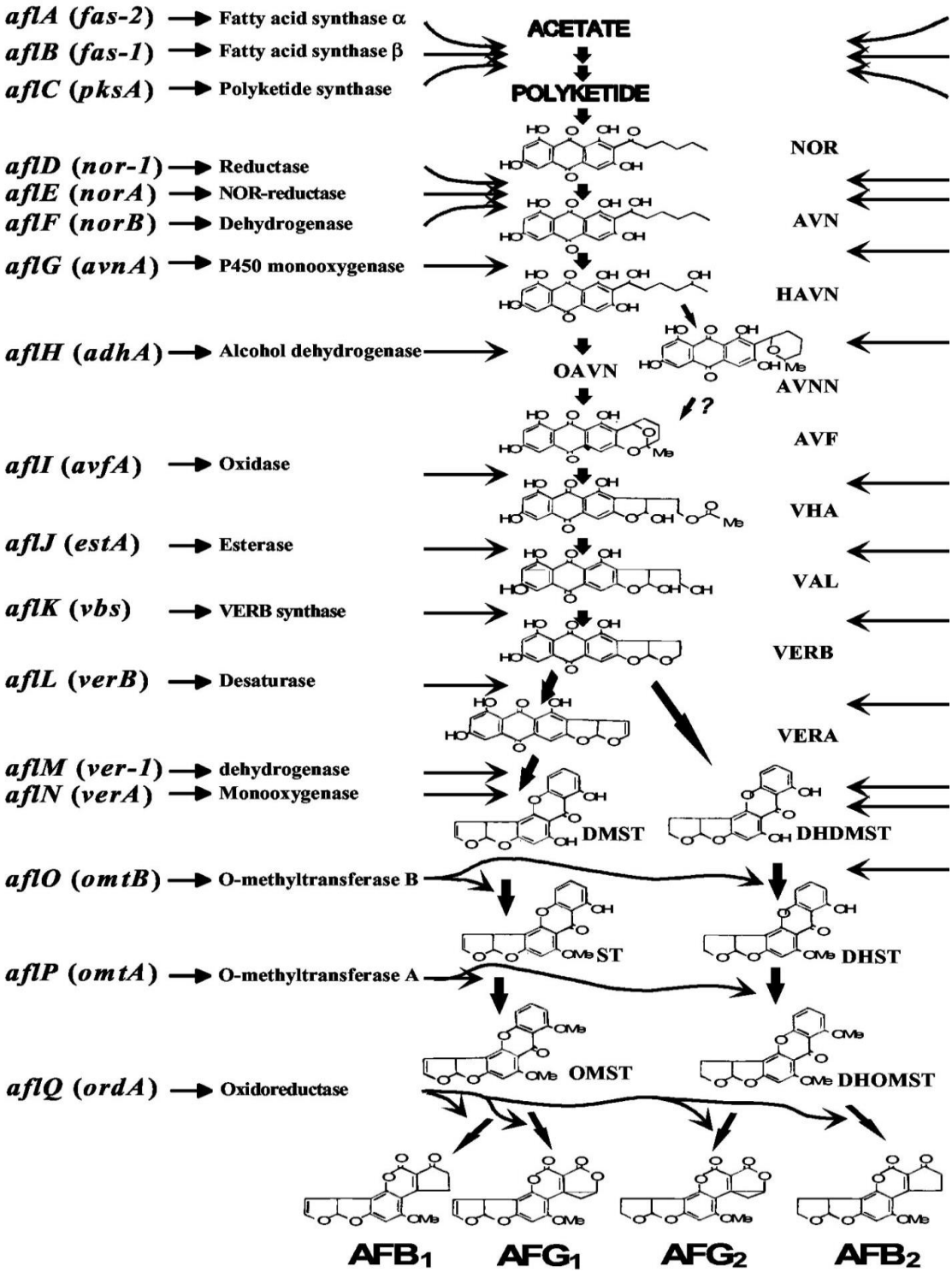


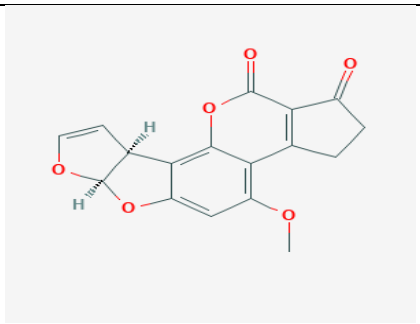
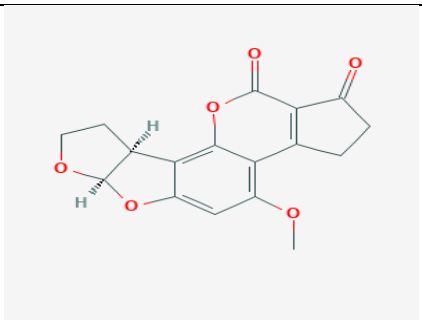
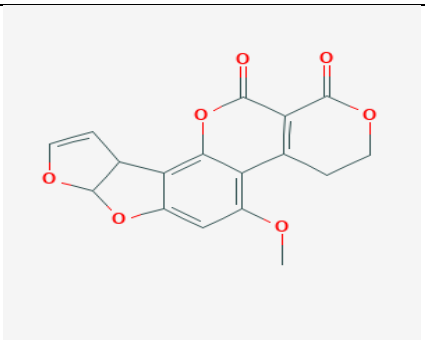
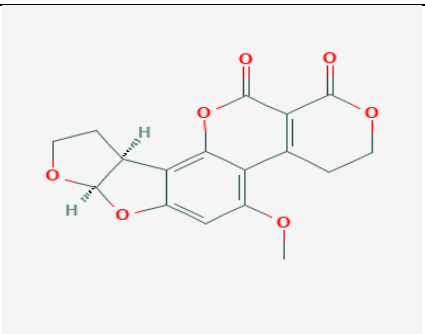
Fig 2. 1 Clustered genes and the biosynthetic pathway of aflatoxin in *A. parasiticus* and *A. flavus* (Yu et al., 2004). Gene encodes enzyme that involves in bioconversion step to make product. (Copyright license number: 4683850707299 from American Society for Microbiology)

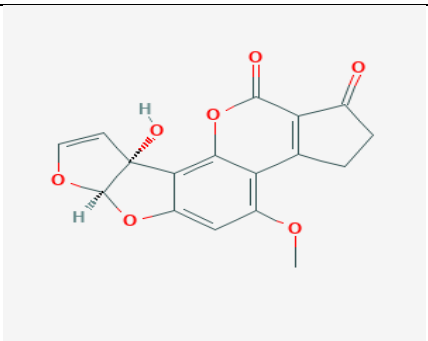
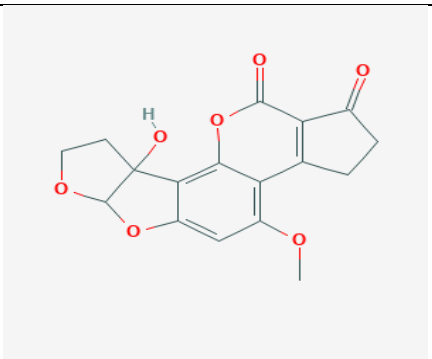
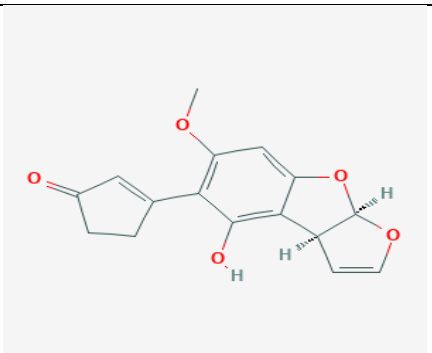
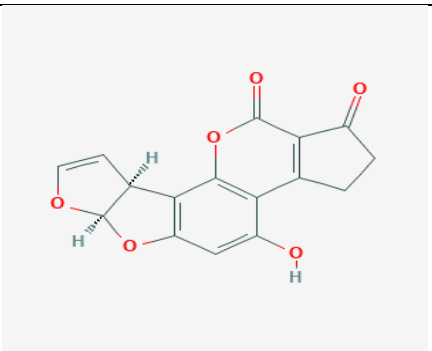
Aflatoxins are a potent carcinogen or carcinogenic in humans and mutagenic in bacteria (affect the DNA) posing a high risk for public health (Fink-Gremmels, 2008). Aflatoxin exposure leads to hepatic failure, encephalopathy and Reye's syndrome as well as influencing the health and development of the fetus and neonates (Bennett & Klich, 2003; Hayes, 1980). AFB1, AFM1 and AFG1 have been categorized as Group 1 carcinogens (IARC, 2002) that are chronic and acute poisons. Most of body organs are targets, particularly the liver and kidneys, resulting in liver cancer and links to other types of cancers that are greater in the presence of hepatitis B virus (HBV). In addition, long term exposure to aflatoxins may lead to birth defects in children, including stunted growth and immunosuppression. A high level of aflatoxins consumption may cause fatality due to liver damage (WHO/NHM/FOS/RAM/18.1, 2018). The lethal dose is 20-120 µg/kg bw per day over 1 - 3 weeks. The symptoms of the disease caused by aflatoxins in humans may depend on various factors including nutritional status, sex, age and/or the exposure to viral Hepatitis (HBV) or parasite infestation (Bennett & Klich, 2003; Berek, Mesterházy Á, & Molnár, 2001).

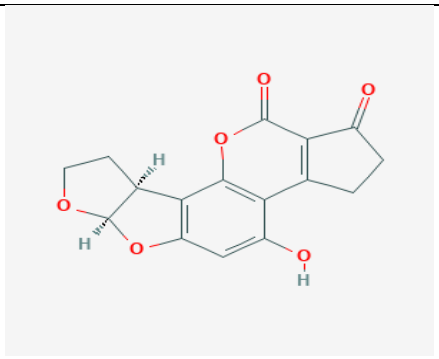
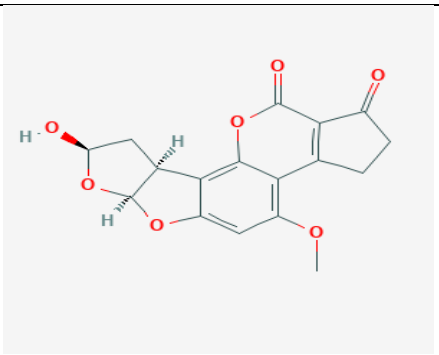
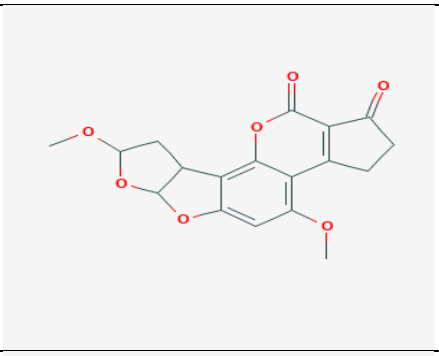
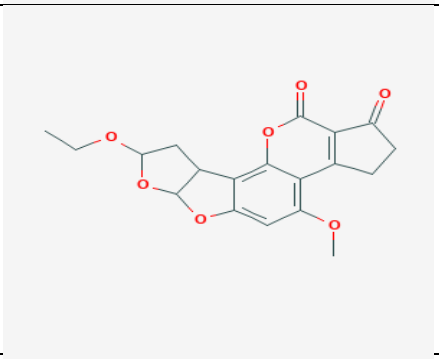
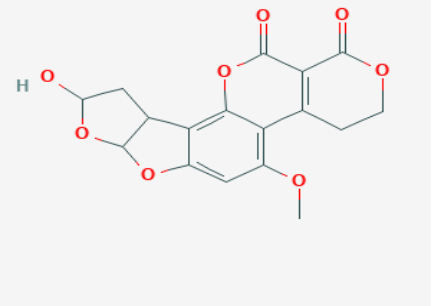
Due to the toxicity of aflatoxins, many countries have established strict regulations for aflatoxins in food including milk, however, these vary in different countries. While the maximum residue level (MRL) of AFM1 in milk is 0.5 µg/L in the United States, Brazil and most Asian countries including Vietnam (8-1:2011/BYT, 2011), it is 0.05 µg/L in most European countries.

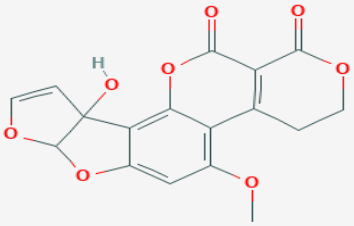
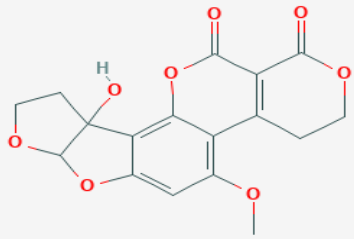
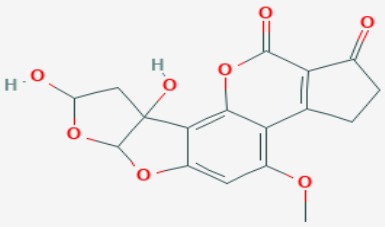
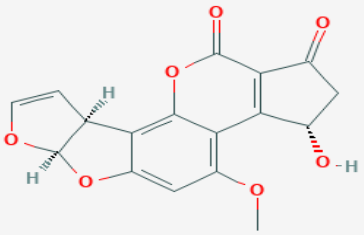
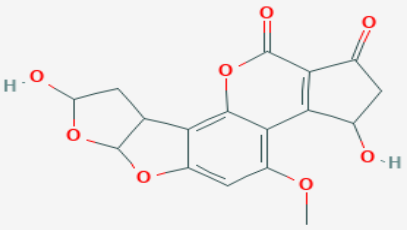
Traditional control involves ensuring the cattle food is kept dry to avoid the growth of toxin producing fungi. However, in some countries, such as Vietnam, where the climate is particularly damp with high humidity, ideal temperatures for fungal growth and where technology is limited, alternative methods of control are needed.

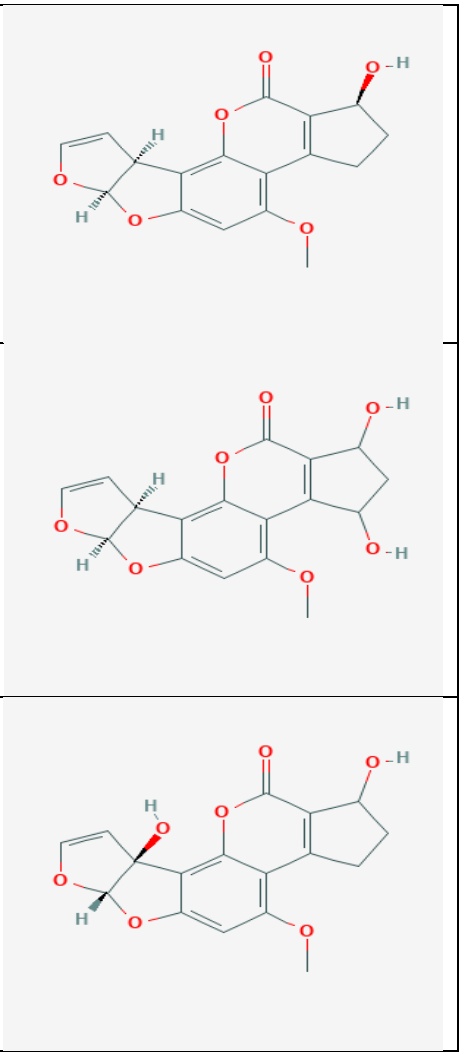
Table 2. 1 Molecular formula, molecular weight and structures of various aflatoxins.

No	Aflatoxin	Molecular Formula	Molecular weight (g/mol)	Structure
1	B1	$C_{17}H_{12}O_6$	312.27	
2	B2	$C_{17}H_{14}O_6$	314.29	
3	G1	$C_{17}H_{12}O_7$	328.27	
4	G2	$C_{17}H_{14}O_7$	330.29	

5	M1	$C_{17}H_{12}O_7$	328.27	
6	M2	$C_{17}H_{14}O_7$	330.29	
7	D1	$C_{16}H_{14}O_5$	286.28	
8	P1	$C_{16}H_{10}O_6$	298.25	

9	P2	$C_{16}H_{12}O_6$	300.26	
10	B_2A	$C_{17}H_{14}O_7$	330.29	
11	Ex_2B_1	$C_{18}H_{16}O_7$	344.3	
12	ExB_2	$C_{19}H_{18}O_7$	358.3	
13	G_2A	$C_{17}H_{14}O_8$	346.3	

14	GM_1	$C_{17}H_{12}O_8$	344.3	
15	GM_2	$C_{17}H_{14}O_8$	346.3	
16	M_2a	$C_{17}H_{14}O_8$	346.3	
17	Q1	$C_{17}H_{12}O_7$	328.27	
18	Q_2a	$C_{17}H_{14}O_8$	346.3	

19	R0	$C_{17}H_{14}O_6$	314.29	
20	Aflatoxicol H1	$C_{17}H_{14}O_7$	330.29	
21	Aflatoxicol M1	$C_{17}H_{14}O_7$	330.29	

2.2 Aflatoxin M1 reduction

2.2.1 Reducing AFM1 by adsorption

2.2.1.1 Yeast treatment

Many studies have investigated the effect of *Saccharomyces cerevisiae* (*SC*) on binding aflatoxin M1 in milk. *SC* Saflager W37/70 was used alone and together with lactic acid bacteria to degrade AFM1 in UHT milk at 37 ^{o}C for 30 and 60 min. AFM1 was reduced by up to 100% by using *SC* in combination with lactic acid bacteria after 60 min treatment. *SC* alone reduced 90.3% of AFM1 during 30 min and 92.7% after 60 min (Corassin, Bovo, Rosim, & Oliveira, 2013). Heat-killed cells were used to avoid fermentation that may influence the binding capacity of the yeast cell walls and enhance the adsorption

ability by altering the cell wall structure of the microorganism (Corassin et al., 2013; Gonçalves, Gonçalves, Rosim, Oliveira, & Corassin, 2017; Ismail, Levin, Riaz, Akhtar, Gong, & de Oliveira, 2017). The binding of aflatoxin with living microorganisms was shown to be a quick process with maximum binding occurring after a few minutes (Bovo, Corassin, Rosim, & de Oliveira, 2013; Serrano-Niño et al., 2013). To improve the aflatoxin binding capacity of *SC*, Ismail et al. (2017) evaluated the effect of various levels of AFM1 on the binding potential of microorganisms. As a consequence, 100% of AFM1 in spiked artificial milk at a level of 0.05 µg/L decreased to undetectable levels by using *S. cerevisiae* alone or combined with lactic acid bacteria. At 0.1 µg/L, *SC* at 10^{10} cell mL^{-1} AFM1 in milk was reduced by 92% (Ismail et al., 2017). *SC* was immobilized on perlite using as a biofilter to reduce AFM1 in milk (Foroughi, Sarabi Jamab, Keramat, & Foroughi, 2018). At an initial level of 0.23 ppb AFM1, 81.3% was reduced within 80 min. Four strains of *SC* including w292, fks1, mnn9 and sc1026 were tested to identify the chemical components involved in binding of mycotoxin and to clarify the nature of the chemical interactions between the yeast cell wall and mycotoxin (zearalenone-ZEN). Interestingly, a correlation between the number of β-D-glucans in the yeast cell walls and complex forming capacity was observed. The binding ability of the yeast cell wall to ZEN was affected by the number of β-D-glucans extracted from cell walls, while chitin decreased the binding capacity, and mannan had no effect (Yiannikouris et al., 2004). The ability of β-D-glucans to complex with ZEN was highly dependent on the pH with a neutral pH having a stabilizing effect on the complexing ability compared with acidic levels (Yiannikouris et al., 2003).

Assaf et al. (2018) used chitin to bind AFM1 in milk and phosphate buffered saline (PBS), and the stability of the complex was accessed after five washes (Assaf, El Khoury, Atoui, Louka, & Chokr, 2018). The binding capacity of chitin depended on treatment

time, level of chitin used, and the AFM1 contamination level in milk. The complex between chitin and AFM1 was more stable after 24 h compared with 30 min incubation. Long time incubation, high levels of chitin and low levels of AFM1 contamination increased the binding capacity of chitin. This is probably explained by the increase in AFM1 exposure to the binders (chitin) coupled with the tight binding resulting from the long exposure time.

The differences in the cell wall components binding to mycotoxins are possibly due to the variety of mycotoxins. Zen's structure is bigger and more complicated than AFM1 and may lead to the differences in the interaction with microbial cell walls.

2.2.1.2 Lactic acid bacteria (LAB) treatment

Lactic acid bacteria (LAB) have been shown to have the ability to capture or bind AFM1 from contaminated liquid media and milk (Table 2.2). The binding capacity of microbe and aflatoxin is generally affected by the microbial strains, incubation time, contamination level, number of the microorganisms, temperature and pH. The highest amount of AFM1 degradation in milk was 90% using a combination of *Lactobacillus plantarum* (DSM 20079), *Lactobacillus acidophilus* (DSM 20079), *Bifidobacterium bifidum* (DSM 20082), *Kluyveromyces lactis* (CBS 2359) and *Saccharomyces cerevisiae* (ATCC 64712) after 72 h incubation with the initial level of AFM1 50 ng/mL (Abdelmotilib, Hamad, Elderea, Salem, & Sohaimy, 2018). *Bacillus pumilus* E-1-1-1 supernatant was also able to reduce 76.9% aflatoxin in solvent (Gu, Sun, Cui, Wang, & Sang, 2018). The reduction of AFM1 in milk by LAB was less than 90% when using a single strain. A high contamination level of AFM1 in milk (50 ng/mL) was reduced from 19-61% by using probiotic bacteria and yeasts including *Pediococcus acidilatici* RC005, *Pediococcus pentosaeus* RC006, *Lactobacillus rhamnosus* RC007, *Saccharomyces*

cerevisiae var. *boulardii* RC009, *Saccharomyces cerevisiae* RC016 and *Kluveromyces marxianus VM003*. The toxicity of the treated samples was assessed by using brine shrimp with approximately 100% of nauplii surviving after 48 h incubation in four testing strains (Martinez, Magnoli, Gonzalez Pereyra, & Cavaglieri, 2019).

Table 2. 2 Bacterial strains used to reduce AFM1 in milk.

Species	Reduction (%)	Contamination level (µg/L)	Sample	Temperature (°C)	Time	Reference
Lactobacillus delbrueckii subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36	27 - 39	10	Reconstituted milk	42	4 h	(Belgin Sarimehmetoglu, 2004)
Bifidobacterium bifidum Bb 13, *Lactobacillus acidophilus* NCC 68	15.92 - 27.31	20	Reconstituted milk	37	4 h	(Kabak & Var, 2008)
Lactobacillus rhamnosus (HOWARU), *Bifidobacterium lactis*, (FLORA-FIT B107) and *Lactobacillus delbrueckii spp. bulgaricus* (LB340)	13 - 37	0.5	UHT skimmed milk	4	15 min	(Bovo, Corassin, Rosim, & de Oliveira, 2013)
Lactobacillus rhamnosus GAF01	15.3 – 95.1	50 - 200	Reconstituted milk	37	6 - 24 h	(Abbes, Salah-Abbes, Sharafi, Jebali, Noghabi, & Oueslati, 2013)
Lactobacillus acidophilus NRRL B-4495 (Ac), *Lactobacillus reuteri* NRRL B-14171 (Lr), Lactobacillus rhamnosus NRRL B-442 (Rha), *Lactobacillus johnsonii* NRRL B-2178 (Jh) and *Bifidobacterium bifidum* NRRL B-41410	19.95 - 25.43	10	PBS	37	12 h	(Serrano-Niño et al., 2013)
Lactobacillus acidophilus ATCC 20552, *Lactobacillus rhamnosus* TISTR 541, *Bidobacterium angulatum* DSMZ 20098, *Lactobacillus plantrium*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*	41 - 46	50	PBS	37	24 h	(Elsanhoty, Salam, Ramadan, & Badr, 2014)
Lactobacillus Acidophilus and *Bifidobacterium lactis*	96.2	0.05	UHT skim milk	4	72 h	(El-kest, Hariri, Khafaga, & Refai, 2015)
Lactobacillus helveticus ATCC 12046	85	0.1	Milk	-	1 h	(Ismail, Levin, Riaz, Akhtar, Gong, & de Oliveira, 2017)
Lactobacillus rhamnosus GG (ATCC53103)	63	50	Liquid media	37	18 h	(Assaf, Atoui, Khoury, Chokr, & Louka, 2018)
Lactobacillus Plantarum (DSM 20079), *Lactobacillus acidophilus* (DSM 20079), *Bifidobacterium bifidum* (DSM 20082), *Kluyveromyces lactis* (CBS 2359) *and Saccharomyces cerevisiae* (ATCC 64712) combination	80 - 90	50	Milk	37	12 - 72 h	(Abdelmotilib, Hamad, Elderea, Salem, & Sohaimy, 2018)
Bacillus pumilus E-1-1-1	89.55	40	Solvent	37	12 h	(Gu, Sun, Cui, Wang, & Sang, 2018)

The AFM1 binding capacity of yoghurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36) was shown to be less in yoghurt manufacture and greater in milk than in PBS (Belgin Sarimehmetoglu, 2004). Similarly, Abdelmotilib et al. (2018) found that the binding of *Lactobacillus plantarum* (DSM 20079), *Lactobacillus acidophilus* (DSM 20079), *Bifidobacterium bifidum* (DSM 20082), *Kluyveromyces lactis* (CBS 2359) and *Saccharomyces cerevisiae* (ATCC 64712) to AFM1 was slightly higher in milk than PBS. Conversely, Bovo et al. (2013) reported that the binding capacity was lower in milk than in PBS when using *L. rhamnosus* and *B. lactis, L. bulgaricus*. There was no noticeable difference in binding ability between milk and PBS (Kabak & Var, 2008) using *Bifidobacterium bifidum* Bb 13 and *L. acidophilus* NCC 68. The variation in the interaction between the microorganism cell walls and aflatoxin may be due to strain variation. Aflatoxin binding was decreased by approximately 9% after the manufacture of yoghurt, and this was explained by the effect of fermentation (Belgin Sarimehmetoglu, 2004) that presumably relates to the acidic pH. However, *L. plantarum* ATCC 10697, *B. animalis* ATCC 27672, and *B. bifidum* ATCC 35914 were found to reduce relatively the same amount of AFM1 in yoghurt and PBS (ranged from 50-70%) with and without the presence of inulin (Sevim, Topal, Tengilimoglu-Metin, Sancak, & Kizil, 2019). El-Nezami. (1998) showed that there was an increase in binding ability in PBS at acidic pH levels. The true explanation for the difference in this binding ability remains unknown.

To reduce the effects of fermentation of the bacteria during the treatment, milk can be treated with heat killed bacteria to improve AFM1 reduction (Assaf, Atoui, Khoury, Chokr, & Louka, 2018; Elsanhoty, Salam, Ramadan, & Badr, 2014; Ismail et al., 2017; Kabak & Var, 2008). The binding ability of non-viable microorganisms has been

shown to be more consistent than the viable ones, regardless of the treatment time (4 h and 24 h) and microbial strain (Elsanhoty et al., 2014). In addition, Kuharic et al. (2018) reported that *Lactobacillus plantarum* KM non-viable cells removed a higher percentage of AFM1 compared with the viable ones (Kuharić et al., 2018). The stability of the complex between aflatoxin and bacteria was evaluated by repeat washing followed by filtration. Up to 87.37% of the AFM1 in complex with bacteria was recovered into the wash solution showing unstable binding (Bovo et al., 2013). Ismail et al. (2017) also determined the stability of aflatoxin M1 and the microbial complex by washing in PBS. Most of the microbial strains released aflatoxin, with only a few strains still releasing aflatoxin after the 3rd washing. The maximum amount of aflatoxin M1 washed from the complex between aflatoxin and *Lactococcus lactis* JF 3102 was 19.5 and 34.8% using 0.05 µg/L and 0.1 µg/L aflatoxin spiked samples, respectively. The release was much greater for other strains (*Lactobacillus plantarum* NRRL B-4496, *Lactobacillus helveticus* ATCC 12046), at 20-70% (Ismail et al., 2017). These results support previous studies, which reported 2-87% aflatoxin M1 release by washing (unspecified number of washes) with PBS (Bovo et al., 2013; Kabak & Var, 2008; Serrano-Niño et al., 2013). This suggests the bond between aflatoxin and the microbes is weak. The binding mechanism is believed to be due to hydrogen bonds and Van der Waals interactions although the exact mechanism of binding has not been fully clarified. Cell wall components of bacteria including both polysaccharides and peptidoglycans are responsible for the absorption of AFM1 (Shetty & Jespersen, 2006). Diverse bacteria strains present different adsorption ability for aflatoxin because of variation in cell wall structure (Pierides, El-Nezami, Peltonen, Salminen, & Ahokas, 2000). That is also a likely explanation for the variation in the AFM1 complex release by washing.

Both viable and heat-killed bacteria show the ability to bind aflatoxin (Belgin Sarimehmetoglu, 2004), in "a short time" (Bovo et al., 2013) suggesting this as a potential method for controlling aflatoxin in milk and food processing. Nonetheless, there are more studies needed to explore the binding principle between AFM1 and microorganisms and factors that may enhance binding capacity and reduce AFM1. In addition, the studies evaluating the flavour of the milk after treatment are also necessary.

2.2.2 Degrading AFM1 by changing structure

2.2.2.1 Enzyme treatment

The nature of the AFM1 reduction using LAB and/or yeast was suggested to be based on adsorption, with the binding between bacteria/yeast cell walls and aflatoxins as a reversible bond, which does not degrade aflatoxins (Guan et al., 2010). The use of microorganisms may affect the organoleptic properties of the samples being treated (Raksha Rao, Vipin, Hariprasad, Anu Appaiah, & Venkateswaran, 2017). Another possible treatment using microorganisms is the effect of enzymes from microbial species on the degradation of aflatoxin (Table 2.3). These enzymes could be extracted for use rather than using the microorganisms themselves. There are many studies using microbial extracts to degrade AFB1, however, the application for AFM1 degradation is limited. Microbial extracts degrade at least 60% of AFM1 with initial levels of 50 - 100 µg/L. Zhao et al. (2011) reported the degradation of aflatoxin using enzyme extracted from *Myxococcus fulvus* ANSM068 was dependent on pH and temperature. Mg^{2+} and H_2O_2 were used to increase the degradation (almost 100%) (Zhao et al., 2011). It is suggested that the addition of Mg^{2+} may improve the enzyme membrane stability which enhances the maintenance of the enzyme's internal structure as well as

stimulates the enzyme active site. The enzyme structure is believed to be changed by using ions such as Zn^{2+} that decrease the interaction between aflatoxin and the active site of enzymes (D'Souza & Brackett, 2000). The mechanism for the degradation of aflatoxin for all these microbiological/enzyme extract treatments is generally unknown. However, a hypothesis was proposed, that the enzymes extracted from microorganisms enhance lactone hydrolysis leading to a structural change in aflatoxin molecules that reduces the biological toxicity of aflatoxin (Loi et al., 2016). This lactone structure is associated with the carcinogenic activity of the aflatoxin molecule (Motomura, Toyomasu, Mizuno, & Shinozawa, 2003), and when destroyed, there is a decrease in mutagenicity (Adebo, Njobeh, & Mavumengwana, 2016; Raksha Rao et al., 2017). A reduction in the toxicology and pathology of samples treated with a microbial enzyme was demonstrated by a decrease in mutagenic activity compared with the untreated samples in the Ames test. An experiment on rat liver indicated no significant difference between the enzyme treated aflatoxin containing samples and untreated aflatoxin samples. Hepatic structure from the treated and control samples was basically the same while the damage of hepatic structure was found in aflatoxin contaminated samples without enzyme treatment (Alberts, Engelbrecht, Steyn, Holzapfel, & van Zyl, 2006; Liu, Yao, Lian, Cheng & Gu, 1998).

This shows enzymatic treatment for aflatoxin is more effective than the reversible binding seen with many microbiological treatments, based on aflatoxin binding to whole cells. Treatments that change the structure of the aflatoxin lactone ring appear to be successful (Motomura et al., 2003). The use of microbial extracts for aflatoxin control is limited in practical application due to the long incubation required to detoxify and complicated procedures to prepare enzyme active extracts (Guan et al., 2010). For instance, the production of myxobacteria aflatoxin degradation enzyme via

myxobacteria fermentation is a challenge because it is easily contaminated during bacterial growth and the enzyme yields are low (Zhao et al., 2011). A mediator can be used as a supplement to reduce the treatment cost, reduce health risks and improve treatment efficiency (Loi et al., 2016).

Table 2. 3 Bacterial species used to produce enzymes that degrade aflatoxins.

Species	*Pleurotus pulmonarius* ACR-16	*Myxococcus fulvus* ANSM068	*Myxococcus fulvus* ANSM068
Reduction (%)	23, with mediators (100 for M1, 90 for B1)	63.82 - 71.89	96.96 and 95.80
Aflatoxin	B1, M1	B1, G1, M1	G1, M1
Contamination level	1 µg/mL B1, 50 µg/L M1	100 µg/L	100 µg/L
Sample	Sodium acetate	Standard solution	Standard solution
Temperature (°C)	25	35	35
pH	5	6	6
Time	72 h	48 h	48 h
Purified enzyme or culture supernatant	Lac2 pure enzyme	Culture supernatant	MADE
Additive	ABTS, AS, SA (10 mM)	Mg^{2+}	Mg^{2+}
Reference	(Loi et al., 2016)	(Zhao et al., 2011)	(Zhao et al., 2011)

2.2.2.2 Ozone Treatment

O_3 is generated electrochemically and described by three equations (McKenzie et al.,1997).

$$2\,H_2O \rightarrow O_2 + 4H^+ + 4e^- \qquad E^o = 1.23\ V \quad [1]$$

$$3\,H_2O \rightarrow O_3 + 6H^+ + 6e^- \qquad E^o = 1.51\ V \quad [2]$$

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad E° = 1.23\ V \text{ [3]}$$

When a direct current is applied, water is oxidized electrochemically at the anode (equation 1 and 2), leading to the production of O_3, while hydrogen is generated at the cathode (equation 3) (Murphy et al., 1994).

Although ozone treatment has been used to reduce AFB1 in various food samples (Chen et al., 2014, King, 2002; Luo, Wang, Wang, Li, Bian, & Chen, 2014), the use of ozone treatment to degrade AFM1 in milk is limited. Approximately 20% of AFM1 in milk was decreased after 60 min ozone treatment with the initial level of 0.233 µg/L (Sert & Mercan, 2021). In another study, ozone treatment for 5 min at 16 mg/L reduced the level of AFM1 in milk containing 0.56 µg/kg by 50%. No noticeable changes in pH or oxidation values of milk were observed, while there was a decrease in ß-carotene content and the total microbial count. The decrease in carotenoid by ozone treatment may result from the reaction of ozone and double bonds in the carotenoid structure (Benevides, Veloso, de Paula Pereira, & Andrade, 2011). Ozone was reported to increase the lightness and decrease the yellowness of the treated samples (Sert & Mercan, 2021). The double bond C8 - C9 of the terminal furan ring of aflatoxins also reacts with molecular oxygen produced by ozone (Agriopoulou, Koliadima, Karaiskakis, & Kapolos, 2016), which results in the formation of 1,2,3-trioxolane (molozonide) derivatives including aldehydes, organic acids and ketones (Diao, Hou, Chen, Shan, & Dong, 2013; Inan, Pala, & Doymaz, 2007; McKenzie KS, 1997) (Fig 2.2). The reaction between the terminal double bond of the furan ring and ozone may reduce the mutagenic potential of aflatoxins, it is suggested that the occurrence of the furan ring is also associated with the toxigenic, carcinogenic, and teratogenic effects of aflatoxin (de Alencar, Faroni, Soares Nde, da Silva, & Carvalho, 2012).

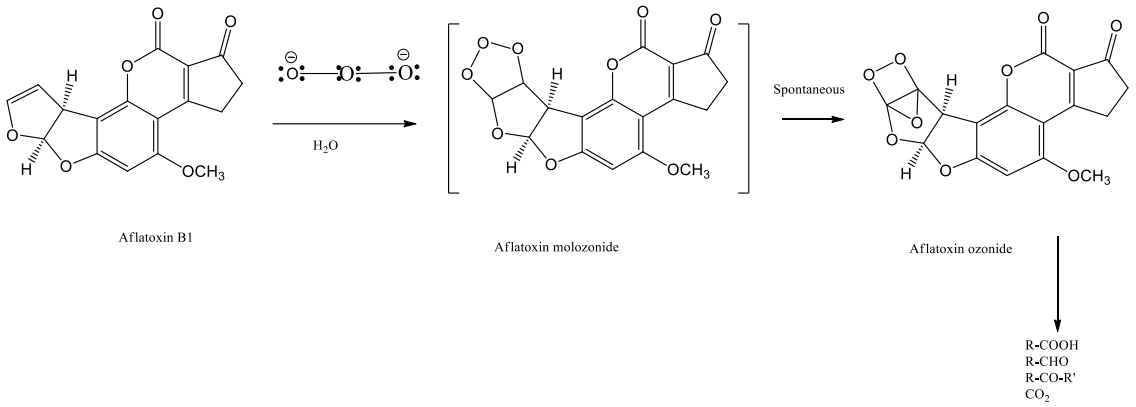


Fig 2. 2 Proposed ozonolysis mechanism for aflatoxin B1 and related products by McKenzie et al. (1997).

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Inactivating aflatoxins by ozone depends on various factors including the nature of aflatoxins, ozone concentration, sample nature and treatment time as well as moisture levels (Agriopoulou et al., 2016; King, 2002; Mohammadi, Mazloomi, Eskandari, Aminlari, & Niakousari, 2017). The reduction is in the order of 50% (Benevides et al., 2011), which is an advantage in the application for milk treatment. The efficacy of aflatoxin reduction in different types of food may vary. Furthermore, the effects of the formation of molozoznide derivatives through ozone treatment need to be assessed in terms of their effect on human health.

2.2.2.3 Peroxide treatment

Applebaum and Marth. (1982) used hydrogen peroxide (H_2O_2), H_2O_2 plus riboflavin (Rib) and H_2O_2 plus lactoperoxidase (LOP) to inactivate AFM1 at a level of 0.7-1.7 µg/L in naturally contaminated raw milk. These treatments ranged in their efficacy from 0-100% (Table 2.4).

Table 2. 4 AFM1 inactivation in naturally contaminated raw milk by H_2O_2, Rib and LOP at various conditions (Marth & Applebaum, 1982). AFM1 in milk was decreased completely by using 4% H_2O_2+ 3.2 mM Rib, while H_2O_2 at low levels (less than 1%), was not able to reduceAFM1.

Treatment	Time (min)	Temp (^oC)	AFM1 Reduction (%)
6% H_2O_2	30	63	71
6% H_2O_2 + 3.2 mM Rib.	15	30	100
4% H_2O_2 + 3.2 mM Rib.	20	30	100
4% H_2O_2 + 1 mM Rib.	25	30	50
0.05% H_2O_2	140	23	0
0.1% H_2O_2	140	23	0
0.05% H_2O_2 + 50 U LOP*	140	23	0
0.1% H_2O_2 + 50 U LOP	140	23	28.6
0.05% H_2O_2 + 5 U LOP	140	23	47.2
0.1% H_2O_2 + 5 U LOP	140	23	52.2
1% of H_2O_2	30	30	0
1% of H_2O_2	30	63	11
1% of H_2O_2 + 1mM Rib. + H	30	63	30
H^e+ 1 % H_2O_2	30	63	12
H + 1% H_2O_2 + 1mM Rib.+ H	30	25	51
H + 1% H_2O_2 + 0.5mM Rib. + H	30	25	66
1% H_2O_2 + 1mM Rib.+ H	30	30	45
1% H_2O_2 + 0.5mM Rib. + H	30	30	98

Rib: riboflavin

U LOP*: units of lactoperoxidase per mL of reaction mixture. One unit of activity is the amount causing an increase in absorbance of 0.001 O.D. per min when measured at 460 nm with a spectrophotometer (Marth & Applebaum, 1982).

Although these peroxide treatments are effective, H_2O_2 use exceeds the permissible level in food and therefore represents a risk to human health. These peroxide treatments are effective for AFM1 degradation and longer shelf - life of milk before processing (Arefin, Sarker, Islam, HarunurRashid, & Islam, 2017). However, the US Food and Drug Administration (FDA or USFDA) regulation for H_2O_2 is 0.5 mg/L in processed food (Özkan, Kırca, & Cemeroğlu, 2004), and a final concentration of 0.05% of the milk weight in the final product (Abbas, Luo, Zhu, Zou, & Tang, 2010). In addition, the protein content as well as methionine content, folic acid and ascorbic acid of milk decreases due to treatment with 0.05% of peroxide, (Elamin, 2001; Lakslunaiah, 1992). Hydrogen peroxide is an oxidizing agent which could cause severe gastrointestinal problems if consumed at high levels (Silva, Montes, Richter, & Munoz, 2012). As a result, the conditions required for treatment would need to be adjusted to optimize the time of treatment as well as ensuring no peroxide residue.

Another peroxide - based product, 0.002% benzoyl peroxide added to milk did not effectively inactivate AFM1 at 25 ^{o}C for 20 min (Yousef & Marth, 1986).

2.2.2.4 UV treatment

Yousef and Marth (1985) used ultraviolet (UV) energy (at a wavelength of 365 nm) to degrade AFM1, with a reduction of up to 100% at the initial level of 0.5 and 1 µg/L. The AFM1 degradation was increased by adding H_2O_2 (0.05-1%) and using a peristaltic pump with treatment time of 2-60 min. The circulation of the milk would improve the exposure of the milk to the UV light and is likely to produce a more

consistent reduction in AFM1 over a thin layer static method. Nevertheless, the milk flavour could be affected by using the pump as well as the length of time for UV treatment (Yousef & Marth, 1985). Recently, UVA LED was used to degrade AFM1 in water with the decrease of 84% at a dose of 1,200 mJ/cm^2. The cytotoxicity in HepG2 cells was investigated to evaluate the toxicity of the degradation product and the cell viability increased from 70.42 to 98.44% after treatment (i.e., less toxic after treatment) (Stanley, Patras, Pendyala, Vergne, & Bansode, 2020). Yousef and Marth (1987) reported that UV inactivation of AFM1 followed first order reaction kinetics. Aflatoxin x (AFM_x) is a product of AFM1 under UV treatment. AFM_x is also presumed to be aflatoxin M2a (AFM_{2a}) based on retention time (co - chromatographed) and the fluorescence ratio between AFM1 and AFM_x when AFM1 was derivatised. AFM_{2a} is suggested to be a product of AFM1 after treating with trifluoroacetic acid (TFA) detected by HPLC (Beebe & Takahashi, 1980). Although, UV treatment has some benefits, problems including the penetration of milk and flavour issues need to be overcome. In addition, more evidence is needed to confirm AFM_x is AFM_{2a} by a more precise method such as mass spectrometry ion fragment confirmation to clarify the mechanism of degradation of AFM1 and the toxicity of AFM_{2a}. Milk quality (organoleptic changes) could change with the length of treatment time and temperature for effective UV treatment (Yousef & Marth, 1987). Although, using high intensity UV irradiation was believed to change the organoleptic properties of milk (Li & Bradley, 1969; Yousef & Marth, 1987), exposure time and UV light wavelength may be optimized to reduce these effects.

2.2.2.5 Cold plasma treatment

Reducing AFB1 in feedstuffs is another option to decrease AFM1 in milk. Cold plasma has been used to degrade AFB1 in corn and hazelnuts (Shi, Ileleji, Stroshine, Keener, & Jensen, 2017; Siciliano et al., 2016).

Ions (H^+, H_3O^+, O^+, H^-, O^-, OH^-, N_2^+) are induced by cold plasma using strong electric field generation including molecular species (N_2, O_2, O_3 H_2O_2), and reactive radicals (O•, H•, OH•, NO•). Stable conversion products (e.g., ozone) and energetic photons (e.g., UV) are also cold plasma products (Bourke, Ziuzina, Han, Cullen, & Gilmore, 2017; Stoffels, Sakiyama, & Graves, 2008) which attack C8 - C9 double bonds in the furan ring of aflatoxins leading to aflatoxin reduction. There are several types of cold plasma producing sources such as dielectric barrier discharge, corona discharge, microwave discharge, radiofrequency discharge and jet plasma (Chizoba Ekezie, Sun, & Cheng, 2017; Coutinho et al., 2018). The applications of each vary. Corona discharge and dielectric barrier discharge, radiofrequency discharge and microwave discharge induce the large surface plasma that are applied to liquid samples while jet plasma is used for solid samples.

Cold plasma has been used for the degradation of various toxins including aflatoxin. A variety of gases are used as the operating gas including argon, nitrogen and nitrogen containing 0.1%, 21% and 65% oxygen, helium and air (Park et al., 2007; Shi et al., 2017; Siciliano et al., 2016; Wang, Huang, Li, Xiao, Zhang, & Jiang, 2015). Degrading AFB1 using cold plasma is believed to be mainly based on ozone or NO_x which are long - life reactive gas species, rather than free radicals, charged particles or ions that are short - lived species (Shi et al., 2017). When high ozone and NO_x concentrations were induced in modified atmosphere gas (MA65: 65% O_2, 30% CO_2, 5% N_2), AFB1 was reduced to a greater extent than with cold plasma induced by air. McKenzie et al.

(1998) suggested that ozone produced by a cold plasma generator attacks the C8-C9 double bonds of aflatoxin, the oxidized site of the aflatoxin molecule, promoting the opening of the terminal furan ring. OH radicals, which are strong oxidizing agents, enhance the reaction between ozone and the furan ring of aflatoxins at the olefinic site (Diao et al., 2013). AFB1 and AFG1 are more sensitive than AFB2 and AFG2 to plasma treatments because there is no terminal double bond at the C8 – C9 position of AFB2 and AFG2.The olefinic double bond in the furan ring of aflatoxins is believed to be associated with the mutagenicity and toxicity of aflatoxins. The disappearance of this double bond would lead to a decrease in aflatoxin toxicity. The cleavage of the lactone ring causes the creation of aflatoxin D1 which is much less toxic than AFB1 (Mendez-Albores, 2008; Samuel, Sivaramakrishna, & Mehta, 2014). Gaseous ozone treatment resulted in AFB1 reduction and the products produced following the treatment were non-toxic (Diao, Hou, Chen, Shan, & Dong, 2013). The breakdown products after plasma treatment of AFB1 as well as degradation pathways were investigated by using high performance liquid-chromatography time-of-flight mass spectrometry (HPLC -TOF- MS) (Shi, Cooper, Stroshine, Ileleji, & Keener, 2017). The disappearance of the double bond at the C8 - C9 position was confirmed, along with the lactone ring modification and the formation of cyclopentanone and methoxyl groups.

The influence of plasma treatment on milk quality has been reported with a slight difference in milk colour during 20 min of plasma treatment (Gurol, Ekinci, Aslan, & Korachi, 2012; Kim, Yong, Park, Kim, Choe, & Jo, 2015; Korachi et al., 2015). A decrease in pH was observed following plasma treatment in one study (Bruggeman et al., 2008) with a minor change in pH from 6.90 to 6.60 in milk after 10 min of treatment in another study (Kim et al., 2015). The concentrations of fatty acids in milk did not

change significantly following plasma treatment although there was some change noted in butyric acid, caprylic acid (Kim et al., 2015) and stearic acid (Korachi et al., 2015) concentrations. Dehydrogenation due to oxygen radicals generated by plasma is believed to cause changes in fatty acids (Korachi et al., 2015). While ketones and alcohols did not show any difference, a change in total aldehyde composition was observed (Korachi et al., 2015). The increase in these aldehydes was suggested because of degradation and/or auto-oxidation of hydroperoxides and several unsaturated fatty acids in milk (Vazquez-Landaverde, Torres & Qian, 2006), which could be caused by reactive species such as N, OH, and NO produced by plasma (Korachi et al., 2015). The aldehyde content increased with the increase in the exposure time to cold plasma (Ragni et al., 2010).

A cold plasma system operating at 30-60 °C at atmospheric pressure, does not noticeably affect the organoleptic characteristics of milk, including colour, pH and total fatty acid content. The treatment is highly effective in degrading aflatoxins over a short time and produces no residue compared with many other aflatoxin degradation procedures (Misra, Tiwari, Raghavarao, & Cullen, 2011; Schluter et al., 2013; Thirumdas, Sarangapani, & Annapure, 2014). In addition, this method is safe for the environment with no residual material left once the plasma power is turned off (Misra et al., 2011). However, this method has not been demonstrated for the degradation of AFM1.

2.3 Conclusions and potential future research

There are plenty of strategies to reduce AFM1 in milk, however, many are unable to completely degrade AFM1, pose an additional food safety risk or are reversible. Peroxide treatment can reduce 100% of AFM1 in milk but requires a high dose of

hydrogen peroxide that may leave a residue that is a concern for human health. Yeast and LAB in combination can reduce up to 100% AFM1 in milk, however, the reduction is based on binding between the microorganisms and AFM1, and this binding is reversible. Ozone, peroxide and UV light degrade AFM1 in milk by changing the toxin structure although the efficacy needs to be improved. In addition, many treatments used to reduce AFM1 may alter the flavour of milk. It is necessary to clarify the mechanism of these methods that change the toxin structure as well as determine the organoleptic effects resulting from milk treatment. Microbial extracts containing enzymes have shown promise for aflatoxin degradation and are unlikely to damage the milk. Enzyme treatment is believed to alter the aflatoxin structure, reducing aflatoxin toxicity. Microbial extracts have been used to inactivate AFM1 in standard solution but have never been used in milk. Cold plasma has never been used for degrading AFM1 in milk either but it has been used to reduce AFB1 in other food samples. The physical and chemical quality of milk following treatment with cold plasma for controlling microorganisms has shown no noticeable changes in pH, colour and fatty acids. These benefits coupled with the environmentally friendly nature of cold plasma suggest this method may be the favoured option to degrade AFM1 in milk and alleviate this food safety issue.

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CHAPTER 3. USING HPLC-MS TO DETERMINE AFLATOXIN M1 IN MILK PRE/POST TREATMENT

Abstract

A method to determine AFM1 pre and post treatment was evaluated. Ethyl acetate was used as an extraction solvent that is less toxic than other organic solvents such as chloroform, methanol or acetonitrile used for liquid-liquid extraction. Sodium chloride was added to increase the ionic strength of the solution to reduce the solubility of AFM1 in the water phase leading to an improvement in AFM1 extraction into the organic solvent phase (ethyl acetate). In addition, liquid-liquid extraction enhanced the method accuracy due to the simplicity of the procedure that reduces the method's error through sample preparation. The procedure combining ethyl acetate and salt is a cheap, simple, and reliable method. Limit of detection (LOD) and limit of quantification (LOQ) of the method were 0.01 and 0.025 µg/L, respectively, that meets the standard to determine a toxin compound in food at low maximum residue level (MRL) (0.05 and 0.5 µg/L). Furthermore, the method has been validated with all precision and recovery values lower than the expectation. These results show that the method appears to be suitable to determine the concentration of AFM1 in milk.

3.1 Literature review of methods to analyze AFM1

3.1.1 Introduction

There are two main stages in analyzing samples for aflatoxin -sample preparation which involves extraction of the aflatoxin and cleaning, followed by detection using techniques such as TLC, ELISA or HPLC.

3.1.2 Pretreatment of sample

3.1.2.1 Immunoaffinity column (IAC)

An immunoaffinity column is used to clean up samples for analysis (Fig 3.1). These columns can be used to filter samples containing aflatoxins using antibodies in the column. All the compounds that are not the analyte pass through the immunoaffinity column as they do not interact with the antibody. An organic solvent releases the aflatoxins from the antibody resulting in the analyte being eluted from the column. Chen et al. (2011) and Alonso et al. (2010) were able to achieve high recovery rates by using immunoaffinity columns. Iha et al. (2013) used immunoassay to purify aflatoxin M1 from dairy products. They used H_2O/MeOH (55/45) to initially extract the aflatoxin from dairy products then the extract was applied to an IAC column for purification. The impurities in the column were washed out with water, then aflatoxin in the column was eluted with MeOH and quantified by HPLC. LOD and LOQ were 3 ng/kg and 10 ng/kg, respectively, with relative standard deviation (RSD) <10% (Iha, Barbosa, Okada, & Trucksess, 2013). Chen and his team measured the aflatoxin in milk with UHPLC-MS/MS using an IAC column by adding 50 ml of fat - free milk to the column without solvent extraction. This method gave a wide linear range ($r > 0.997$) from 0.05 to 500 µg/L, LOD was 0.18 ng/L, RSD <15% (Chen et al., 2011). The main drawback of this method is that it is a long process to clean the sample. A

column with high selectivity cannot be used for analysis with other compounds and the columns are costly. In addition, the use of immunoaffinity columns has the disadvantage of not being able to be used for samples containing the impurities in the sample matrix that will compete with the target compound to be the antigen and bind to the antibody, affecting the productivity of extraction and analysis (Castegnaro, Tozlovanu, Wild, Molinie, Sylla, & Pfohl-Leszkowicz, 2006).

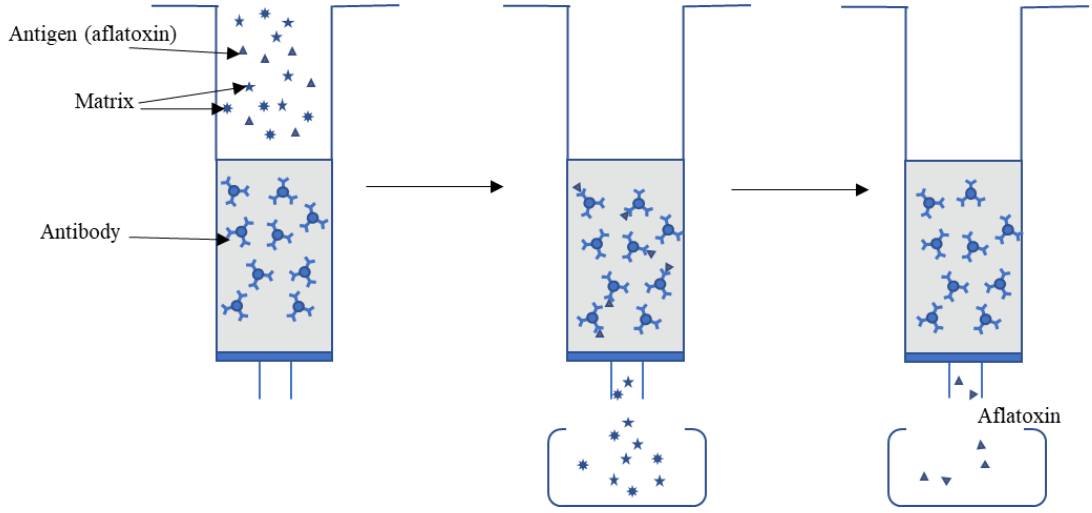

Fig 3. 1 Aflatoxin immunoaffinity column for sample pretreatment (clean - up and enrichment) based on Peiwu Li & Zhang. (2011).

3.1.2.2 Solid phase extraction (SPE)

One of the improvements in sample cleaning for aflatoxin analysis, is solid phase extraction (Fig 3.2). Samples are cleaned by a column containing C-8 or C-18, water is passed through the column and the mycotoxins are retained within the column and other contaminating materials are washed away. Finally, the target compounds are eluted with a polar solvent such as methanol or acetonitrile. The advantage of this method as opposed to the IAC method is less solvent use and more automation.

An experiment was conducted by Bijl et al. (1987), analyzing aflatoxin in cheese samples extracted with a water/acetone mix (3/1). Acetone was then evaporated and the water phase was passed through a disposable C - 18 column. After the column was washed with a mixture of acetonitrile/water (1/9), the target compound was eluted with acetonitrile. Finally, the quantification was done by TLC or HPLC with a C-18 column. The recovery rate was higher than 90% and the coefficient of variation was 6%. The LOD of the method was about 10 ng/kg (Bijl et al., 1987).

Manetta et al. (2005) used C-8 columns in the sample purification step, followed by HPLC analysis. Pyridinium hydrobromide perbromide was used as a post-column derivatizing agent to treat the sample before determining aflatoxin in milk and cheese. The LOD of AFM1 in milk and cheese using this method was 1 ng/kg and 5 ng/kg, respectively. The linear range of the calibration curve was 0.001-0.1 ng/kg. The average recoveries of AFM1 in milk and cheese were 90 and 76%, respectively.

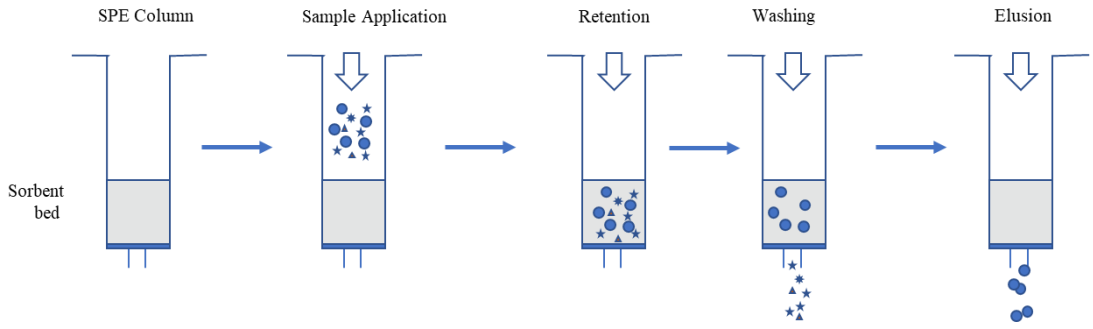

Fig 3. 2 A schematic of the SPE column modes and functionality based on Wang, Vitha, & Kay (2014).

3.1.2.3 Liquid-Liquid Extraction (LLE)

This method is based on the distribution of compounds between the water phase and the organic solvent phase (soluble or insoluble in water and solvent such as acetone, chloroform or methanol) (Fig 3.3).

In 2011, Wang and colleagues used acetonitrile to extract AFM1 from milk. This method had a LOD of 0.005 µg/L and LOQ of 0.02 µg/L with an average recovery rate of 88.8 to 100.6 % and RSD < 15 % (Wang, Zhou, Liu, Yang, & Guo, 2011). Campone et al. (2013) used liquid-liquid extraction with small volumes of acetonitrile to extract AFM1 from milk, resulting in a reliable, low cost, simple and rapid extraction method (Campone, Piccinelli, Celano, Russo, & Rastrelli, 2013). In Parma, Italy, Biancardi and his colleagues used ethyl acetate as a solvent for liquid-liquid extraction of AFM1 from milk with an average recovery of 95 %, a confidence interval of 1.9 % and a coefficient of variance (CV) of 4.5 % (Biancardi et al., 2013).

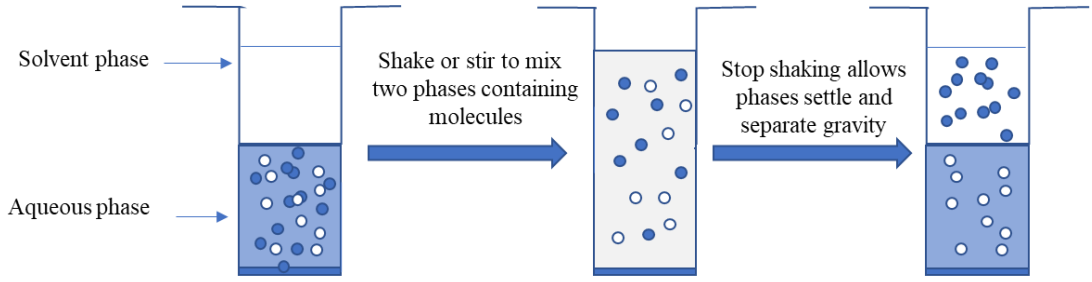

Fig 3. 3 Principle of liquid-liquid extraction

Biancardi reported no significant differences observed between LLE and IAC for the extraction of AFM1 in milk, which can be compared with a conventional method (IAC) based method ISO 14501. However, LLE is believed to have better accuracy, with all mean values close to each other (Biancardi et al., 2013). Salt was added to the extraction process (Biancardi et al., 2013; Campone et al., 2013; Michlig et al., 2015) to increase the extraction capacity by increasing the ionic strength.

3.1.3 Detection techniques

3.1.3.1 Thin - layer chromatography (TLC)

Thin layer chromatography is one of the techniques widely used in aflatoxin analysis for separating and evaluating purity (Van Egmond et al., 1978) (Fig 3.4). Thin layer chromatography is a stationary phase spread on a glass or plastic plate together with a mobile phase that is an organic solvent. Samples are liquid or are dissolved in a volatile solvent that is dotted onto the stationary phase spread on the plates and placed in a container of organic solvent. The samples move up the plate together with solvent due to the capillary action. The plate will be taken out of the solvent container when the solvent reaches the plate limit. The separation of components in the sample is then observed under ultraviolet light or by spraying appropriate reagents. The nature of the aflatoxin can be determined by running a standard parallel to the sample. Different compounds in a mixture move different rates on the chromatograms due to differences in their partitioning between the mobile and stationary phases (Fig 3.4). The Rf value of each spot is the ratio of the distance (cm) from the beginning to the center of the sample point and the distance (cm) from the beginning to the limit of the chromatogram (stop of the solvent). The Rf is the retardation factor or ratio of fronts. Consequently, the Rf is specific for a compound moving via the mobile phase on the stationary phase. The Rf value of the standard is compared to the sample for identification. The colour of the analyte in the sample under UV light is compared to the standard at various concentrations to determine the concentration (Hussain, 2011). Aflatoxin can easily be detected by fluorescent light (detected at λ = 365 nm or λ = 430 nm emission). Thin layer chromatography has been used as an AOAC method for aflatoxin determination since 1971, with the initial application of TLC for aflatoxin determination used by Van Egmond et al. (1978). They identified AFM1 in a thin chromatographic plate by the reaction of AFM1 and trifluoroacetic acid (TFA) with a

mobile phase of methanol/acetic acid/water (92/8/2). But this method is not often used for quantification due to limitations in accuracy.

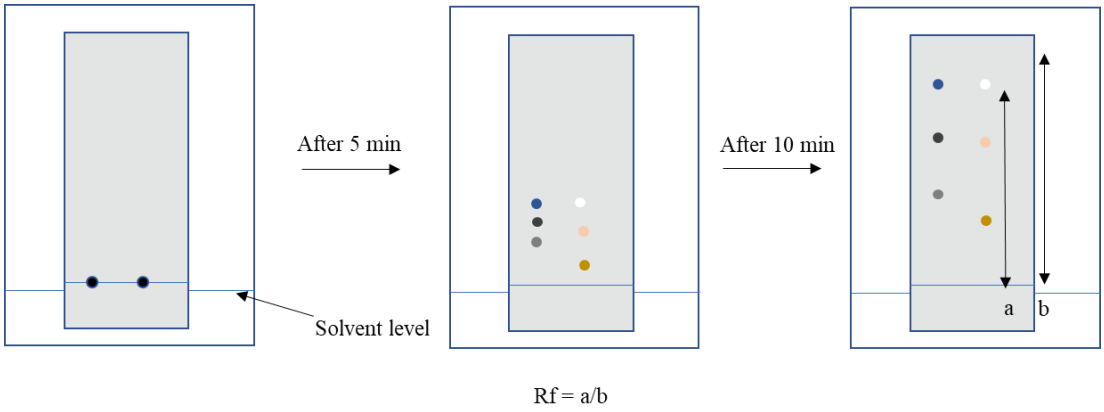

Fig 3. 4 Steps of thin layer chromatography. The distance from the beginning to the center of the sample point and the distance (a), the beginning to the limit of the chromatogram (stop of the solvent) (b).

3.1.3.2 Enzyme - linked immunosorbent assay (ELISA)

This method is used for aflatoxin analysis with main advantages being simplicity, ease of use, sensitivity and compatibility. There are several ELISA type assays including direct competition with immune binding enzymes (antibodies are coated on a solid phase microtiter plate) which is the most common format, and indirect competition with immunoglobulin enzymes (protein - toxin complexes are coated on the microtiter plate), sandwich and competitive ELISA. Aflatoxin in the sample is detected and quantified by using an enzyme and specific antibody. Enzyme linkages are based on antigen - antibody responses. The antigen, aflatoxin, is added to bind with the wells in the microtiter strip. The antibody is then added to the well forming the complex with antigen and an antibody labelled conjugate enzyme is added. The wells are washed between every step to remove unbound antigen and conjugate enzyme before a

substrate is added to react with the conjugate enzyme producing a colour change that can be read in a spectrophotometer and is semi - quantitative indicating a positive (aflatoxin) reaction (Fig 3.5).

The disadvantage of this method is that it is susceptible to cross-contamination (cross reaction) resulting in false negative reactions (Elke Anklam, 2002). The ELISA is most used as a screening test before HPLC quantification.

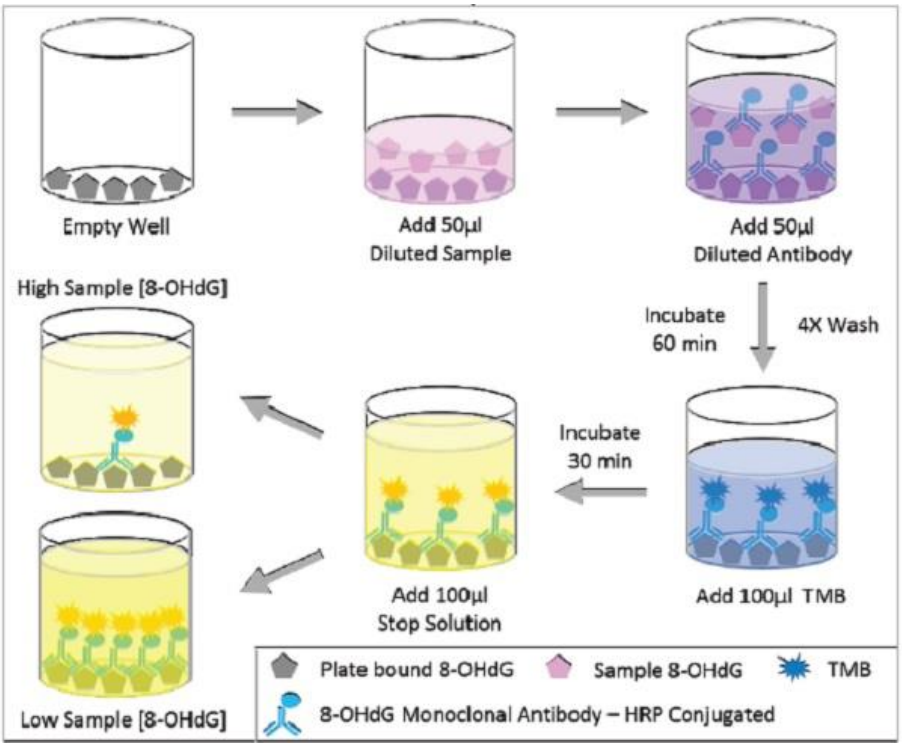

Fig 3. 5 ELISA Kit Schematic

(http://www.abcam.com/8-hydroxy-2-deoxyguanosine-elisa-kit-ab201734.html)

3.1.3.3 High pressure liquid chromatography (HPLC) (Fig 3.6)

This method is increasingly used because of its accuracy, sensitivity, selectivity, recovery, and reliability compared to TLC. In HPLC, samples are moved in a solvent or mobile phase through a stationary phase fixed in a column. The analytes will be

partitioned in the mobile phase and the stationary phase leading to the separation of the compounds.

The two columns commonly used in HPLC are the normal phase column and the reserve - phase (RP) column. Normal phase chromatography is the use of a polarized stationary phase, for example silica gel and a non - polar solvent such as hexane. With RP - HPLC, columns contain a non-polar stationary phase (such as C-8 or C-18) and a polarized solvent (water, methanol or acetonitrile). Aflatoxin is derivatized to form a fluorescent compound and is detected by fluorescence when fluorescence detector is used as a measurement. RP-HPLC is usually used to determine aflatoxin in food and the derivative is the use of strong acids or oxidants such as Br_2, I_2 or trifluoroacetic acid. The peaks in the chromatogram provide two types of analytical information: qualitative (based on retention time) and quantification (based on peak area). The common detectors used in HPLC include Ultraviolet (UV), Diode Array Detector (DAD), Fluorescence detector (FLD) and Mass Spectrometry (MS). The MS is believed to be the best for analyzing low MRL compounds.

Liquid chromatography with mass spectrometry (LC-MS) has recently been developed for detecting aflatoxin. In the MS, the sample travels to the ionization chamber through a sprayer, and fragmentation takes place in the collision chamber. The fragments of the target compound then enter the deep vacuum area and the probe where the signal is read. This method quantifies compounds based on fragments of molecules (along with retention time) which increases the accuracy that enables this method to fully meet the requirements for toxin compound analysis such as sensitivity, high accuracy and low MRL requirements. In addition, according to the Commission Decision 2002/657/EC (2002), a method based on mass spectrometry (MS) would be best suited to provide sufficient information for aflatoxin analysis. The drawbacks of this

approach are the expense for the initial investment, and technical requirements of the user.

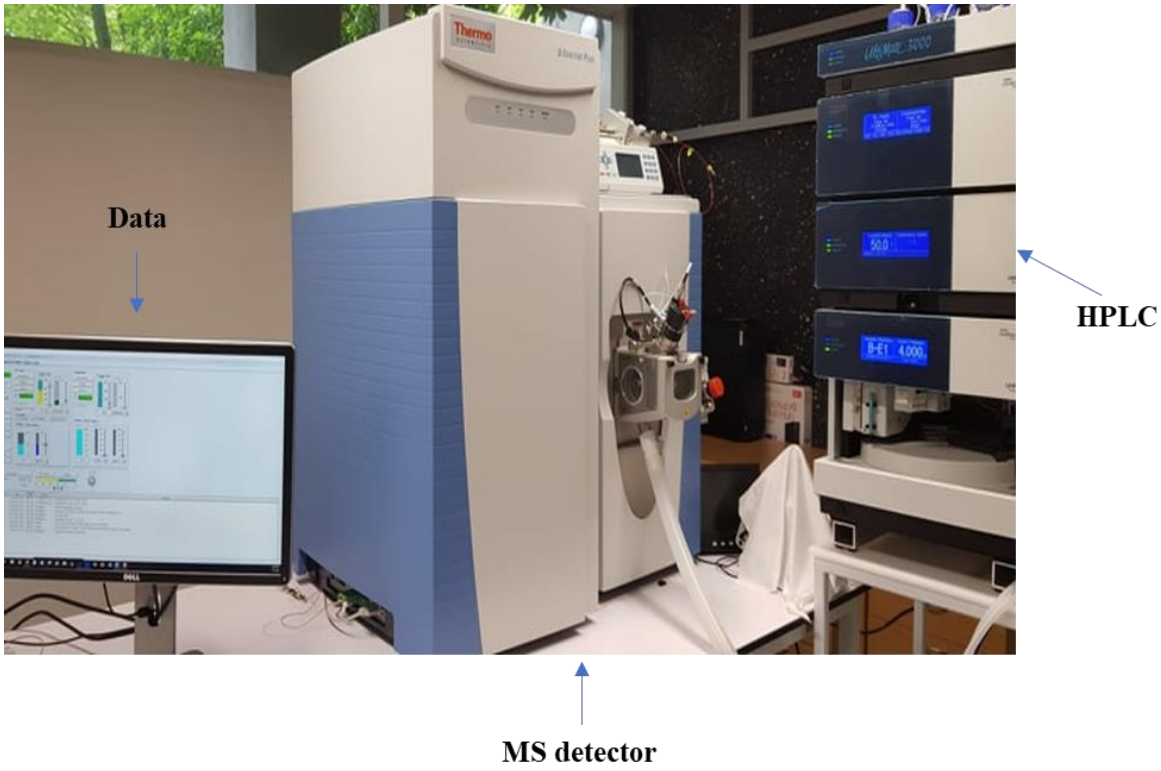

Fig 3. 6 HPLC - MS system

3.1.4 Conclusion

Liquid - liquid extraction is a reliable, low cost, simple and rapid method together with better accuracy compared with the multi-step procedures. These benefits make it a good method for analysis of AFM1 in milk. In addition, LC-MS is likely to be the most useful technique to determine AFM1 in milk due to its selectivity, sensitivity and accuracy that are the key requirements for analyzing compounds at low levels. Therefore, LLE and LC-MS were used for AFM1 determination in this study.

3.2 Methodology

3.2.1 Chemicals and standard

The Orbitrap LC-MS Q-Exactive (Thermo Fisher Scientific, San Jose, USA) was used.

The chemicals used were:

1. Aflatoxin M1 standard 50 µg/mL (Sigma-Aldrich, USA).

2. Water W6 - 4 Optima LC-MS water.

3. Methanol Optima LC-MS grade EcoSafPak 4L.

4. Ammonium acetate Optima LC-MS grade 50g (FSBA114 - 50).

5. Formic Acid Optima for LC-MS grade 50mL (FSBA117 - 50).

6. NaCl (Univar).

7. Ethyl acetate (Scharlau, Spain).

8. Acetonitrile (HPLC grade).

The standards were prepared as follows:

50 µg AFM1 standard was mixed with 1 ml acetonitrile 100% (stock standard 50 µg/ml = 50 mg/L) (A).

100 µL (A) + 9900 µL acetonitrile → 10000 µL (10 mL) AFM1 500 µg/L (B)

10 µL (B) + 990 µL H_2O/ACN (85/15) 0.1% Formic Acid → 1000 µL AFM1 5 µg/L (C)

200 µL (C) + 800 µL H_2O/ACN (85/15) 0.1% Formic Acid → 1000 µL AFM1 1 µg/L (D)

100 µL (C) + 900 µL H_2O/ACN (85/15) 0.1% Formic Acid → 1000 µL AFM1 0.5 µg/L (E)

100 µL (D) + 900 µL H_2O/ACN (85/15) 0.1% Formic Acid → 1000 µL AFM1 0.01 µg/L

100 µL (E) + 900 µL H_2O/ACN (85/15) 0.1% Formic Acid → 1000 µL AFM1 0.05 µg/L (F)

100 µL (F) + 900 µL H_2O/ACN (85/15) 0.1% Formic Acid → 1000 µL AFM1 0.005 µg/L

3.2.2 Validating method to determine aflatoxin M1 in milk before and after treatment by LC-MS.

Milk samples were contaminated artificially with AFM1 and tested by the following procedure (Fig .3.7) which was modified from the method developed by Biancardi et al. (2013). Four mL of milk (after fat extraction) was mixed with 0.4 g of NaCl and 12 mL of ethyl acetate on a shaker for 10 min at 300 rpm. The mixture was centrifuged for 10 min at 8400 g to separate the organic layer. Three mL of supernatant was evaporated under nitrogen flow (40 ^oC) and re-dissolved in 1 mL of H_2O/ACN 85/15 (v/v) with 0.1 % formic acid.

4 ml milk, fat was extracted by centrifugation at

4 ^{o}C, 8400 x g for 10 min

↓

+ 12 ml ethyl acetate + 0.4 g NaCl

↓

Shaking for 10 min at 300 rpm

↓

Centrifugation at 8400 x g for 10 min

↓

3 mL supernatant is evaporated by N_2 (at 63 ^{o}C in water)

↓

Dissolve by 1 ml H_2O/ACN (85/15) 0.1% acid formic

↓

Filter → LC-MS/MS

Fig 3. 7 Milk testing procedure

3.2.3 Statistical analysis

All the data were statistically evaluated by Analysis of Variance (ANOVA), performed with the Statistical Analysis Tool of Microsoft Excel 2016, Microsoft Office.

3.3 Results and discussion

The following were the validations used for the determination of aflatoxin M1 in milk before and after treatment by LC-MS.

- Linearity

Seven (7) levels: 0.005; 0.01; 0.05; 0.1; 0.5; 1.0 and 5.0 µg/L were diluted from a stock standard 50 mg/L in acetonitrile for a solvent curve (Fig 3.8 a) and in the sample matrix (milk) (Fig 3.8 b). These are standard curves used for future assays.

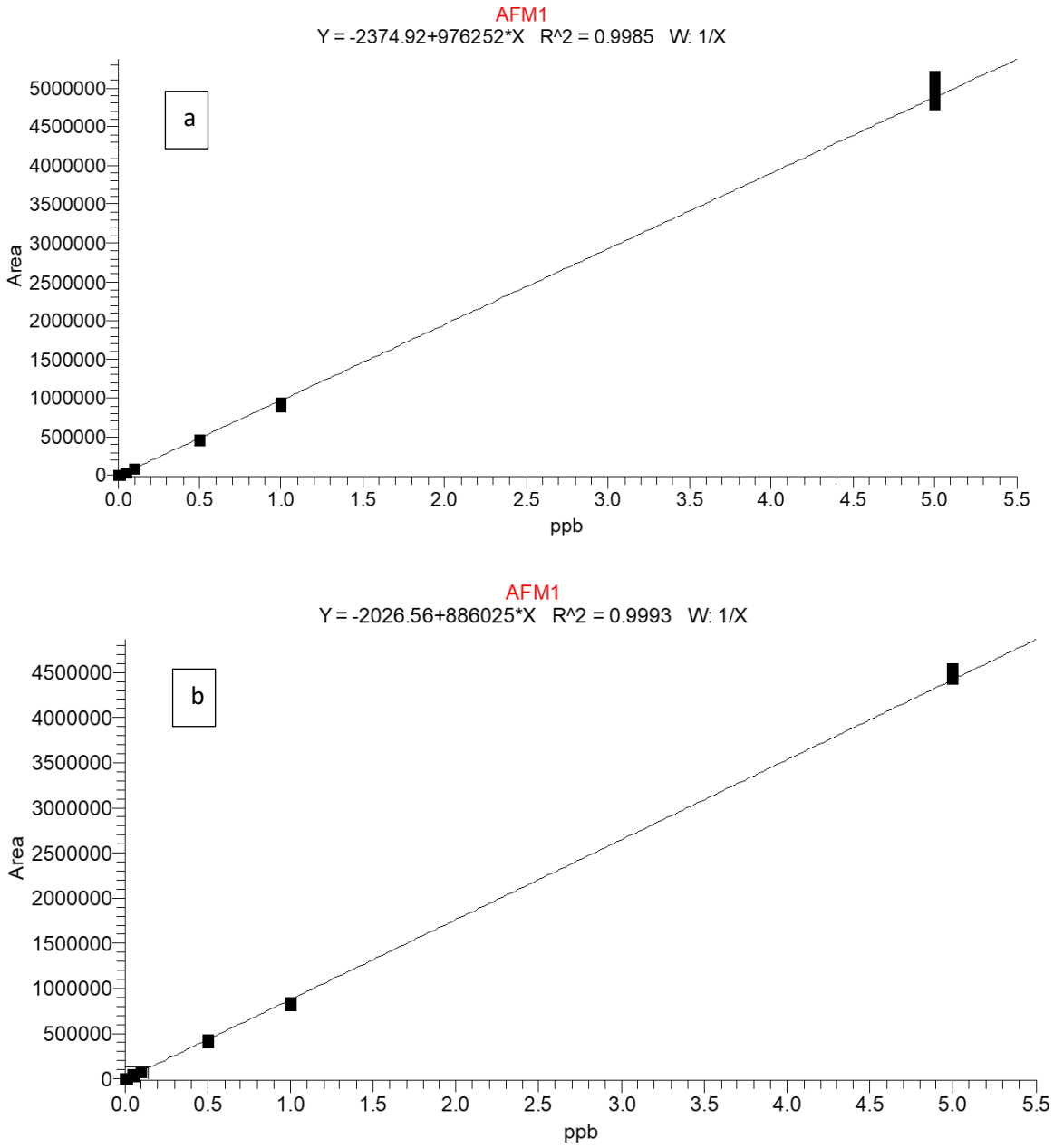

Fig 3. 8 Linearity of AFM1 in acetonitrile (a) and milk (b)

Linearity ranged from 0.005 - 5 µg/L with R^2 = 0.999 and 0.998 corresponding to the standard in the sample matrix (milk) and solvent, respectively.

- LOD, LOQ

0.025 µg/L was estimated as the LOQ of the method. Seven spiked samples at the level of 0.025 µg/L were analyzed for repeatability, recovery and relative standard deviation (Table 3.1).

Table 3. 1 Repeatability, recovery and relative standard deviation at 0.025 µg/L.

No	Concentration (µg/L)	Recovery (%)	Mean (%)	RSD (%)
1	0.0130	52.00	51.61	7.01
2	0.0133	53.20		
3	0.0124	49.72		
4	0.0118	47.24		
5	0.0140	55.92		
6	0.0119	47.44		
7	0.0139	55.76		

LOD of the method is estimated from LOQ → LOD = 0.01 µg/L (LOD = LOQ/3).

- Accuracy: Precision + Trueness

Seven samples containing AFM1 at the following levels 0.025; 0.05; 0.5 and 1 µg/L, were analysed to evaluate the method accuracy (Table 3,1; 3.2; 3.3 and 3.4)

Table 3. 2 Repeatability, recovery and relative standard deviation at 0.05 µg/L.

No	Concentration (µg/L)	Recovery (%)	Mean (%)	RSD (%)
1	0.0316	63.30		
2	0.0302	60.48		
3	0.0384	76.84		
4	0.0383	76.62	67.87	10.19
5	0.0309	61.80		
6	0.0355	70.96		
7	0.0325	65.08		

Table 3. 3 Repeatability, recovery and relative standard deviation at 0.5 µg/L.

No	Concentration (µg/L)	Recovery (%)	Mean (%)	RSD (%)
1	0.2916	58.33		
2	0.3142	62.84		
3	0.3286	65.72		
4	0.2609	52.18	61.80	8.53
5	0.3215	64.30		
6	0.3058	61.16		
7	0.3402	68.04		

Table 3. 4 Repeatability, recovery and relative standard deviation at 1 µg/L.

No	Concentration (µg/L)	Recovery (%)	Mean (%)	RSD (%)
1	0.55	54.73	61.60	6.29
2	0.60	59.92		
3	0.63	63.27		
4	0.66	66.15		
5	0.64	64.00		
6	0.59	59.23		
7	0.64	63.92		

Relative standard deviations (% RSD) were 7.01; 10.19; 8.53 and 6.29 at the level of 0.025; 0.05; 0.5 and 1 µg/L, respectively. All relative standard deviations were lower than 30% and the recoveries at all concentrations were higher than 40 % that meet the standards for chemical methods at low concentrations (1 µg/L), (Table 3.5) (AOAC, 2016). The expected recovery as a function of analyte concentration is shown in Table 3.6.

Table 3. 5 Expected precision (repeatability) as a function of analyte concentration (AOAC, 2016)

No	Concentration		RSD (%)
1	1	100 %	1.3
2	10^{-1}	10 %	1.8
3	10^{-2}	1 %	2.7
4	10^{-3}	0,1 %	3.7
5	10^{-4}	100 ppm	5.3
6	10^{-5}	10 ppm	7.3
7	10^{-6}	1 ppm	11
8	10^{-7}	100 ppb	15
9	10^{-8}	10 ppb	21
10	10^{-9}	1 ppb	30

Table 3. 6 Expected recovery as a function of analyte concentration (AOAC, 2016)

No	Concentration		Recovery (%)
1	1	100 %	98-102
2	10^{-1}	10 %	98-102
3	10^{-2}	1 %	97-103
4	10^{-3}	0,1 %	95-105
5	10^{-4}	100 ppm	90-107
6	10^{-5}	10 ppm	80-110
7	10^{-6}	1 ppm	80-110
8	10^{-7}	100 ppb	80-110
9	10^{-8}	10 ppb	60-115
10	10^{-9}	1 ppb	40-120

Seven milk samples contaminated at 0.05 µg/L were analysed on another day to evaluate within - lab repeatability (precision). These results were compared with those from Table 3.2 by using a one-way analysis of variance (ANOVA) (Appendix A).

$RSD_R = 4.11 < 25.12$ (expected RSD_R at 0.05 µg/L calculated by Horwitz: CV = 2(1-0.5logC), C is analyte concentration).

3.4 Conclusion

A simple method based on liquid-liquid extraction and MS detector to determine AFM1 before and after treatment was modified and validated with all the linearity, LOD, LOQ and accuracy values meeting the standards for analysing chemical compounds at low concentrations. Liquid-liquid extraction using ethyl acetate was

reported as a simple, cheap and reliable method because of its advantages including time efficiency, low cost and good recovery. In addition, HPLC - MS quantifies chemical compounds based on retention time confirmed by molecular fragmentation which enhances the accuracy of the method that allows this method fulfils the requirements of toxin analysis such as sensitivity, high accuracy and low MRL.

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CHAPTER 4. INVESTIGATION OF ULTRAVIOLET (UVC) (254 nm) FOR THE REDUCTION OF AFM1 IN SKIM MILK

Abstract

This study investigated the reduction of AFM1 in skim milk by using UVC light at 254 nm and the effects of factors influencing the efficacy including treatment time (min), depth of samples (mm), contamination level (μg/L), stirring, temperature and fat content in milk. The degradation product of AFM1 after UVC treatment was identified. The colour and pH of milk samples were measured to evaluate the influence of the treatment on these values. UVC reduced up to 50% of AFM1 in milk after 20 min of treatment regardless of the initial AFM1 contamination level. Treatment time, depth of sample and stirring were all found to significantly ($P < 0.05$) enhance the reduction of AFM1. The milk colour was affected but there was no influence in the pH of milk samples at all durations of UV exposure. The double bond of furan ring of AFM1 molecules was oxidized forming the degradation product, which, in theory, is expected to reduce the toxicity of the toxin.

4.1 Introduction

4.1.1 UV light introduction

Ultraviolet light consists of the electromagnetic radiation in the non-ionising region ranging from 100 to 400 nm (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000). It is divided into three categories. (1) The UVC region ranging from 200 to 280 nm (short-wave) is capable of inactivating microorganisms including bacteria, viruses and fungi. (2) The UVB region ranging from 280-315 nm (medium-wave) may cause skin burning and potentially lead to skin cancer. (3) The UVA region ranging from 315-400 nm (long-wave) causing tanning or human skin changes. The UV light ranging from 100-200 nm is called vacuum UV because it is absorbed by all material but vacuum (Go´mez-Lo´pez, Koutchma, & Linden, 2012).

Energy (E) is emitted in the form of photons of light when electrons of atoms/ions transfer from a higher energy state (E2) to a lower one (E1). The energy E (J) is calculated by the Planck equation:

$$E = E2 - E1 = hc/\lambda$$

Where h is Planck's constant = 6.23310234 (Js)

c is the speed of light = 2.9983108 (m/s)

λ is the wavelength of radiation (m)

Each chemical element has a unique spectrum of light emission because the energy levels of the atoms or ions representing a specific element are driven by the number of neutrons, protons and electrons in the atom or ion of that element and the interaction between them and the external force fields. For instance, there are some elements with specific differences between energy levels leading to photon emission in the UVC range. Similarly, it requires an input of energy for electrons to transfer from the lower

energy level to the higher one. This energy may be taken from the collision between atoms or atoms with ions, electrons or photons. The transmission of energy to atoms may lead to a change in the kinetic energy of atoms, the ionization of atoms caused by the removal of the electron and the transfer of electrons between energy levels. The ionization induces either cations or free charged electrons. When a free electron recombines with a cation, the light is emitted. The maximum wavelength of the emitting light can be a continuum or a range and be calculated by the equation:

$$\lambda_{max} = hc/E_o$$

Where E_o is the ionization energy

The photon is likely to be the smallest unit of radiation energy. The unit of energy of a single photon is eV (electronvolt), which is the energy required for an electron passing through 1 volt (1.6×10^{-19} J). Kilocalories/Einstein is used to represent the unit of the energy of 6.02×10^{23} photons (one mole photon). The energy per Einstein (E_E) is calculated by:

$$E_E = (hc \div \lambda)A$$

Where A is Avogadro's number = 6.02×10^{23} mol^{-1}

(Go´mez-Lo´pez, Koutchma, & Linden, 2012).

4.1.2 Reduction of aflatoxins by UV

UV light has been used to degrade AFB1 in various food samples, including ground nut (Arachis hypogea) and tree nuts (Juglans regia and Pistachio vera), peanut oil, peanuts and red chilli powder (Diao, Shen, Zhang, Ji, Ma, & Dong, 2014; Farhat, Ijaz, Muhammad, Zahoor-Ul-Hassan, & Muhammad, 2012; Jubeen, Bhatti, Khan, Zahoor-Ul-Hassan, & Shahid, 2012; Liu, Chang, Jin, Huang, Liu, & Wang, 2013; Liu, Jin, Huang, Liu, Wang, & Mao, 2011; Neelima, Manjeet, Saleem, & Rakesh, 2013;

Tripathi & Mishra, 2010). However, its application in degrading AFM1 in milk is limited. Yousef and Marth. (1985) used UV irradiation (365 nm) to degrade AFM1 in milk for the first time, with a reduction of up to 100% after 60 min with an initial level of 1 µg/ml. The treatment time was sufficiently long that it may affect the organoleptic properties of the milk. In an attempt to reduce the treatment time, a pump was used to circulate the milk sample during treatment and added 1% of H_2O_2 together with UV was used in another experiment (Yousef & Marth, 1986). The degradation time was shorter although the treatment effects on milk quality remained, which may have been exacerbated the by pumping, or possible residue of peroxide.

Generally, the efficacy of aflatoxin degradation by UV is believed to be dependent on various factors including treatment time, intensity, the wavelength of irradiation, contamination levels and experimental parameters such as temperature, the distance from the UV light to the sample surface and the penetration into the milk (Enjie, Xiangyang, Zheng, Wenwen, Ning, & Haizhou, 2015; Hassan & Hussein, 2017; Yousef & Marth, 1985). UVC is commonly used to decrease aflatoxins by reducing the growth of the fungi while UVA is used to degrade aflatoxins directly (Enjie et al., 2015). The aflatoxin reduction by higher energy UVC is greater than UVA. Increasing the UV treatment time improves aflatoxin reduction. Yousef and Marth. (1986) reported that the AFM1 reduction under UV light increased from 3.6% to 100% after 2 and 60 min of irradiation. Similarly, a significant increase in aflatoxin reduction in red chili power was observed when the exposure time rose from 30 to 60 min (Tripathi & Mishra, 2010). However, AFM1 degradation in milk was no different after 15 and 30 min of UV treatment (Hassan & Hussein, 2017). While the decrease of aflatoxin in peanut oil increased from 86.08% to 88.74% after 10 and 40 min of UV irradiation (Diao et al., 2014). A decline in aflatoxin was found in the first 10 min of treatment

and the efficacy did not improve significantly after 20 min of treatment. Recently, UVA LED was used to degrade AFM1 in water with a decrease of 84% at a dose of 1,200 mJ/cm^2. HepG2 cells were used to evaluate the cytotoxicity of the degradation products, and 98.44% of the cells survived after treatment compared with 70.42% of cell viability in the control samples containing aflatoxin (Stanley, Patras, Pendyala, Vergne, & Bansode, 2020). The differences in these findings on AFM1 degradation by UV treatment are probably due to various factors including temperature, types of aflatoxin, the nature of the samples and the level of aflatoxin contamination.

The intensity of UV light is one of the important factors affecting the aflatoxin degradation by UV treatment. The intensity of 800 μw/cm^2 was shown to produce more AFB1 degradation compared with 200 and 400 μw/cm^2 (Liu et al., 2013). These findings agree with a previous study reporting the photodegradation of AFB1 in peanuts oil under UV treatment after 20 -100 min was in the order: 800 > 400 > 200 μw/cm^2 (Liu et al., 2011). This was confirmed by another study that found higher aflatoxin degradation at higher UV intensity compared with the lower one (Patras, Julakanti, Yannam, Bansode, Burns, & Vergne, 2017). However, the intensity of UV light was believed to impact the sensory of samples, particularly in milk, with an unpleasant organoleptic result in high intensity UV treated samples (Li & Bradley, 1969; Weckel, Jackson, Haman, & Steenbock, 1936). Optimization is necessary to balance the sample quality and aflatoxin degradation.

Contamination levels were shown not to influence the degradation of AFB1 in peanuts. The contamination levels used were 0.2 to 5 μg/g (Liu et al., 2011; Liu et al., 2010), and 48-128 μg/kg (Mao et al., 2016). However, the efficacy of AFM1 degradation may be different in milk samples.

The depth of the sample for milk experiments is probably one of the most important factors affecting aflatoxin degradation using UV treatment. Yousef and Marth (1986) used UV light to reduce AFM1 in milk with the 10 mm layer or depth of the milk sample. The increase in temperature under UV light treatment was shown to cause a decrease in the aflatoxin degradation. The reduction in AFM1 was greater at lower temperature experiments (5 ^{o}C) and reduced gradually at 25 and 65 ^{o}C compared to 5°C (Yousef & Marth, 1986). In addition, UV treatment at lower temperatures was believed to avoid the impact of high intensity UV treatment on the organoleptic quality of milk compared with higher temperatures. However, the research on the temperature effects on aflatoxin degradation is limited. Interestingly, Hassan & Hussein (2017). found that increasing the distance between the UV light and milk surface resulted in an improvement in AFM1 degradation. The 90 cm distance produced the highest AFM1 reduction compared with 30 and 60 cm. Neelima et al. (2013) reported that using UV at 30 cm reduced aflatoxin in peanuts more than 15 cm (Neelima et al., 2013). Surprisingly, the volume of samples did not show an effect on degradation. A similar reduction in AFM1 in milk was obtained in the 25 and 50 – mL volumes (Hassan & Hussein, 2017). However, information on the experimental set up is required and further experiments may be needed to clarify this finding because in milk, UV transmittance is low.

4.2 Materials and Methods

4.2.1 Chemicals and equipment

Aflatoxin M1 standard 10 μg/mL in acetonitrile (Sigma-Aldrich, USA) was used in this study. Chemicals used for analysis were water W6-4 Optima LC-MS, methanol

Optima LC/MS EcoSafPak 4L, ammonium acetate Optima LC/MS (FSBA114-50), formic acid Optima for LC/MS grade (FSBA117-50), sodium chloride, ethyl acetate and, acetonitrile (HPLC grade). Plastic petri dishes, 60 mm x 15 mm (NEST, Cat no: 705001) were used to hold the samples for treatment with the lids removed for the treatment time. The UV light used was a Philips TUV 260W XPT HO DIM UNP/20 (diameter 32 mm, length 1400 mm).

4.2.2 Aflatoxin M1 contaminated milk samples

Skim milk (trim milk-Countdown) with 99.5% fat free purchased in local supermarket (Countdown) was used in this study.

AFM1 10 μg/mL was used as a standard stock. An intermediate standard (500 μg/L) was made by diluting 50 μL of AFM1 10 μg/mL into 950 μL of acetonitrile. Milk samples containing 10 μg/L (high level of contamination) were prepared by spiking 30 μL (10 μg/mL) into 30 mL milk. Milk samples with the levels of 0.05, 0.5 and 1 μg/L (low and average levels of contamination) AFM1 were spiked with 30 μL of 50μg/L; 30 and 60 μL of 500 μg/L into 30 mL milk respectively. All spiked samples were mixed.

4.2.3 Effects of different factors on the AFM1 reduction

Experiments were performed on the milk samples and the effects of different factors were investigated. These factors included treatment time, depth of sample, stirring, contamination level, room temperature and fat content in milk (Table 4.1). A small petri dish was used to hold the milk samples for treatment. These were placed on the base of a fully sealed container, with highly reflective surfaces, containing the UV tube

which was positioned 6 cm above the samples. The UVC tube was turned on for 2 minutes to warm it up before exposure to milk samples. Milk samples were exposed to UVC for 5, 10, 15 and 20 min. Three different volumes (3, 5 and 10 mL) were used to make a depth of 3, 4 and 6 mm of milk samples in the small petri dishes to investigate the effects of depth of sample on the efficacy of treatment. The temperature of the samples during the experiment was monitored in trials with and without using ice. Ice was placed on the base of the container containing the bulb to a depth of approximately 5 mm underneath the milk samples.

Table 4. 1 Different parameters investigated for UVC (254 nm) treatment of skim milk to reduce AFM1.

Factors	Treatment time (min)	Depth (mm)	Contamination level (μg/L)	Using ice
Treatment time (min)	5, 10, 15, 20	4	1	yes
Depth (mm)	5, 10, 15, 20	3, 4 and 6	1	yes
Contamination level (μg/L)	5, 10, 15, 20	4	0.05, 0.5, 1, 10	yes
Stirring	10	4	1	yes
Without using ice	5, 10, 15, 20	4	1	-
Full cream milk	5, 10, 15, 20	4	1	yes

4.2.4 Determination of AFM1 concentration in milk (details in Chapter 3)

AFM1 concentration was determined by HPLC-MS. The extraction method was developed by Biancardi, Piro, Dall'asta, & Galaverna (2013) with modifications. A 4 mL of skim milk were mixed with 0.4 g of sodium chloride and 12 mL of ethyl acetate on a shaker for 10 min at 300 rpm. The mixture was centrifuged for 10 min at 8400 x g to separate the organic layer. A 3 mL of supernatant was evaporated under nitrogen flow (40 ^{o}C) and redissolved in 1 mL of CH_3CN/H_2O 15/85 (v/v) with 0.1% formic acid and mixed by vortex.

For HPLC-MS analysis. A 10 µL of sample was injected into the Orbitrap LC-MS Q-Exactive (Thermo Fisher Scientific, San Jose, USA). The gradient of the mobile phase was set up as follows: 0-5 min from 15% of B, 5-7 min from 15% to 90% of B, 7-15 min 90% to 15% of B with flow rate of 0.2 mL/min (A: 2 mM ammonium acetate, 98% H_2O, 2% MeOH 0.1% formic acid, B: 2 mM ammonium acetate, 2% H_2O, 98% MeOH, 0.1% formic acid). The column temperature was 40 ^{o}C and the ion mode was positive. The analytical column was Accucore-150-C18 100 x 2.1 (mm) and particle size was 2.6 µm (Thermoscientific).

4.2.5 pH analysis

The pH of the milk samples was measured by pH meter (FP20, Mettler Toledo, USA). Buffers (pH 4, 7 and 10) provided by the manufacturer, were used to the calibrate pH meter at room temperature.

4.2.6 Colour measurement tests

The colour of milk samples (treated and control) was measured on 25 mL samples using the colour values including L (lightness), a^* (redness), and b* (yellowness) using

a Minolta Chroma Meter CR-400 (USA). The colour meter was calibrated with a standard black and white plate before using for testing the samples.

L, a* and b* values were measured to evaluate the colour of milk samples before and after treatment at four different treatment times. The L value represents for lightness with the numbers from 0-50 indicating dark and the range from 50-100 indicating light. The a* scale indicates red with positive numbers and green with negative numbers. The b* scale stands for yellow with positive numbers and blue with negative ones.

4.2.7 Milk composition analysis

Milk samples (30 mL) were heated to approximately 40 ^{o}C after removing from 4 ^{o}C to analyse the protein, total solids and lactose using MIR (Milkoscan FT1, FOSS, Denmark). The Milkoscan FT1 is an application of mid infrared spectroscopy with the transmission through 37-50 um cells using Fourier transform infrared (FTIR) at a wavelength range 10000-40000 nm.

4.2.8 Identification of AFM1 degradation products after UVC treatment

- Degradation of AFM1 by UVC

Aflatoxin M1 standard in acetonitrile (50 μg/mL) was used to prepare a working standard (0.5 μg/mL) in 85/15 H_2O/ACN. Working standard (2 mL) was held in a small petri dish and placed in a sealed container (4.2.1.3). Treatment time was 2, 5, 10 and 20 min as the purpose was to monitor the production of the degradation products at different times corresponding to the decrease of the original compound (AFM1).

- HPLC analysis

The Orbitrap LC-MS Q-Exactive (Thermo Fisher Scientific, San Jose, USA) was used to monitor the degradation of AFM1 by UVC over time.

For HPLC-MS analysis, 10 µL of samples were injected into the Orbitrap LC-MS Q-Exactive. The gradient of the mobile phases was set up as follows: 0-1 min from 10% of B, 2-17 min from 10% to 90% of B, 18-25 min 90% to 10% of B with flow rate of 0.3 mL/min (A: H_2O, 0.1% formic acid, B: ACN 0.1% formic acid). The column temperature was 40 ^oC and the ion mode was positive. The analytical column was Accucore-150-C18 100 x 2.1 (mm) and particle size was 2.6 µm (Thermoscientific).

The Agilent UPLC-QTOF (MSMS) was used to fragment the precursor ion to form the structure of the degradation product, with condition details in Chapter 5 (Session 5.3.1.7.2).

4.2.9 Statistical analysis

All experiments were done in triplicate. The significance analysis was carried out using ANOVA by running Minitab (Minitab reference manual: Macintosh version, release 19, 2019). Statistical significance was $P < 0.05$.

4.3 Results and discussion

4.3.1 Factors influencing AFM1 reduction

Fig 4.1 shows the degradation of AFM1 in milk by UVC (254 nm) at different treatment times (5, 10, 15 and 20 min) and the effects of various depths (3, 4 and 6 mm) of sample on the efficacy of the treatment. The degradation gradually increased with treatment time at all depths of sample. A reduction of approximately 20 % AFM1

was achieved after 5 min of UV irradiation and up to 50 % after 20 min treatment with the depth of 4 mm. The explanation for the significant improvement ($P < 0.05$) in the treatment's efficacy that corresponded to the exposure time is likely to be the UV dose dependence. The dose is directly proportional to treatment time (Patras, Julakanti, Yannam, Bansode, Burns, & Vergne, 2017). Longer exposure also enhanced the treatment efficacy in other studies. In another study using UVA, Yousef and Marth. (1986) reported that the AFM1 reduction under UV at 365 nm increased from 3.6% to 100% after 2 and 60 min of exposure respectively. Similarly, a significant ($P< 0.05$) aflatoxin B1 reduction was observed in red chili powder when the exposure time increased from 30 to 60 min (Tripathi & Mishra, 2010). UVA was able to reduce AFM1 in milk from a concentration of 50 μg/L to 15 μg/L after 15 min and to an undetectable level after 30 min (Hassan & Hussein, 2017). The increase in UV treatment time increased the degradation of other aflatoxins including B2, G1 and G2 in ground nut and tree nuts (Jubeen et al., 2012). The aflatoxin reduction in peanut oil increased from 86.08% to 88.74% after 10 and 40 min of UV irradiation, respectively. A large reduction in aflatoxin was found in the first 10 min of exposure, with a much smaller reduction after 20 min of treatment (Diao et al., 2014). The difference in these findings is probably due to various factors including temperature, types of aflatoxin, the nature of the samples and the level of aflatoxin contamination.

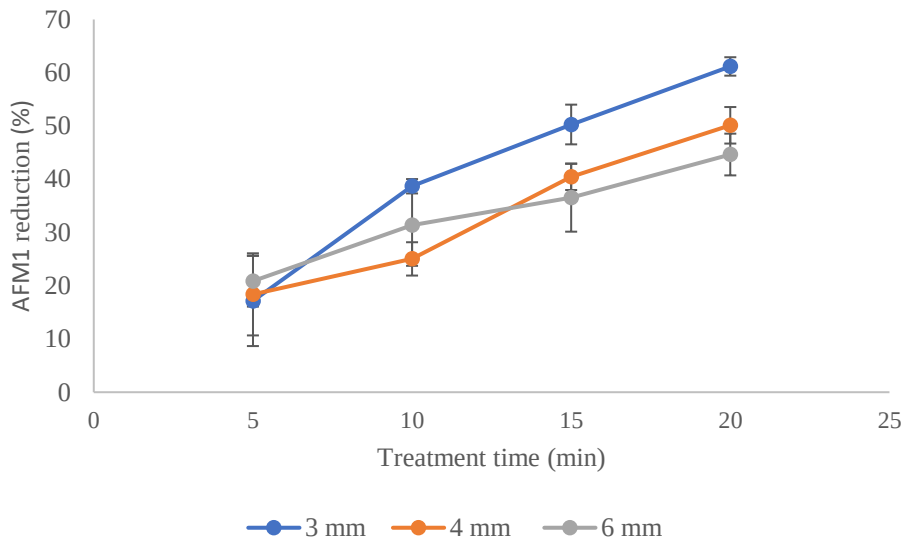

Fig 4. 1 Effect of milk sample depth on the reduction of AFM1 in milk by UV (254 nm) treatment at various treatment time (Mean and SD).

The efficacy of the treatment was also enhanced significantly ($P < 0.05$) by reducing the depth of the milk samples. Milk has low UV transmissivity due to a high absorption coefficient compared to other fluids (Go´mez-Lo´pez, Koutchma, & Linden, 2012) (Table 4.2) which limits the penetration of UV into the milk sample. Therefore, the decrease in the depth of the sample may lead to an improvement in UV penetration into the sample and an increase in the efficacy of the treatment. The possible reason is due to the penetration of UV into samples as milk has a high absorption coefficient, therefore, longer treatment time and thin layers probably improve the relative penetration.

Table 4. 2 Absorption coefficients of milk and other liquid foods at 254 nm (Go´mez-Lo´pez, Koutchma, & Linden, 2012).

Substance	Absorption Coefficient (cm^{-1})
Water	0.01
Liquid sucrose	0.45
Clear syrup	2 - 5
Liquid fructose	3.0 - 5.0
White wine	10
Beer	10 - 20
Red wine	30
Clear apple juice	7.1 - 39
Apple cider	˜40
Dark syrup	20 - 50
Orange juice	>60
Egg white	104
Raw milk	290

In the present study, in general, AFM1 in the samples with the depth of 3 mm degraded more than 4 mm and 6 mm (Fig 4.1) although the reduction after 5 min treatment did not follow that trend, probably due to the reproducibility of results at the short treatment time. The reproducibility tended to increase at longer treatment times. The AFM1 reduction increased gradually corresponding to the decrease in the depth of the sample after 15 and 20 min of treatment. In addition, slight stirring is believed to enhance the efficacy significantly ($P < 0.05$) as a reduction of 43.96% was found in

stirred samples compared with 25.04% in non-stirred samples after 10 min of UV irradiation. This is explained by an increase in the exposure of milk to UV light.

The effects of AFM1 concentration on the efficacy of UVC were investigated by using four different levels 0.05; 0.5; 1 and 10 µg/L of AFM1 (Fig 4.2). The reduction in AFM1 in milk at the four initial levels was not significantly different ($P > 0.05$) regardless of the treatment time, which indicated that UV treatment showed the same efficacy at all concentrations of AMF1 in milk (low, medium and high levels). Contamination levels were shown not to affect the degradation of AFB1 in aqueous medium and peanut oil by using UVA, at contamination levels of 0.2 to 5 mg/kg (Liu et al., 2011; Liu et al., 2010) and 48-128 µg/L (Mao et al., 2016). These findings are helpful to adjust the treatment time when the contamination level is available. For instance, in the present trial, samples contaminated AFM1 slightly higher than the level of 0.05 and 0.5 µg/L need to be irradiated for 5-10 min by UVC to be able to meet the regulation (0.05 and 0.5 µg/L for most of European countries and others including Vietnam, respectively).

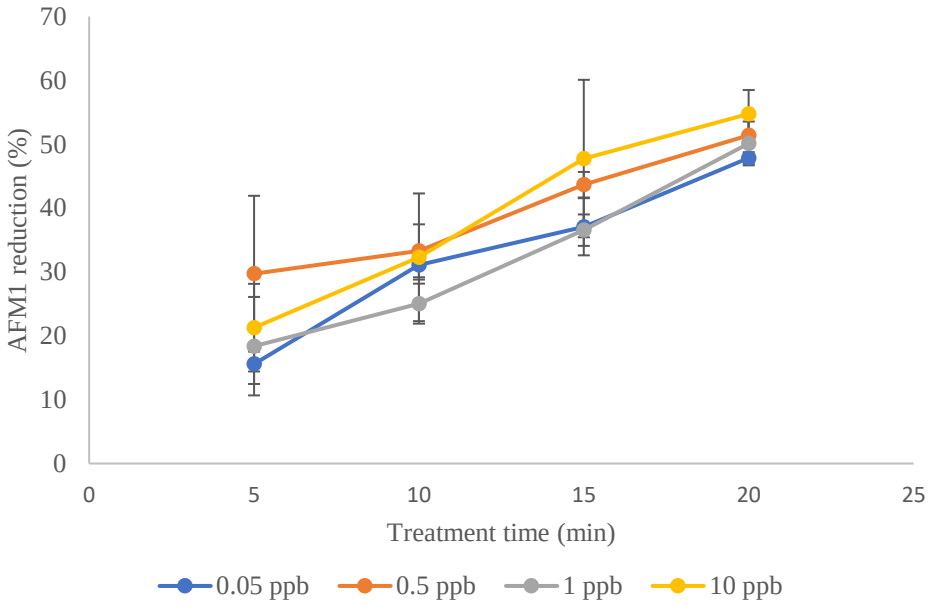

Fig 4. 2 The reduction of AFM1 in milk at different contamination levels by UV (254 nm) at four different treatment times ($P > 0.05$) (Mean and SD).

The occurrence of AFM1 in milk and milk products in some countries in the world is summarized by Iqbal et al. (2015). The highest concentration was below the maximum residue level in many countries, however, it exceeded the limit in some countries including Italy with 0.098 µg/L (MRL in Italy is 0.05 µg/L), Iran with 0.528 µg/L, 0.76 µg/L in Brazil and 1 µg/L in Pakistan (Akbar et al., 2019) where the MRL in these countries is 0.5 µg/L. Therefore, using UVC is likely to be able to reduce AFM1 in milk to below the MRL.

Milk is rich in nutrition that favours the growth of bacteria, therefore, maintaining the low temperature of the samples during the treatment to prevent the growth of microorganisms is important. In addition, irradiation at lower temperatures is believed to alleviate the impacts of high intensity UV treatment on organoleptic issues with milk compared with higher temperatures (Yousef & Marth, 1986). In the present study, ice was used as a layer underneath the samples to keep them cool and the temperature

of the samples during the treatment was monitored (Fig 4.3). The temperature was 25 ^{o}C before treatment and went up to above 40 ^{o}C in the samples without using ice while it ranged from 10-15 ^{o}C when using ice after 20 min of treatment. The AFM1 in milk was reduced by 50.13% and 73.66% in the samples with ice and without ice, respectively, after 20 min of exposure (Fig 4.4). Samples without ice showed a significant ($P < 0.05$) improvement in the efficacy in this study. A different trend was seen in another study using UVA light (365 nm) with a high reduction in AFM1 obtained at 5 ^{o}C which reduced gradually at 25 and 65 ^{o}C (Yousef & Marth, 1986). The difference between these findings and our trial is hard to explain as the only obvious difference is the wavelength of UV light.

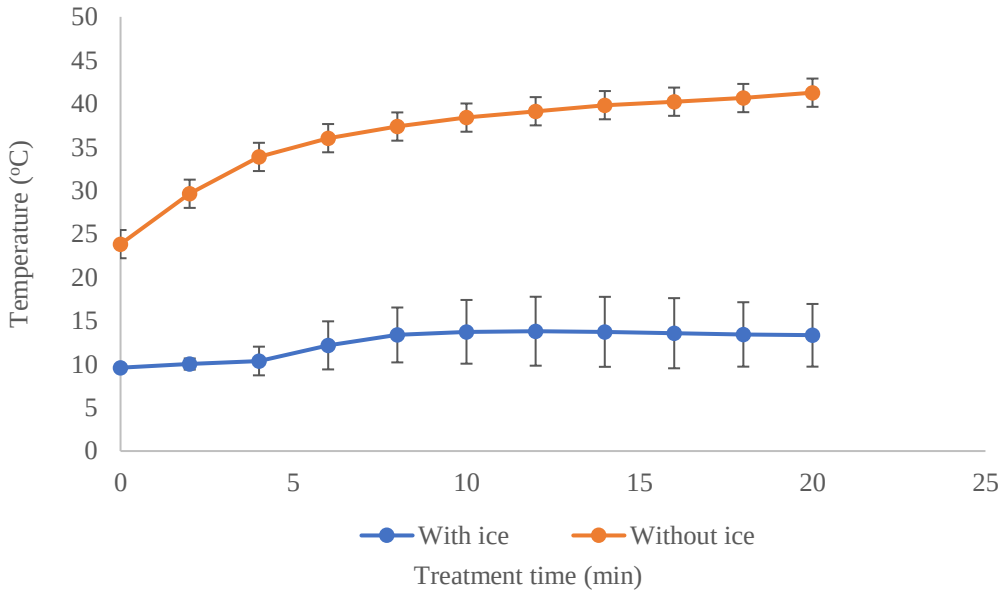

Fig 4. 3 Temperature monitoring during UV treatment with and without using ice (Mean and SD).

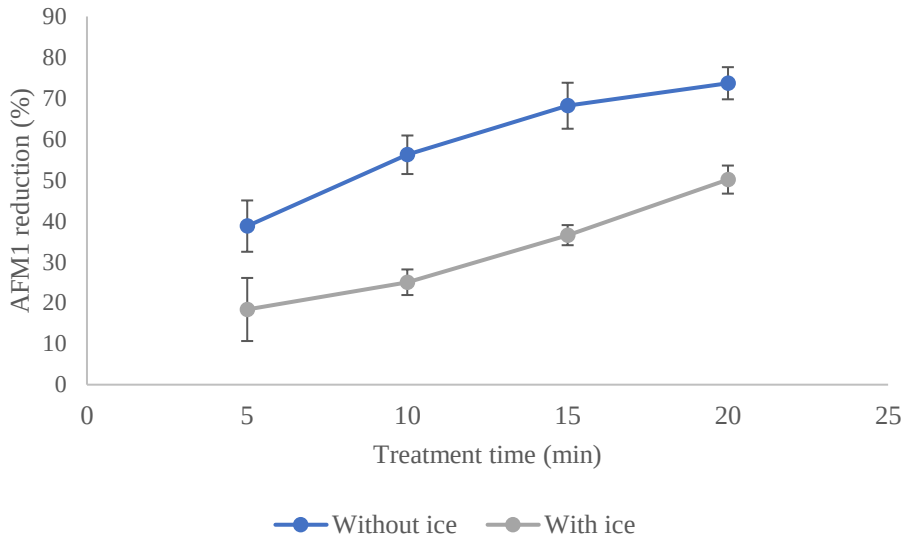

Fig 4. 4 The reduction of AFM1 in milk with and without using ice (Mean and SD).

4.3.2 The influence of treatment on the weight of samples

The weight of the milk samples before and after treatment was monitored to evaluate the evaporation of the samples with the increase in temperature during treatment (Table 4.3 and Table 4.4). Evaporation increased from 0.52% to 1.97% in the samples with 3 mm depth treated for 5 to 20 min. Evaporation increased from 0.38% to 1.66% and 0.25% to 1.02% in the 4 and 6 mm deep samples, respectively, over the 20 min treatment. The evaporation rate tended to be less in the deeper samples (6 mm) compared with the thinner ones. Full cream milk showed less evaporation, presumably due to fat protecting the samples from evaporation. Evaporation ranged from 0.09 to 0.65% after 5 and 20 min of irradiation in the 4 mm deep samples. The maximum evaporation of 3.6% occurred after 20 min treatment when the temperature of the samples was about 40 ^{o}C compared with 1.66% when the temperature was below 20 ^{o}C in 4 mm deep samples.

Table 4. 3 The difference in sample weight during UVC (254 nm) treatment (with ice).

	The decrease in weigh of sample after treatment (%)			
	Skim milk			Full cream milk
Treatment time	3 mm	4 mm	6 mm	4 mm
5 min	0.52 ± 0.20	0.38 ± 0.01	0.25 ± 0.02	0.09 ± 0.0462
10 min	0.83 ± 0.73	0.48 ± 0.07	0.42 ± 0.29	0.31 ± 0.1293
15 min	1.24 ± 0.80	1.22 ± 0.31	0.80 ± 0.1	0.38 ± 0.1306
20 min	1.97 ± 0.13	1.66 ± 0.20	1.02 ± 0.25	0.65 ± 0.1343

Table 4. 4 The difference of sample weight (skim milk) during UV (254 nm) treatment (without ice) at 4 mm depth.

Treatment time (min)	Evaporation (%)
20	3.6 ± 1.5
15	3 ± 0.2
10	1.7 ± 0.1
5	0.8 ± 0.1

4.3.3 The influence of fat content in milk on the efficacy of UVC treatment on AFM1 reduction

Full cream milk was used to investigate the influence of fat content in milk on the efficacy of AFM1 reduction by UVC irradiation. No significant difference between

the AFM1 reduction in skim and full cream milk was observed at all treatment times ($P > 0.05$) (Fig 4.5). This can be explained by the affinity of AFM1 to the milk protein and the fat component does not appear to block the UV access to the milk protein. The bond between AFM1 and milk protein is controversial. On the one hand, Brackett and Marth. (1982) used an equilibrium dialysis experiments to show that casein binds to AFM1 in milk and the bond tends to increase when the level of AFM1 in milk increases. Similarly, approximately 60% of AFM1 in milk was believed to bind with casein and found in curd with much of the rest found in whey (Deveci, 2007; Motawee & McMahon, 2009). The binding of AFM1 to casein was also supported by the high concentration of AFM1 commonly found in cheese (Colak, Hampikyan, Bingol, Cetin, Akhan, & Turgay, 2012; Oruc, 2006). In addition, AFM1 is a semi-polar compound that makes it less likely to interact with serum proteins leading to the lower partition seen in whey (Applebaum, Brackett, Wiseman, & Marth, 1982). On the other hand, the amount of AFM1 found in whey ranges from 50-100% in some other studies (Blanco, Dominguez, Gomezlucia, Garayzabal, Goyache, & Suarez, 1988; Grant & Carlson, 1971; Stoloff et al., 1975). The possible reason for the contradictory findings is the difference in some factors including methods used for AFM1 detection, the extraction methods, quality of milk, type and level of milk contamination and cheese manufacture method (Galvano, Galofaro, & Galvano, 1996).

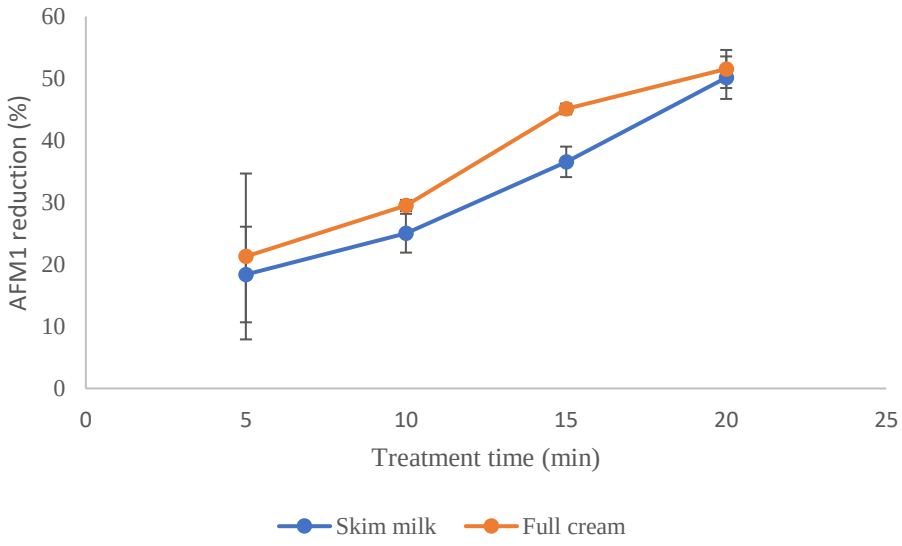

Fig 4. 5 The reduction of AFM1 in skim and full cream milk by UV (254 nm) (Mean and SD).

4.3.4 pH and milk colour

The increase in treatment time tends to have more effect on the L, a^* and b^* values of UV treated samples compared with the controls. The colour of treated milk samples is likely to appear brown, with less red and more yellow indicated by the scale with longer treatment times indicated by a decrease in the L value and the increase in a^* and b^* values. Short UV treatment times had less impact on milk colour. All the values for samples with greater depth (6 mm) and short treatment time (5 min) were close to the ones of the control. UVC treatment did not change the pH of the milk (Table 4.5, 4.6 and 4.7). This result is in line with some other studies which showed that UVC treatment did not affect the pH of the samples (Orlowska, Koutchma, Grapperhaus, Gallagher, Schaefer, & Defelice, 2013; Rafia et al., 2020).

Table 4. 5 Colour values and pH of milk sample (3mm) treated by UVC (254 nm). Levels connected by different letter (in one column) are significantly different ($P < 0.05$).

Treatment time	L	a^*	b^*	pH
Control	$80.19^{a} \pm 0.19$	$-6.39^{a} \pm 0.07$	$2.41^{a} \pm 0.30$	$6.73^{a} \pm 0.01$
5 min	$78.60^{b} \pm 0.17$	$-5.08^{b} \pm 0.05$	$3.72^{b} \pm 0.17$	$6.74^{ab} \pm 0.00$
10 min	$77.57^{c} \pm 0.29$	$-4.17^{c} \pm 0.22$	$4.51^{bc} \pm 0.25$	$6.72^{ab} \pm 0.01$
15 min	$76.83^{cd} \pm 0.53$	$-3.63^{cd} \pm 0.30$	$4.96^{c} \pm 0.42$	$6.72^{ab} \pm 0.01$
20 min	$76.20^{d} \pm 0.42$	$-3.39^{d} \pm 0.27$	$5.21^{c} \pm 0.55$	$6.70^{b} \pm 0.02$

Table 4. 6 Colour values and pH of milk sample (4 mm) treated by UVC (254 nm). Levels connected by different letter (in one column) are significantly different ($P < 0.05$).

Treatment time	L	a^*	b^*	pH
Control	$80.19^{a} \pm 0.19$	$-6.39^{a} \pm 0.07$	$2.41^{a} \pm 0.30$	$6.73^{a} \pm 0.01$
5 min	$79.34^{b} \pm 0.12$	$-5.39^{b} \pm 0.03$	$3.12^{b} \pm 0.22$	$6.73^{a} \pm 0.01$
10 min	$79.34^{b} \pm 0.08$	$-4.93^{c} \pm 0.05$	$3.71^{bc} \pm 0.07$	$6.72^{a} \pm 0.01$
15 min	$79.48^{b} \pm 0.12$	$-4.19^{d} \pm 0.12$	$3.78^{c} \pm 0.26$	$6.72^{a} \pm 0.01$
20 min	$77.91^{c} \pm 0.16$	$-3.89^{e} \pm 0.18$	$3.98^{c} \pm 0.24$	$6.71^{a} \pm 0.01$

Table 4. 7 Colour values and pH of milk samples (6 mm) treated by UVC (254 nm). Levels connected by different letter (in one column) are significantly different ($P < 0.05$).

Treatment time	L	a^*	b^*	pH
Control	$80.19^{a} \pm 0.19$	$-6.39^{a} \pm 0.07$	$2.41^{a} \pm 0.30$	$6.73^{a} \pm 0.01$
5 min	$79.98^{a} \pm 0.04$	$-5.86^{b} \pm 0.04$	$2.63^{ab} \pm 0.17$	$6.73^{a} \pm 0.00$
10 min	$79.84^{ab} \pm 0.14$	$-5.33^{c} \pm 0.05$	$2.85^{b} \pm 0.05$	$6.72^{a} \pm 0.01$
15 min	$79.49^{bc} \pm 0.15$	$-4.92^{d} \pm 0.10$	$2.93^{b} \pm 0.04$	$6.72^{a} \pm 0.01$
20 min	$79.22^{c} \pm 0.12$	$-4.71^{d} \pm 0.14$	$2.97^{b} \pm 0.02$	$6.73^{a} \pm 0.01$

4.3.5 Milk composition

Table 4.8 shows the difference in the composition (protein, total solids and lactose) of skim milk before and after UVC treatment. The change of all three parameters increased with the increase in treatment time with the greatest percentage increase seen in the thinnest sample (3 mm). The most likely explanation for this is evaporation as shown in Tables 4.3 and 4.4.

Table 4. 8 The difference (%) of chemical composition (protein, total solid and lactose) in milk after UVC (254 nm) treatment from untreated sample.

Sample		Protein	Total solids	Lactose
3 mm	5 min	1.01 ± 0.39	1.03 ± 0.10	1.13 ±0.45
	10 min	1.82 ± 0.99	1.71 ± 0.92	2.48 ± 0.98
	20 min	4.25 ± 0.13	3.97 ± 0.31	4.74 ± 0.00
4 mm	5 min	0.84 ± 0.26	0.68 ± 0.31	0.98 ± 0.13
	10 min	1.90 ± 0.13	1.68 ± 0.16	2.33 ± 0.35
	20 min	3.19 ± 0.00	2.94 ± 0.06	3.76 ± 0.47
6 mm	5 min	0.38 ± 0.13	0.17 ± 0.06	0.53 ± 0.13
	10 min	0.61 ± 0.47	0.55 ± 0.3	1.28 ± 0.13
	20 min	1.37 ± 0.00	1.44 ± 0.00	1.66 ± 0.34

4.3.6 Degradant structure elucidation

Fig 4.6 shows the chromatogram of UV treated (5 min) and untreated (0 min) samples. There was one peak in the untreated sample while there was an extra peak in the treated one (Fig. 4.7). The change of this degradation product was monitored over time (2, 5, 10 and 20 min) (Fig 4.8).

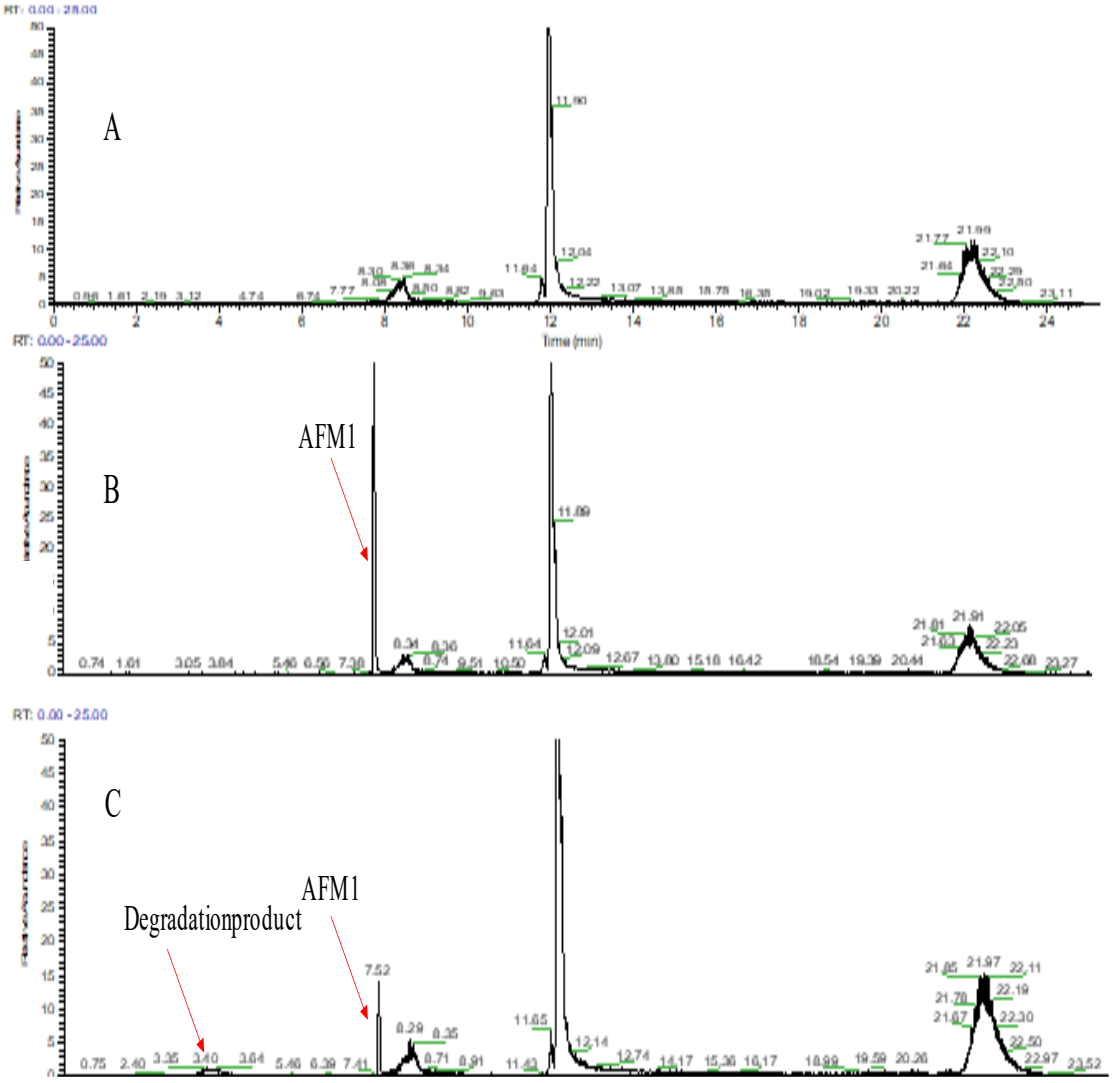

Fig 4. 6 Chromatogram of Solvent (A), AFM1 standard 500 ppb (B) and AFM1 standard after 5 min UV treatment (C).

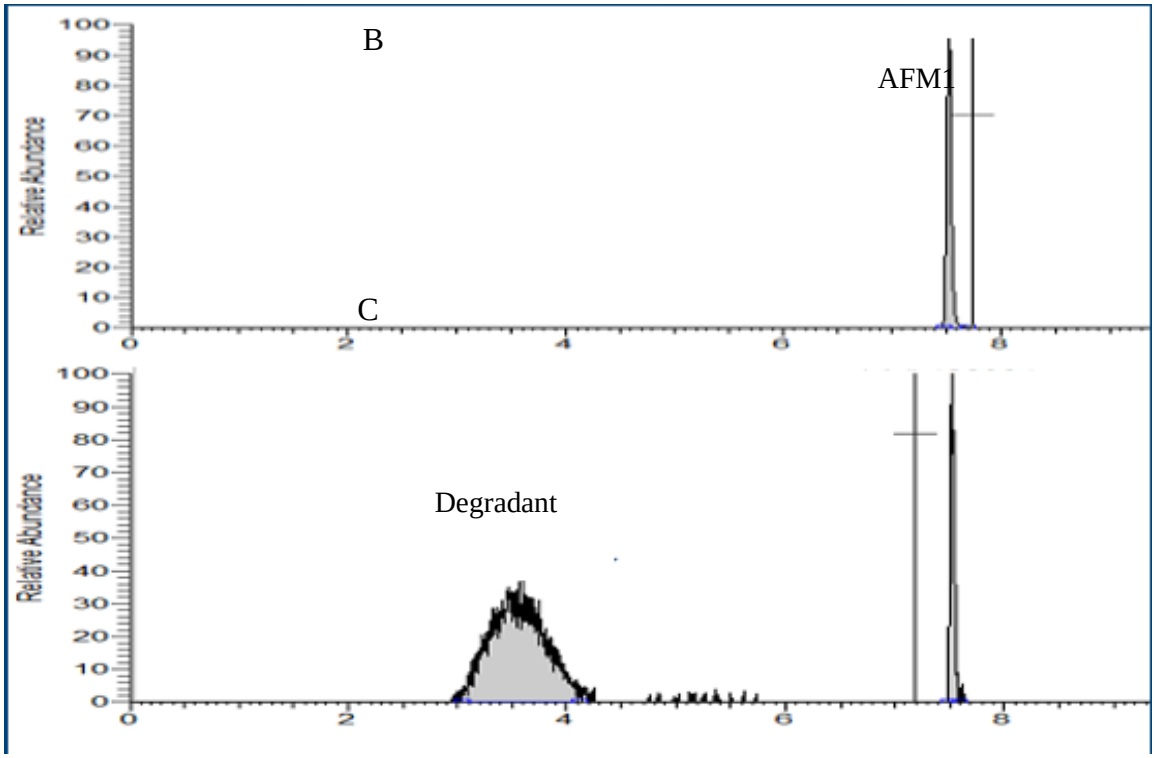

Fig 4. 7 Chromatogram of AFM1 standard 500 ppb (B) and AFM1 standard after 5 min UV treatment (C).

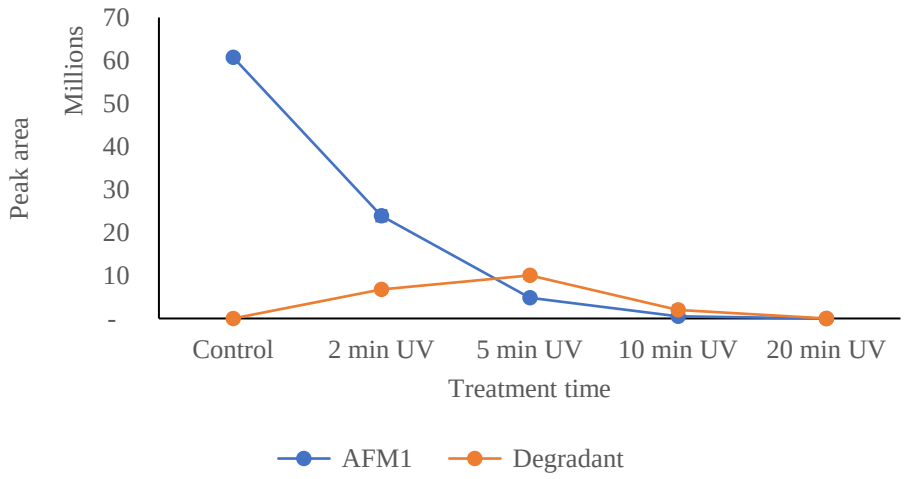

Fig 4. 8 Relative change in the peak area of degradation product responding to the reduction of AFM1 over treatment time.

The AFM1 was reduced gradually responding to the increase in the treatment time while the amount of the degradation product reached the peak after 5 min treatment and decreased to an undetectable level. This could be because the degradant changed to the other compounds that were too small to be detected or had different characteristics which cannot be detected by the same conditions as the original one.

Table 4.9 shows the possible formula for the degradation product by using LCMS - orbitrap.

Table 4. 9 The proposed formulas of the degradation products of AFM1 after treatment

No	RT	Proposed formula	Ion mass (m/z)	Diff (ppm)	Score (%)
1	3.81	$C_{17}H_{14}O_8$	347.0763	3.51	95.51
2	7.53	$C_{17}H_{12}O_7$ (AFM1)	329.0689	4.19	95.27

Note: The m/z of $[M+H]^+$. Diff: difference between observed and theoretical mass

The METLIN database was used to search for possible structures of $C_{17}H_{14}O_8$. There were more than 50 compounds with similar molecular weight, but 4 of them had the aflatoxin's structure. MSMS fragmentation was used to confirm the possible structure amongst the four similar structures.

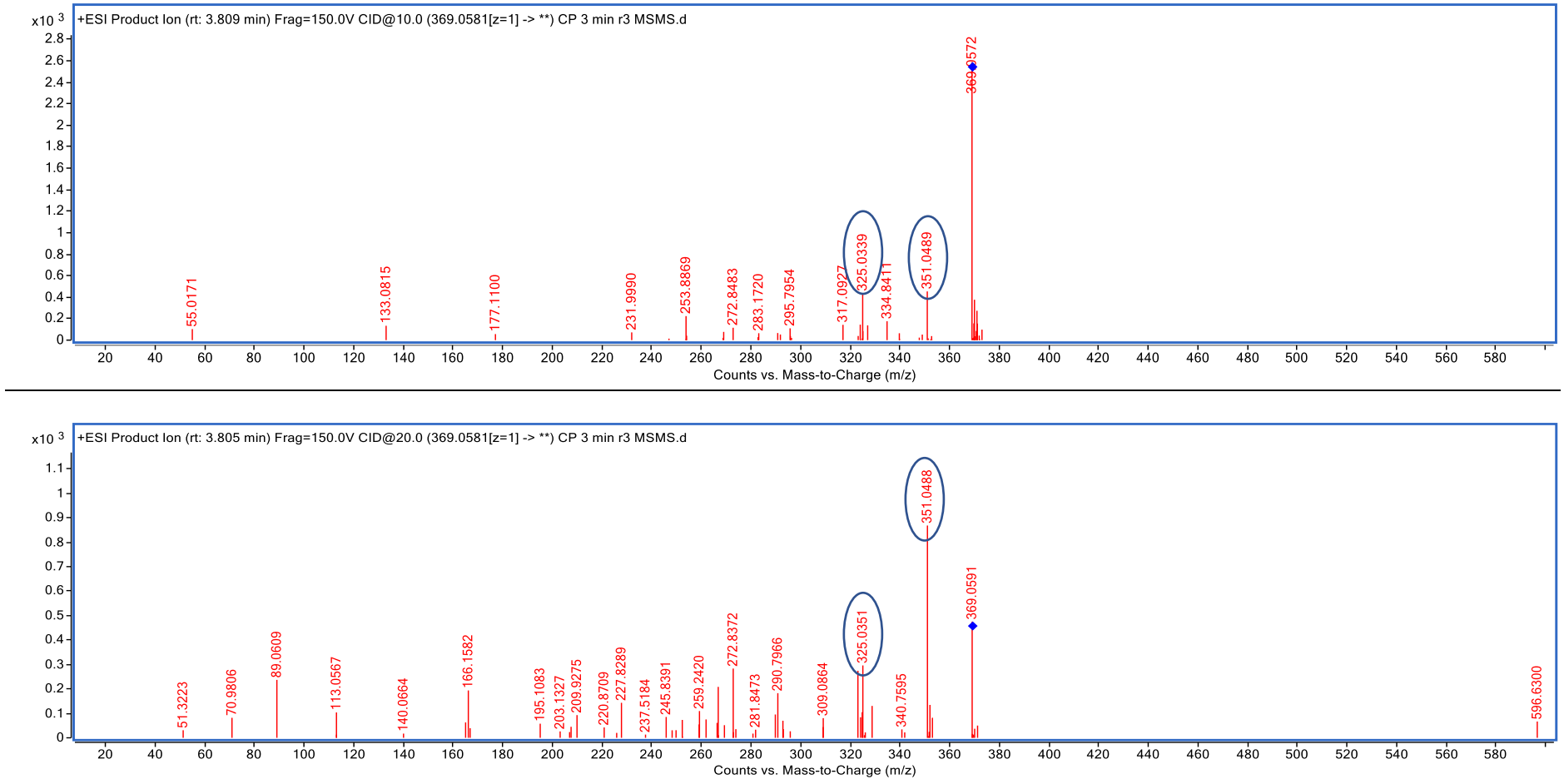

Fig 4. 9 MSMS spectra at CID 10 and 20 of ion 347.0763 with $[M+Na]^+$ = 369.05.

The increase in the energy of collision - induced dissociation (CID) from 10 to 20 decreased the intensity of ion 369.05 and increased the intensity of ions 325.03 and 351.04 (Fig 4.9). This indicated that ion 325.03 and ion 351.04 were broken from ion 369.05 by collision. The structures of the MSMS fragments were suggested (Fig 4.10). Different ionizations of all three ions are shown below:

$[M+Na]^+$	$[M+H]^+$	[M]
369.05	**347.05**	346.05
351.04	329.04	328.04
325.03	302.03	301.03

Note: 347.05 is the targeted ion (the degradation product)

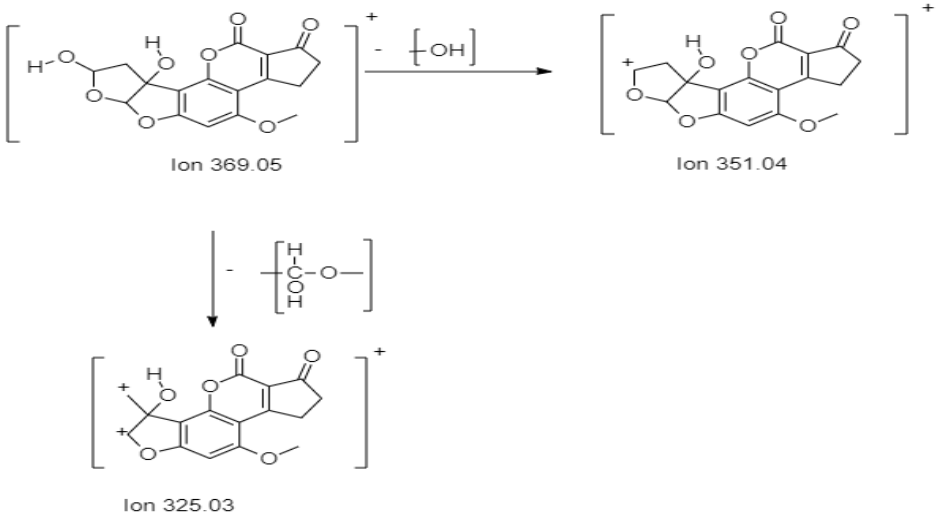

Fig 4. 10 The structures of the fragmentation of the degradation product confirming its structure.

The retention time of the degradant peak was also used to confirm its structure. The LogP value of AFM1 and the degradant are 0.9 and 0.08, respectively. The relation between LogP of a compound and its retention time is reported to be proportional (Zheng & West, 2009) which was illustrated with the retention of the AFM1 (t_r at 7.52 min) being longer than the degradant's retention time (t_r at 3.81 min).

4.4 Conclusion

UVC degrades AFM1 in milk by 50-70% after 20 min treatment with no change in pH but a noticeable change in colour. UVC (254 mm) treatment can be used to reduce AFM1 in milk to below the MRL (0.5 µg/L) from the average contamination level (1 µg/L). Treatment time can be reduced in samples with decreased depth. The concentration of AFM1 does not influence the rate of UVC degradation of AFM1. UVC degradation of AFM1 increases with temperature but is also effective at refrigeration temperatures. Milkfat does not affect the reduction in AFM1 following UVC treatment. Changes in colour can be reduced by shortening the treatment time. Treatment conditions can be optimised to provide AFM1 reduction to below the MRL with minimal damage to the milk. The double bond of the furan ring in AFM1 molecules was oxidized forming the degradation product, which reduced the toxicity of AFM1 as the furan ring structure responsible for the toxicity of the toxin was destroyed. However, a toxicity test is necessary to confirm.

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CHAPTER 5. USING COLD PLASMA TO REDUCE AFLATOXIN M1 IN SKIM MILK

Abstract

The application of high voltage atmospheric cold plasma (HVACP) in the reduction of AFM1 in skim milk was explored with different influencing factors including treatment times (5, 10 and 20 min), operating gases (air and MA65 - 65% O_2, 30% CO_2, 5% N_2), three voltages (60, 70 and 80 kV), using direct and indirect treatment, AFM1 contamination levels (0.1; 1 and 50 µg/L) and the volume of the sample (10, 20 and 30 mL). The reduction of AFM1 in skim was 64.99 and 78.86% by using air and MA65 as operating gases, respectively, after 20 min treatment without changing milk colour although a small change in pH was observed. Later then, a dielectric barrier discharge (DBD) cold plasma set up which was like the HVACP patent was built in order to continue investigating the effects of other operating gases with different mixtures (5, 10 and 20% of air, pure oxygen and nitrogen in helium), and milk components (casein, lactose and whey protein) on AFM1 reduction and degradation products of AFM1 after cold plasma treatment. The plasma chemistry induced by these two systems may vary as the voltage generator used for the DBD cold plasma system was lower and the gap between two aluminium electrodes was smaller compared with HVACP. However, this small DBD system reduced approximately 70-100% of AFM1 in water after 3 and 10 min treatment by using air/helium (10/90). The reduction of AFM1 was much less in skim milk, whey protein and casein while almost 70% AFM1 reduction was achieved in lactose. The increase in the concentration of air/pure oxygen in the mixture with helium increased the reduction of AFM1 while it was unchanged in the nitrogen/helium mixture. The structure of three different degradation products was elucidated.

5.1 Introduction

5.1.1 Fundamentals of cold plasma

Plasma, a quasi-neutral gas, is used to describe the fourth state of matter where high energy transforms a solid to a liquid then to gas and eventually to an ionized state termed plasma (Fig 5.1). Plasma consists of ions (H^+, H_3O^+, O^+, H- , O- , OH- , N_2^+), molecular species (N_2, O_2, O_3, H_2O_2), and reactive radicals (O•, H•, OH•, NO•), stable conversion products (e.g., ozone), and energetic photons (e.g., UV) (Bourke, Ziuzina, Han, Cullen, & Gilmore, 2017; Stoffels, Sakiyama, & Graves, 2008). Photons and electrons are light species, the others are heavy species (Misra, Tiwari, Raghavarao, & Cullen, 2011). The overall charge in the plasma phase is neutral due to the equal number of positive and negative charges (Chizoba Ekezie, Sun, & Cheng, 2017; Pankaj & Keener, 2017b). Plasma can exist in both excited and stable states. The excited state is induced by supplying sufficient energy for the neutral gas to be energized under different temperatures and pressures (Chizoba Ekezie et al., 2017). Therefore, the classification of plasma is based on the type of plasma generation conditions such as low-, atmospheric- or high pressure and thermal or non - thermal (cold). The processing parameters and operating gas characterise the nature of the plasma (Phan, Phan, Brennan, & Phimolsiripol, 2017). Cold plasma can be induced at all three pressures with the reactive species and electron density range similar for all types of plasma generation (Zhang, Oh, Cisneros-Zevallos, & Akbulut, 2013). Cold plasma is also considered as a non - equilibrium plasma as there is no localized thermodynamic equilibrium between electrons and heavy species (Thirumdas, Sarangapani, & Annapure, 2015). It is generated at 30-60 °C and needs less energy than thermal plasma which benefits the food industry, especially for processing heat sensitive products. In contrast, thermal plasma consumes higher levels of energy to

produce the plasma at high temperature which maybe thousands of degrees Kelvin (Scholtz, Pazlarova, Souskova, Khun, & Julak, 2015) and a local thermodynamic equilibrium exists in thermal plasma so it is designated as “equilibrium plasma” (Coutinho et al., 2018).

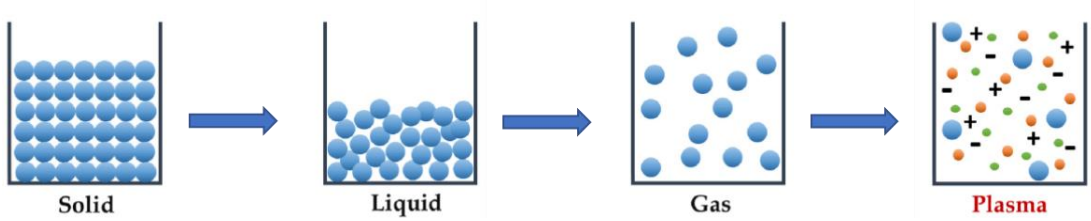


Fig 5. 1 Plasma production through different states by energy increase (Hojnik, Cvelbar, Tavcar-Kalcher, Walsh, & Krizaj, 2017).

5.1.2 Plasma technological applications

Cold plasma is a novel and emerging technology for the food industry. Applications include food pesticide degradation, food waste cleaning and food processing (Sarangapani, Patange, Bourke, Keener, & Cullen, 2018). Various plasma induced systems such as corona discharge, dielectric barrier discharge, atmospheric pressure plasma jet and microwave discharge have been developed (Fig 5.2).

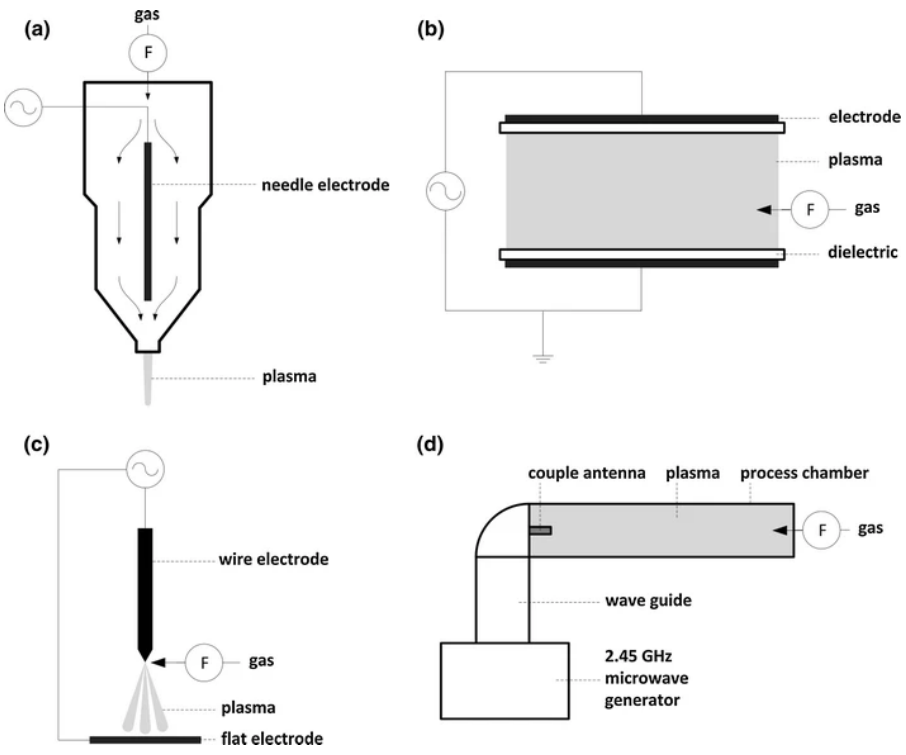


Fig 5. 2 Different cold plasma generation systems with a: Atmospheric pressure plasma jet, b: Dielectric barrier discharge (DBD), c: Corona discharge, d: Microwave discharge (Surowsky, Schlüter, & Knorr, 2015) (Copyright license number: 5198580954055 from Springer Nature)

Generally, electrical, optical (UV light), radioactive (gamma radiation), thermal and X-ray electromagnetic radiation can be the source of energy to generate plasma, however, electric or electromagnetic fields are common (Pankaj & Keener, 2017a). Non - equilibrium plasma at atmospheric pressure is also of interest due to the potential in the food industry. Plasma generated at low temperatures reduces the impact on food quality after treatment and can be used for heat sensitive products. In addition, less energy is required by operating at atmospheric pressure.

5.1.3 Application of cold plasma on aflatoxin reduction

The application of cold plasma on aflatoxin reduction is limited, however, this approach is likely to have potential (Table 5.1). Cold plasma induced by many sources degraded 62-100% of AFB1 in a standard solution as well as in contaminated food samples (corn, hazelnuts) from high initial levels (200 - 420 ppb). Park et al. (2007) used microwaves as a source of plasma to completely inactivate AFB1 on a glass slide after only 5 s. However, in other studies treatment time was in the range of 10-15 min (Sakudo, Toyokawa, Misawa, & Imanishi, 2017; Siciliano et al., 2016; Surowsky et al., 2015; Wang, Huang, Li, Xiao, Zhang, & Jiang, 2015)

High Voltage Atmospheric Cold Plasma (HVACP) that is Dielectric Barrier Discharge (DBD) based plasma production was used in this study to inactivate AFM1 in milk. These systems produce large volumes of plasma that have potential for treating liquid samples. The experiment temperature is low (22-28^{o}C) avoiding any heat damage to the milk. Furthermore, air can be used as the operating gas for HVACP making this method cost effective.

Table 5. 1 Cold plasma applications in use on aflatoxin B1 inactivation.

	Microwaves induced plasma	Pulsed power supply	DBD CP	HVACP	Radio frequency
Degradation reduction (%)	100	90	100% - standard, 70 % - hazelnuts	62 and 82, (76% in 5 mins)	88.3
Contaminated level	-	200 (ppb)	10 ppb	420 (ppb)	
Temperature (^oC)	75-130	<80	28.9	22	40
Operating gas	Argon	N_2	21% O_2, 1%O_2, 0.1% O_2 and N_2	Air, MA 65	-
Treatment time	5s	15 min	12 min	1 and 10 mins	10 min
Sample	Standard	Standard	Standard (10 ppb), hazelnuts (20 ppb)	Corn	Standard
Reference	(Park et al., 2007)	(Wang et al., 2015)	(Siciliano et al., 2016)	(Shi, Ileleji, Stroshine, Keener, & Jensen, 2017)	(Sakudo et al., 2017)

5.2 Materials and Methods (this experiment was done in Iowa State University, USA)

5.2.1 Methodology

5.2.1.1 Chemical

Aflatoxin M1 standard 10 µg/mL in acetonitrile, in an ampule of 1 mL (Sigma-Aldrich) was used as a standard to inoculate milk. LC-MS was used for the analysis of AFM1. The reagents used for LC-MS detection were water (LCMS grade, ThermoFisher), methanol (LCMS grade, ThermoFisher), acetonitrile (LCMS grade, ThermoFisher), formic acid (LC/MS grade, Sigma) and ammonium acetate (LCMS grade, Sigma). NaCl, ethyl acetate and acetonitrile (HPLC grade) were used for sample extraction.

For HVACP treatment of milk, sterile Petri dishes, (ID: 47 mm and 100mm) (Millipore, Merck) were used. The gas for the HVACP treatment, MA65 (65% O_2, 30% CO_2, 5% N_2) was purchased from a local gas supplier (Ames, Iowa).

5.2.1.2 HVACP system

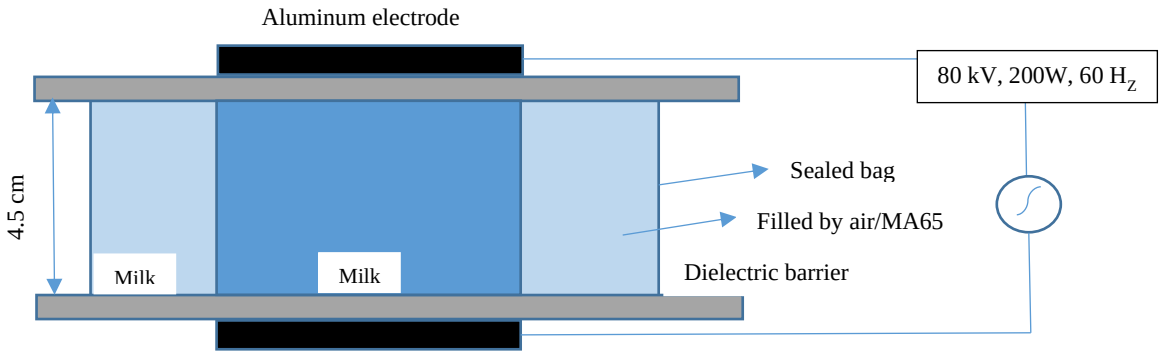


Fig 5. 3 Experimental set-up of HVACP for treatment of AFM1 in milk.

The HVACP system (Fig 5.3) was developed by researchers at Purdue University (Keener & Jensen, 2014) utilizing a high voltage generator BK-130 (Phenix Technologies, Accident, MD). The system was operated at 80 kV, 200 W and 60 H_Z. Milk samples were placed inside a translucent plastic box that was sealed by Cryovac B2630 film (sealed bag) to avoid gas and plasma leaking. The box was in line with two aluminium electrodes (high voltage and ground) for direct treatment and out of the direct plasma field for indirect treatment. The sealed bag and plastic box were filled completely with MA65 or air in 5 min with many purges to avoid air contamination when using MA65. The experiments were carried out at room temperature (18-22 ^oC).

5.2.1.3 Aflatoxin M1 contaminated milk samples

A 2.2 L box of skim milk, fat free from Great Value was purchased from a local supermarket (Ames, Iowa State, USA) and stored at 4 ^oC. The milk sample was mixed before using. Sample preparation and all experiments were carried out at room temperature.

AFM1 10 μg/mL (ppm) was used as stock standard. Fifty (50) μL of AFM1 10 μg/mL was diluted in 950 μL of acetonitrile to prepare an intermediate standard of 500 μg/L. Milk samples contaminated with 0.1 μg/L were prepared by spiking 60 μL (50 μg/L) into 30 mL skim milk. Milk samples with the levels of 1 and 50 μg/L AFM1 were spiked by adding 60 μL (500 μg/L) and 150 μL (10 μg/mL), respectively, into 30 mL skim milk.

5.2.1.4 The application of HVACP for AFM1 reduction

AFM1 (2 mL) dissolved in water/acetonitrile 85/10, 50 µg/L was pipetted to a small petri dish for HVACP treatment and was placed in the middle of the reaction box (direct mode). Treatment times were 5 and 10 min, with a 0 min sample used as a control.

5.2.1.5 Effect of different factors on detoxification

Treatment time and different operating gases (air and MA65) were investigated using a small petri dish containing 5 mL of milk exposed to treatment at different treatment times (5, 10 and 20 min). Air and MA 65 were used as operating gases.

Treatment mode, voltage and levels of contamination were investigated using a small dish of milk placed in the centre of the plastic box for direct treatment and out of the field for indirect treatment. Three voltages of 60, 70, 80 kV and contamination levels of 0.1; 1 and 50 µg/L AFM1 in milk were used.

Volumes of 10, 20 and 30 mL milk were poured into big petri dishes (the depth of the samples changed 3, 4 and 6 mm due to the increase in the volume of the samples). These dishes were placed in the direct section of the reaction box.

5.2.1.6 Optical absorbance spectroscopy (OAS) of plasma

A UV-Vis (190-1100 nm) Ocean Optics HR2000 + ES spectrometer, resolution 0.9 nm, and a deuterium - halogen lamp with emission in the same UV-Vis range were used to determine the reactive species of cold plasma (Shi et al., 2017). The optical fiber was placed inside the reactive species box and connected to the spectrometer and the computer. The empty box, box with milk in air and a box with milk in MA65 were used as untreated controls.

The set-up of OAS for cold plasma inside the package is illustrated in Fig 5.4. The OAS used a deuterium-halogen UV-VIS-NIR light source (model DH-mini, Ocean Optics Inc, USA) and a spectrometer connected with two optic fibers. The experimental set-up measures the incident and transmitted spectral intensity I0 (λ = 200 - 800 nm) and IT (λ = 200-800 nm), respectively, of a beam from the light source and after crossing the post-discharge gas inside the package. The relationship between the intensities and the species concentration is given by the Lambert - Beer law:

$$I_T(\lambda) = I_0(\lambda)e^{-\sum_i \sigma_i(\lambda)C_i L} \qquad \text{Eq 1}$$

where, L is the path length (cm), σ_i (λ) is the wavelength dependent absorption cross-section of each species (cm^2/molecule), and C_i is the density of each species (cm^{-3}). The path length L for the OAS experiment was set at 2.1 cm. The absorption cross-section of each species σ_i (λ) was adapted from the MPI-Mainz UV/VIS Spectral Atlas Database and was interpolated to match the spectrometer resolution (Moiseev et al., 2014). The concentrations of reactive gas species were determined via numerical direct deconvolution as detailed by (Moiseev et al., 2014). The reactive oxygen and nitrogen species were measured by the OAS include ozone (O_3) and multiple nitrogen oxides (NO_2, NO_3, N_2O_4, and N_2O_5).

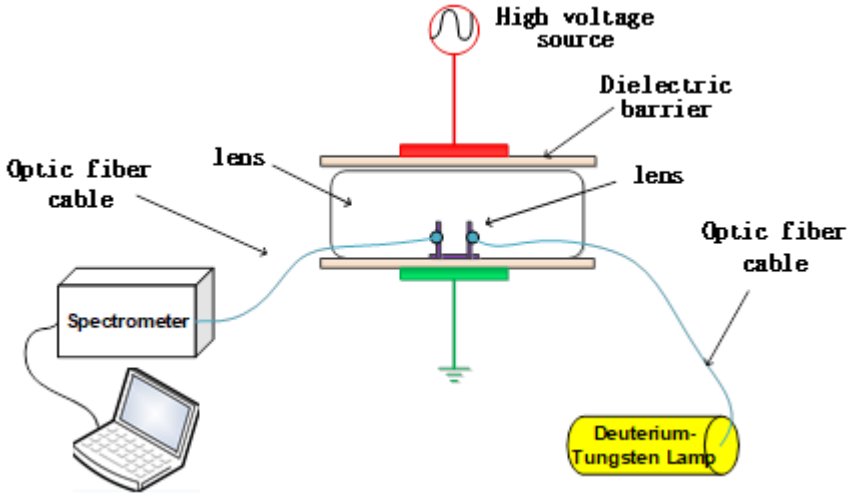


Fig 5. 4 Experimental set-up of optical absorption spectroscopy.

5.2.1.7 Determination of AFM1 concentration in skim milk (detail in Chapter 3)

The extraction method was developed by Biancardi, Piro, Dall'asta, & Galaverna (2013) with the following modifications. Four mL of milk was mixed with 0.4 g of NaCl and 12 mL of ethyl acetate on a shaker for 10 min at 300 rpm. The mixture was centrifuged for 10 min at 8400 x g to separate the organic layer. Three mL of supernatant was evaporated under nitrogen flow (40 ^{o}C) and redissolved in 1 mL of CH_3CN/H_2O 15/85 (v/v) with 0.1% HCOOH.

For HPLC-MS analysis, 10 µL of sample were injected into an Orbitrap LC-MS Q-Exactive (Thermo Fisher Scientific, San Jose, USA). The gradient of the mobile phase was set up: 0-5 min at 15% of B, 5-7 min from 15% to 90% of B, 7-15 min 90% to 15% of B with flow rate of 0.2 mL/min (A: 2 mM ammonium acetate, 98% H_2O, 2% MeOH, 0.1% formic acid, B: 2 mM ammonium acetate, 2% H_2O, 98% MeOH, 0.1% formic acid). The column temperature was 40 ^{o}C, and the ion mode was positive. The analytical column was Accucore-150-C18 100 x 2.1 (mm) with a particle size of 2.6 µm (Thermoscientific).

The analysis method performance was evaluated as described by Biancardi et al. (2013) including linearity, specificity and LOQ, accuracy (trueness and precision (RSD%).

5.2.1.8 pH analysis

The pH of milk samples was measured by pH meter (model: Fisherbrand™ Traceable™ pH/ORP/Temperature Meter, Fisher scientific). The manufacturer provided buffers (4, 7 and 10) were used to calibrate the pH meter at room temperature.

5.2.1.9 Colour measurement tests

The colour test followed a previous study with modification (Kim et al., 2015). Milk samples (25 mL) (treatment and controls) were measured against colour values including L* (lightness), a* (redness), and b* (yellowness) by using a ColorFlex EZ Spectrophotometer (HunterLab, USA). The colour meter was calibrated with a standard black and white plate before testing samples.

5.2.1.10 Statistical analysis

All experiments were replicated three times. The significance analysis was carried out using ANOVA by running Minitab (Minitab reference manual: Macintosh version, release 19, 2019).

5.2.2 Results and discussion

5.2.2.1 Reduction of AFM1 in milk by air HVACP and effect of treatment time

The reduction of AFM1 in milk samples (1 µg/L) and standard solutions (50 µg/L) by using HVACP at different treatment times is shown in Fig 5.5.

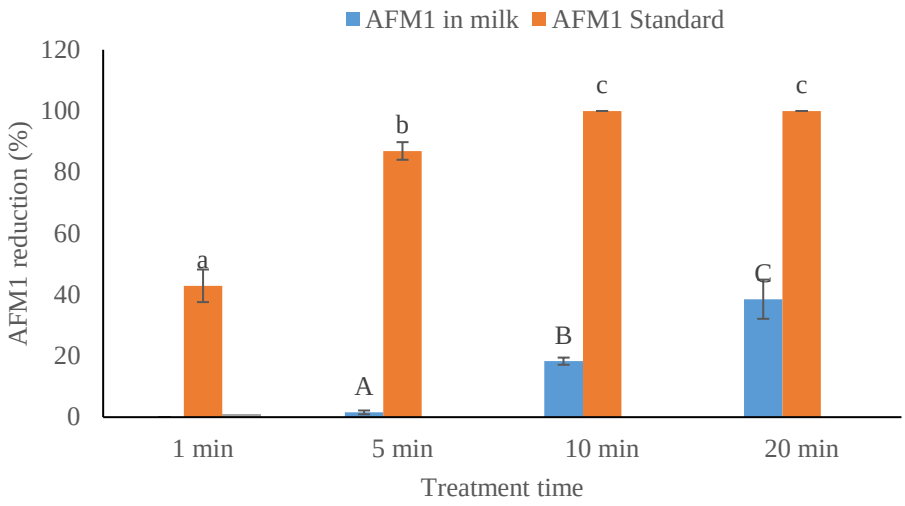


Fig 5. 5 Reduction of AFM1 in milk samples and standard solutions. The orange bars represent AFM1 standard and the blue bars AFM1 in milk. The bars with different letters show significant differences in each category (standard or milk) ($P < 0.05$).

Cold plasma has been shown not to affect milk colour after 20 min of treatment (Korachi et al., 2015; Gurol, Ekinci, Aslan, & Korachi, 2012), therefore, in the milk experiment, the maximum treatment time was 20 min to maintain the sample quality as well as save time and energy. Air was used as the operating gas with a voltage of 80 kV. Increasing the treatment time improved AFM1 reduction, which was believed to be due to either the increase in time of exposure to the reactive species of the samples or enhancing the concentration of reactive species in cold plasma (Moiseev et al., 2014). Fig 5.7 shows that the reactive species were not detected at 0 min, then increased gradually when the treatment time was increased, finally reaching a peak before decreasing after 20 min. Therefore, the treatment time of 20 min was deemed

optimal. The reduction in AFM1 with longer treatment may be due to a change in the reactive chemical species (ozone, NO_2, NO_3, N_2O_4, N_2O_5 + H_2O_2) produced by the plasma (Fig 5.5 and Fig 5.7), however testing for all other reactive species was not available. The concentrations of ozone and NO_x rose during the experiment from 0 to 20 min and became saturated after 20 min (Shi et al., 2017).

The results in this study are like previous studies using cold plasma for the treatment of AFB1 although these were in different types of generally solid food products not liquids (Shi et al., 2017; Siciliano et al., 2016). Siciliano et al. (2016) investigated the detoxification of aflatoxins in hazelnuts using a dielectric barrier discharge (DBD) cold atmospheric plasma system. Aflatoxins were completely removed from standard solutions as the treatment time increased from 1 to 4 min with N_2 as the operating gas, while more than 70% of AFB1 was degraded from hazelnuts after 12 min exposure to N_2 cold plasma. AFB1 degradation of 88% was obtained within 10 min of cold plasma treatment using a radio frequency of 300W to generate the plasma (Wang, Huang, Li, Xiao, Zhang, & Jiang, 2015). Nitrogen gas cold plasma reduced 90% of AFB1 in a standard solution after 15 min treatment from an initial level of 200 μg/L. The reduction was close to 100% if the treatment time was increased to 30 min (Sakudo, Toyokawa, Misawa, & Imanishi, 2017). Shi et al. (2017) used high voltage atmospheric cold plasma (HVACP) to detoxify aflatoxin in corn, with 62% and 82% reduction of AFB1 after 1 and 10 min treatment, respectively. It should be noted that the efficacy of cold plasma on toxin degradation can vary due to the difference in the type of sample (solid and liquid) and the aflatoxins (aflatoxin B1, B2, G1, G2 and M1).

Based on the results from the optimization experiment (Fig 5.5), a treatment time of 20 min was used for further experiments investigating the effects of different influencing factors.

5.2.2.2 Effect of different operating gases (air and MA65)

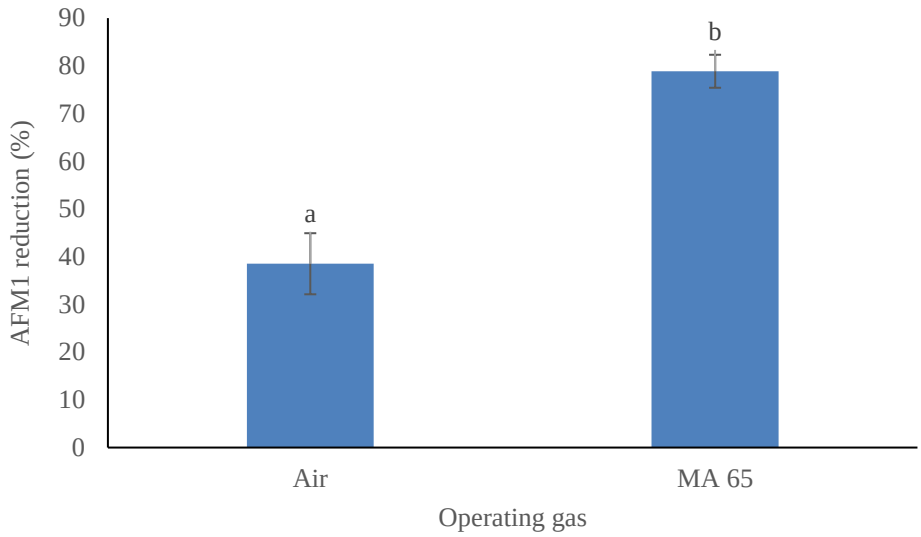


Fig 5. 6 The reduction of AFM1 in skim milk by HVACP induced by air and MA65, values with different letters representing a significant difference ($P < 0.05$).

Using MA 65 as the operating gas significantly ($P < 0.05$) reduced AFM1 in milk to 78.86% instead of 38.52% when using air as the operating gas (Fig 5.6). This is in line with a recent study for AFB1 in corn where treatment of corn using MA65 and air for 1 min reduced the AFB1 by 76% and 62%, respectively (Shi et al., 2017). This difference in the efficacy was explained by the variation in concentrations of reactive species in HVACP induced by MA65 and air (Shi et al., 2017). This was supported by the OAS experiment in the present study (Fig 5.7), where the concentration of ozone generated by MA65 was twice as much as that in air. The nitrogen reactive species were also produced at higher concentration in MA65. The plasma chemistry is believed to be driven by the operating gases (Whitehead, 2016). In the present study, the concentrations of ozone and NO_x generated by MA65 were higher than in air (Fig 5.7) coinciding with the reduction of AFM1. This is most likely due to the increased

oxygen in MA65. The generation of reactive species by cold plasma follows the following steps (Whitehead, 2016): (1) generation of free electrons (2) the dissociation of molecular oxygen into two oxygen atoms. The energy required to ionize an atom or molecule ranges from 10-20 eV while the particle translational energy at room temperature is approximately 0.04 eV (One eV is the input energy to accelerate an electron going through 1-volt electric potential difference) Therefore, the dissociation forming reactive species happens between these two levels of energy.

$$O_2 + e \rightarrow O + O$$

The recombination of an oxygen atom and molecular oxygen is successive, with an O_2 molecule as the third body.

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

The O atoms react with nitrogen species producing NO_x,

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

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

$$NO_2 +O+M \rightarrow NO_3 +M$$

$$NO_3 +NO_2+M \rightarrow N_2O_5 +M$$

M: any gas molecule

Therefore, the increase in O atoms from the dissociation reaction is likely to generate ozone and NO_x at high concentration (Moiseev, et al., 2014; Shi et al., 2017).

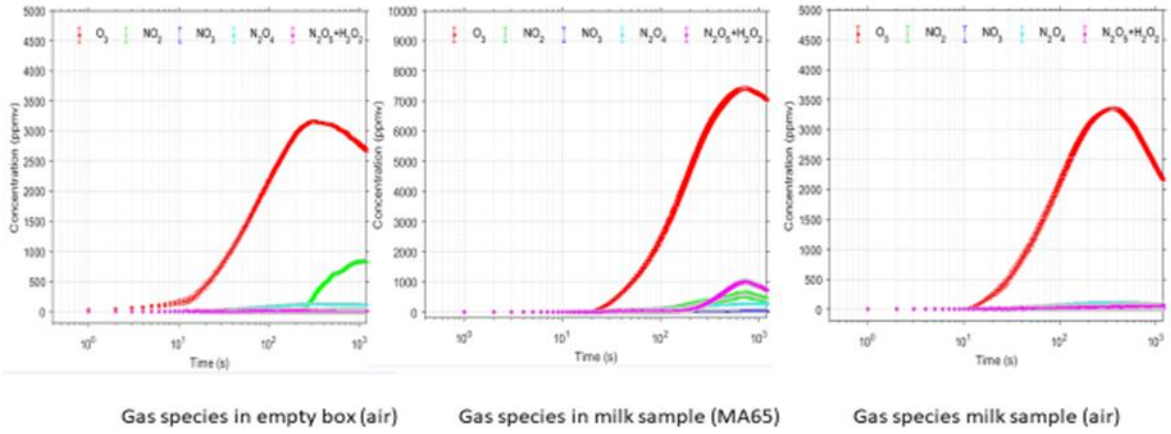


Fig 5. 7 The concentration of ozone and NO_x generated by HVACP in an empty box (filled by air) and milk samples (filled by MA65 and air) with red line representing of ozone, green - NO_2, blue - NO_3, cyan - N_2O_4, plum - N_2O_5+H_2O_2.

The concentration of ozone was more than 3 times higher in MA65 than in air after 5 min treatment at a relative humidity (RH) 40% (Shi et al., 2017). In our study, the amount of ozone generated in MA65 was twice as much as in air after 20 min treatment at relative humidity 70% (Fig 5.7). The difference in concentration of reactive species induced in MA65 and in air between our study and the previous one is probably due to variations in relative humidity. Shi et al. (2017) reported that the increase in relative humidity (5, 40 and 80%) decreases the concentration of reactive species (ozone and NO_x) in cold plasma. In addition, the presence of water during the cold plasma generation also affects these reactive species concentrations. The ozone in the plasma may react with water forming the hydroxyl radicals that led to the decomposition of ozone and the decrease in the ozone concentration (Staehelin & Hoigne, 1985).

The degradation of AFM1 by HVACP is likely to result from the damage to the double bond of the furan ring or the lactone ring. The double bonds of the furan ring and lactone ring structures are the reactive sites of the molecule (Siciliano et al., 2016) and

are believed to be responsible for the toxicity and mutagenicity (Wogan, Edwards, & Newberne, 1971). The molecular structures of AFB1 and AFM1 are similar in that they both have a double bond at $C_8 = C_9$ and a lactone ring that once destroyed reduces the toxicity (Iram, Anjum, Iqbal, Ghaffar, & Abbas, 2015; Lee, Dunn, DeLucca, & Ciegler, 1981; Wang, Xie, Xue, Wang, Fan, & Ha, 2011). AFB2 and AFG2 are less sensitive than AFB1 and AFG1 to plasma treatments because there is no terminal double bond at the C8 = C9 position of AFB2 and AFG2 (Siciliano et al., 2016). The terminal furan ring of the AFB1 molecule is attacked during many treatments including UV, plant extracts, ozone and gamma ray radiation (Iram, Anjum, Iqbal, Ghaffar, & Abbas, 2015; Luo, Wang, Wang, Li, Bian, & Chen, 2014; McKenzie et al., 1998; F. Wang, Xie, Xue, Wang, Fan, & Ha, 2011) and reacts with the reactive species in cold plasma (Shi et al., 2017).

5.2.2.3 Effect of different voltage and treatment mode

The voltage is one of the key factors influencing reactive species generation by HVACP that can improve the efficacy of the treatment (Keener & Jensen, 2014). Three different levels of voltage generation between the two electrodes of the HVACP system were used to evaluate their effect on AFM1 degradation. A high voltage (80 kV) boosted the efficacy of the treatment (Fig 5.8). Siciliano et al. (2016) observed similar results when increasing the power of the cold plasma system for AFB1 treatment with 12 min treatment reducing AFB1 by 45.7 and 70.7% with the power of 400 and 1150 W, respectively.

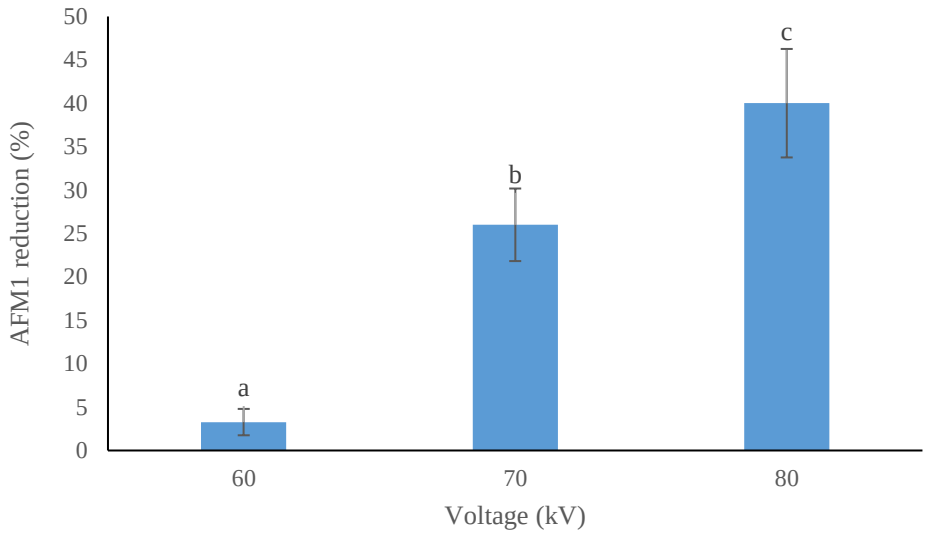


Fig 5. 8 The reduction of AFM1 in milk by HVACP induced by air at different voltages, values with different letters are significantly different ($P < 0.05$).

Together with voltage, reaction mode (direct/indirect) is also believed to be one of the factors affecting the efficacy of cold plasma treatment (Keener & Jensen, 2014). This is believed to be due to the samples reacting with short or long - lived reactive species. The reactive species such as O_2^-, $^1O^2$, ·OH, N_2^+ and N_2O^+ have half-lives of less than 0.5 s and are responsible for the cold plasma efficacy in direct treatment rather than indirect treatment (Wan, Pankaj, Mosher, & Keener, 2019) because some would transfer to their original state before reaching and reacting with samples in indirect treatment. Conversely, longer surviving reactive species (O_3, O_2, NO_2, NO and CO) play the key role in the interaction with samples in the indirect mode (Laroussi, 2009). In this study, the difference in AFM1 degradation during direct and indirect experiments was significant ($P < 0.05$) (Fig 5.9) and suggests that short - lived species contribute most to the reaction between cold plasma and AFM1 molecules. However, the mechanism of the degradation needs to be confirmed as well as the optimisation of the treatment mode. Wan et al. (2019) reported direct plasma generated by HVACP

was more effective than indirect plasma for the inactivation of *Listeria innocua* on Queso Fresco cheese. Conversely, the efficacy of reaction modes using HVACP did not show a significant difference in controlling *Salmonella enteritidis* in chicken eggs (Wan, Chen, Pankaj, & Keener, 2017). Shi et al. (2017) reported that aflatoxin degradation was mainly based on the reaction between long-life reactive species and the aflatoxin molecule. This means that the aflatoxin is exposed to reactive species regardless of the reaction modes (direct or indirect). The difference in the efficacy of reaction modes of HVACP between studies appears to be due to the differences in samples being treated.

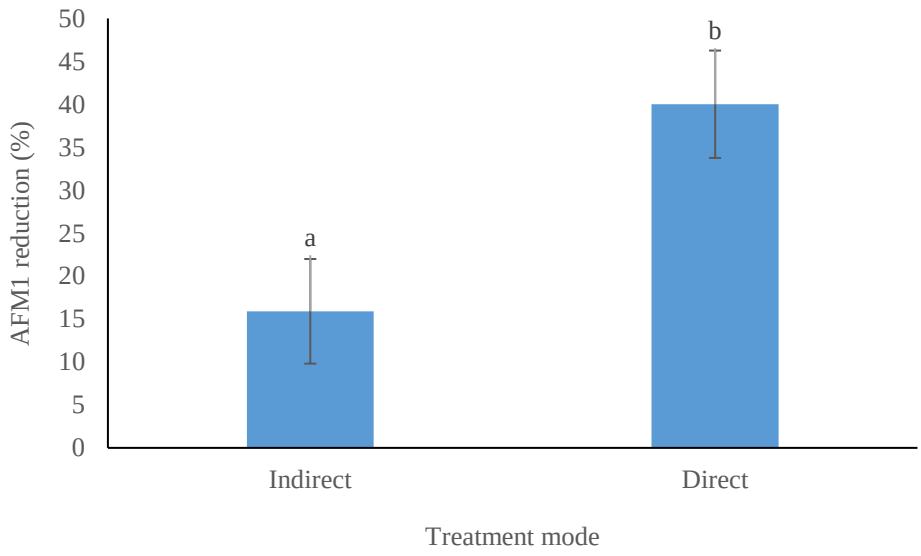


Fig 5. 9 The effect of reaction mode on the reduction of AFM1 in milk by HVACP induced by air. Values with different letters are significantly different ($P < 0.05$).

5.2.2.4 Effect of contamination levels and volume of sample

The reduction of AFM1 in milk contaminated with 0.1-50 µg/L ranged from 40 to approximately 50% (Fig 5.10). The difference in AFM1 degradation in milk

containing three different levels of AFM1 was insignificant (P > 0.05) suggesting that the concentration of AFM1 does not influence the efficacy of cold plasma.

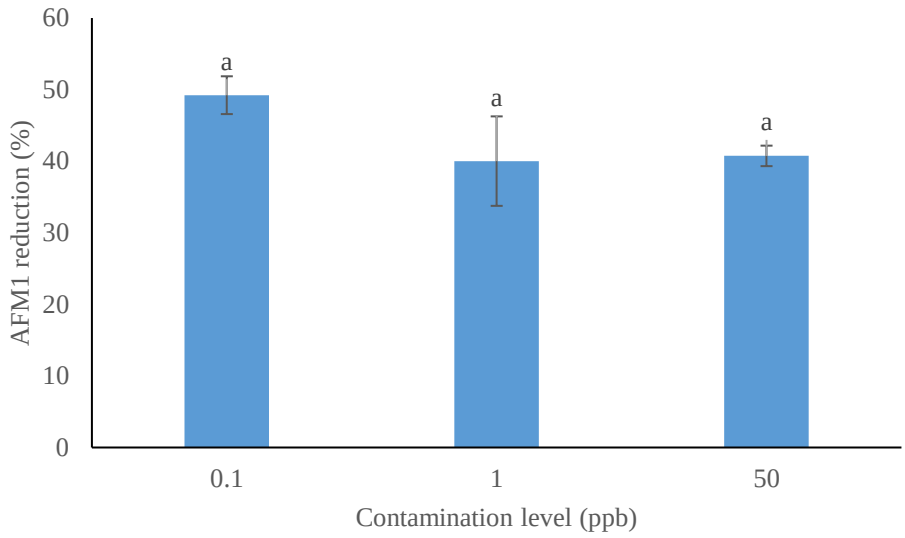


Fig 5. 10 Reduction of AFM1 in milk by HVACP induced by air various contamination levels. Values with different letters are significantly different (P < 0.05).

The reduction of AFM1 in milk was different between three volumes of sample (10, 20 and 30 mL). The decrease in the volume of milk increased the efficacy of HVACP on AFM1 reduction and vice versa (Fig 5.11). The penetration of the reactive species induced by plasma is probably an explanation for the difference in the AFM1 reduction in different volumes of milk. The reactive species tend to absorb more into the smaller amount of sample leading to the improvement in the efficacy of HVACP.

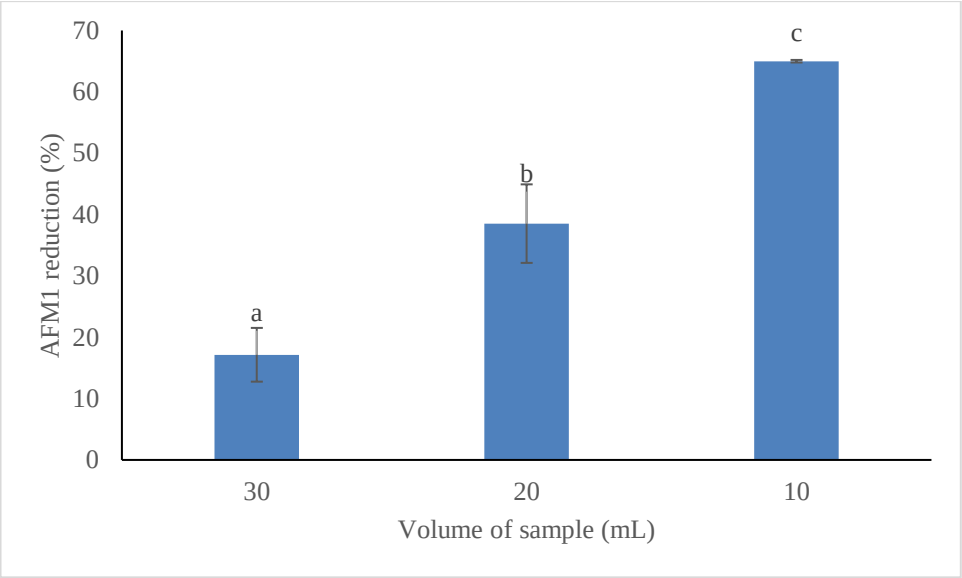


Fig 5. 11 Reduction of AFM1 in milk by HVACP in various volumes of sample.

Values with different letters are significantly different ($P < 0.05$).

5.2.2.5 Evaluation of milk colour and pH after HVACP treatment

The colour values for milk after 20 min HVACP treatment compared with the untreated control sample are shown in Table 5.2. L^*, a* and b* values in the treated sample increased slightly, however, these changes were insignificant ($P > 0.05$) and there was no change in milk colour by eye. The influence of plasma treatment on milk quality has been reported with no significant difference in whole milk colour during 20 min of plasma treatment (Gurol et al., 2012; Kim, Yong, Park, Kim, Choe, & Jo, 2015; Korachi et al., 2015). The a* values declined, while L* and b* values increased, which may be explained by a boost in the number of fat globules diffracting light (Walstra, Walstra, Wouters, & Geurts, 2005) in fullcream milk. However, the explanation for change in the colour of skim milk following cold plasma treatment has not been determined.

Table 5. 2 Hunter colour values of skim milk samples and pH after 20 min HVACP induced by air. Values with different letters in a column are significantly different ($P < 0.05$).

Sample	L*	a*	b*	pH
Control	$86.64^{a} \pm 0.038$	$-4.5^{a} \pm 0.35$	$6.34^{a} \pm 0.035$	$6.93^{a} \pm 0.03$
Sample	$87.82^{a} \pm 0.46$	$-4.04^{a} \pm 0.092$	$6.41^{a} \pm 0.15$	$6.52^{b} \pm 0.05$

NO_2 generated by air in the empty box, disappeared when the milk sample was present (Fig 5.7), NO_2 was possibly absorbed by milk and may form either HNO_3 or HO_2NO_2 by the reaction of NO_2 and other species such as OH in cold plasma (Misra, 2016; Whitehead, 2016)

$$OH + NO_2 \rightarrow HNO_3$$

$$HO_2 + NO_2 + M \leftrightarrow HO_2NO_2 + M$$

leading to an increase in the acidity and decrease in the pH of the milk sample (Table 5.2). The change in pH was significant ($P < 0.05$) between the control and treated samples. Similarly, Kim et al. (2015) reported that the pH of milk samples gradually decreased from 6.9 to 6.6 after 0 and 10 min of cold plasma treatment, respectively and the production of acid has been reported (Bruggeman et al., 2008). Cold plasma treatment also caused a decrease in pH in fruit and vegetable samples by reacting with the water on surface of the samples (Chen, 2008). Conversely, there was no significant change in the pH of strawberries, cherry tomatoes and orange juice treated with cold plasma (plasma-activated water for strawberries, 98% Ar + 2% O_2 and dielectric barrier discharge air generated plasma for cherry tomatoes and orange juice) (Ma, Wang, Tian, Wang, Zhang, & Fang, 2015; Misra, Keener, Bourke, Mosnier, & Cullen,

2014; Shi, Zhang, Wu, Li, Ma, & Shao, 2011). Nitrogen was absent in the operating gas used for the treatment of strawberries and that is likely to explain the lack of change in pH. Buffering counteracted the plasma chemistry in the treatment of orange juice. Generally, the pH change of treated samples by cold plasma may be influenced by several factors including buffering action, composition of the operating gas, and sample characteristics (Misra, 2016).

5.2.3 Conclusions – HVACP Study

HVACP can be used to degrade AFM1 in skim milk with more than 60% reduction after 20 min treatment without significant change in milk colour although a slight change in pH was observed. Cold plasma can be used to reduce AFM1 in skim milk to below MRL (0.5 µg/L) from an average contamination level of 1 µg/L. The contamination levels were shown to have an insignificant impact on the efficacy of HVACP treatment (P values > 0.05). Other factors including voltage, volume of sample, treatment time, treatment mode and different operating gases had a significant effect on the reduction of AFM1 in milk (P values < 0.05) as these related to the concentration of reactive species (ozone and NO_x) generated in cold plasma. Increasing the amounts of reactive species in cold plasma enhances the degradation of AFM1 in milk treated with HVACP.

5.3 Reduction of AFM1 in milk by Dielectric barrier discharge (DBD) cold plasma (cold plasma)

5.3.1 Methodology

5.3.1.1 Chemicals

For the cold plasma treatment, the following gases were used:

- Helium (technical grade, compressed - BOC, 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 probe of the ozone detector was placed inside the reaction bag after treatment and the bag resealed. The meter went up once put inside the bag and it was read at the peak before it went down.

The sample treatment was conducted in a plastic bag (heavy duty poly bags 300x450mm, 70 micron, clear, OfficeMax).

Aflatoxin M1 standard 10 μg/mL in acetonitrile, in an ampule of 1 mL (Sigma-Aldrich) was used for cold plasma trials to determine the effect on aflatoxin reduction.

To detect some of the reactive species, Quantofix peroxide test sticks, 6 mm x 95 mm (pack of 100), ranged 1-100 mg/L (Germany); Quantofix® nitrite, 6 mm x 95 mm (pack of 100), ranged 1-80 mg/L and 0.1-0.3 g/L, 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) used as controls.

For LC-MS testing, water (LCMS grade, ThermoFisher), methanol (LCMS grade, ThermoFisher), acetonitrile (LCMS grade, ThermoFisher), formic acid (LC/MS grade, Sigma) and ammonium acetate (LCMS grade, Sigma) were used.

For sample extraction NaCl, ethyl acetate and acetonitrile (HPLC grade) were used for sample extraction.

Gas flow meter, 1 and 10 L/min (Aalborg-USA) used to mix the gases.

2 µm PTFE filter (Sartorius, Germany) used to filter the samples before analysing by LCMS.

5.3.1.2 Dielectric barrier discharge system

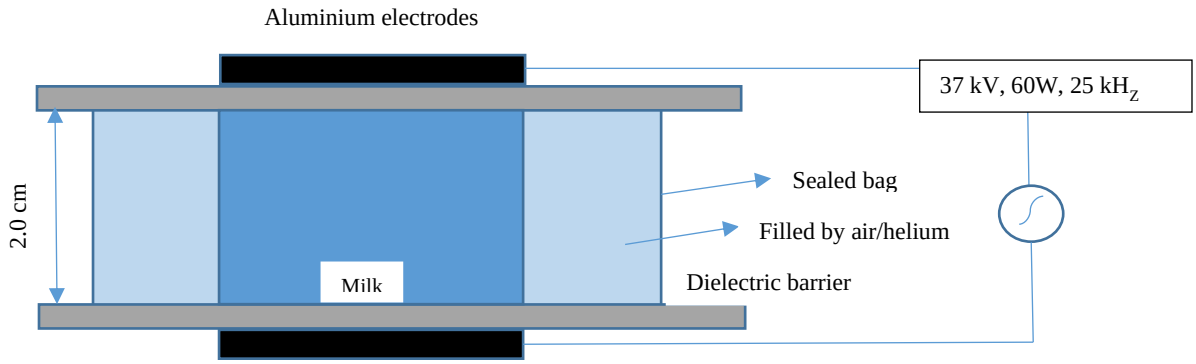


Fig 5. 12 Experimental set-up of DBD cold plasma for treatment of AFM1 in milk.

To investigate the effects of different operating gases, various ratios of operating gases, the influence of milk components on AFM1 reduction using cold plasma and the degradation products of AFM1 after treatment, a DBD cold plasma system was built at Massey University with a similar set-up to the HVACP at Iowa State University (Fig 5.12). The system was built with support from Dr Kevin Keener (technical advice) and John Pedley (setting up the system, trial runs and trouble shouting). It was finished in one year and a half with struggles due to technical issues and delays caused by the world pandemic. As the technique was new to New Zealand and the commercial supplies were not available, we started by looking for suitable power supply within budgetary constraints and ordering a power supply including stepdown transformer, high voltage cable, electrodes and ancillary equipment such as cutting boards (dielectric barriers) (Dec. 2019). It took us two months to collect all the items, then the lockdown due to the Covid-19 pandemic happened so assembling the items was

postponed until May 2020 when the lockdown was lifted. The equipment required modifications to meet NZ Electrical/Safety Standards including:

- stepdown transformer earthing improved and hard-wired output cable
- plasma supply key isolation switch fitted, and rear panel added for safety
- high voltage cables fitted with additional plastic tubing for insulation
- emergency stop switch fitted
- clipsal 10mA RCD (residual current device) used to provide protection for equipment
- all equipment electrically tested

Later the equipment was mounted on a base board with a regulated airline and tubing for cooling the high voltage coil. A K-type thermocouple was also added to monitor the high voltage coil temperature. When all was done, the standard operating procedure (SOP) was made and approval to use the equipment had to be obtained prior to use. This took two months (till August. 2020). Initial testing of the equipment highlighted the problem that the supplied equipment could not be used in resonance mode as advertised and required a different transformer to be ordered and fitted. The initial testing using air proved difficult as the plasma supply was operating beyond its capabilities causing electrical breakdown around the high voltage coil. This required insulating the high voltage coil and the primary coil and dry nitrogen gas was flushed into the high voltage area to help reduce arcing. With a change to Helium gas this reduced the high voltage required and the system ran for some time reliably, until we had a failure of the high voltage coil after a long testing day. The coil was replaced but

due to the potting material (insulation) we had to order replacement ferrite cores before fitting a replacement coil.

The testing of AFM1 and treated products was delayed due to a LC-MS/MS break down. This instrument was used to determine AFM1 before and after cold plasma treatment to evaluate the efficacy of treatment. Because of the pandemic, shipping items took longer than expected, but it was fixed in April. 2021 (it took 4 months to get fixed). Testing continued until we had a further high voltage coil failure. On cutting the coil open and examining it we found that the material used to pot the coil had good electrical insulation properties but was not very thermally conductive. The outside of the coil would only feel warm due to the thermal insulation, but the internal coil would be extremely hot causing the failure. On placing an order for some replacement high voltage coils they advised they had no stock and that their supplier in China had stopped making them and a local option was explored. Lead times of 3-4 months were indicted due to the pandemic delay. We tried making a high voltage coil ourselves, this proved very difficult and time consuming due to its construction and while we did get one working the capacitance of the coil was different (this wasted considerable time). After numerous calls and emails to the USA we found the supplier had made a mistake and did have the high voltage coils we required, these were ordered and a new coil fitted with an air supply to inject air between the ferrite core and the coil. A K-type thermocouple was added (between the ferrite core and the high voltage coil) so the temperature could be checked with the power off. The system has been reliable since the latest modifications.

5.3.1.3 Effect of different gas ratios on the efficacy

The mixtures of 5, 10 and 20 % of air, pure oxygen and nitrogen with helium (Table 5.3) were used to evaluate the effect of different operating gases on the reduction of AFM1 (10 µg/L in water/acetonitrile 85/15 v/v) after 3 min cold plasma treatment. Gas flow meters were used to mix the gases.

Table 5. 3 Different operating gas mixtures

Helium (%)	Air (%)	Oxygen (%)	Nitrogen (%)
80	20	20	20
90	10	10	10
95	5	5	5

5.3.1.4 Effect of different treatment times

AFM1 standard (10 µg/L in 85/15 water/acetonitrile) (2 mL) was held in a small petri dish and exposed to the cold plasma for the treatment times of 3, 5 and 10 min. Air/helium (10/90) was used.

5.3.1.5 Effect of milk components on the AFM1 reduction in milk

Lactose (5 g), casein (2.7 g), and whey protein (0.7 g) were each dissolved in 100 mL of water. A 3- mL of each with AFM1 added (final concentration of 10 µg/L) was poured into the petri dish and sealed in the reaction bag then placed in line with the 2

aluminium electrodes to treat with cold plasma for 5 min (Fig 5.12). Air/helium (10/90) was used.

5.3.1.6 Measurement of reactive species including hydrogen peroxide and nitrite in the samples after treatment using test strips

Before testing the samples, the colour of the strips was tested by using positive controls. For hydrogen peroxide testing, 83.33 µL H_2O_2 30% was pipetted to 25-mL volumetric flask and filled by distilled water to make the solution with the concentration of 1000 ppm. 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 samples.

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/mL). 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 30 s. The change in the colour of the strip indicators for the positive control was confirmed before testing treated samples.

5.3.1.7 Degradation products of AFM1 after cold plasma treatment

- Treating AFM1 with cold plasma

AFM1 standard (2 mL) in 85% water in acetonitrile (1 µg/mL) was held in a small glass petri dish (ID 40 mm) placed in line with two electrodes in the reaction bag (Fig

5.11). 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 60 W, 20 kHz and 30 kV. The samples after plasma treatment were filtered through a 2 µm PTFE filter before LC-MS/MS injection.

- **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:

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

LC gradient details and the MS parameter are shown below (Tables 5.4):

Table 5. 4 The gradient of the mobile phase of LC-MS/MS

Time (min)	A (water + 0.1% FA) %	B (MeOH + 0.1% FA) %
0	70	30
15	0	100
17	0	100

Details of the MS Instrument Parameters are in the Appendix B and Appendix C.

5.3.2 Results and discussion

5.3.2.1 Testing of strip indicator colour

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 darkness of the strip indicators. Similarly, they turned pink-red when dipped into nitrite solutions while negative control strip indicators remained white. The darkness of the strip indicators increased gradually from 1 to 100 ppm of nitrite then turned red at the concentration of 2011 ppm (Fig 5.13).

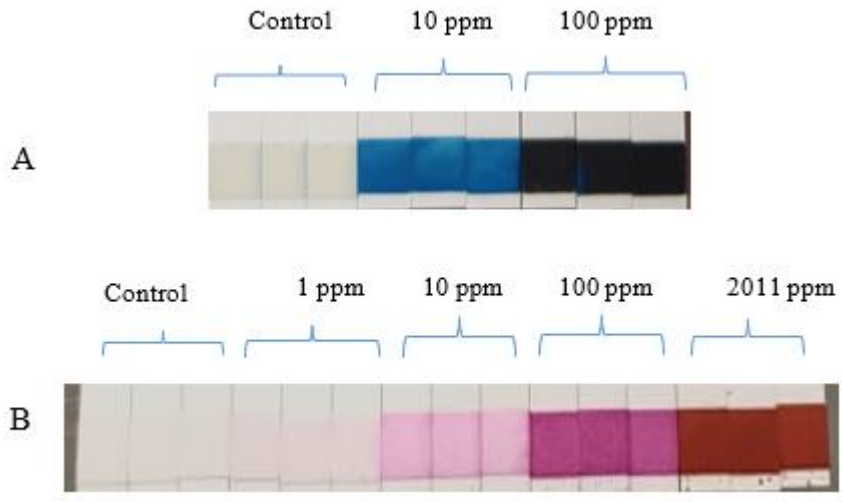


Fig 5. 13 The colour of strip indicators: Hydrogen peroxide (A) at 0, 10 and 100 ppm (triplicate). Nitrite (B), 0, 1, 10, 100 and 2011 ppm (triplicate).

5.3.2.2 Effect of different operating gases

The reduction (%) and residue (µg/L) of AFM1 by cold plasma with various operating gas mixtures are shown in Fig 5.15 and Table 5.5. These results indicate that different gases and the ratio of the gases have significant effects on AFM1 degradation by cold plasma. Helium was used to initiate the ionization of air/oxygen/nitrogen during treatment. It produced zero reduction of AFM1 when 100% helium was used as an operating gas although a relatively low concentration of hydrogen peroxide (3-10 ppm) and nitrite (approximately 1 ppm) were generated (Fig 5.14) which was probably either insufficient to reduce AFM1 in the standard solution or lacking the reactive species responsible for AFM1 reduction in helium induced plasma. Approximately 4 ppm of ozone was produced by 100% helium after 3 min treatment. Low levels of reactive species (hydrogen peroxide, nitrite and ozone) were produced by 100 % helium plasma which was likely to be caused by a trace of air dissolved in the solution (Fig 5.14).

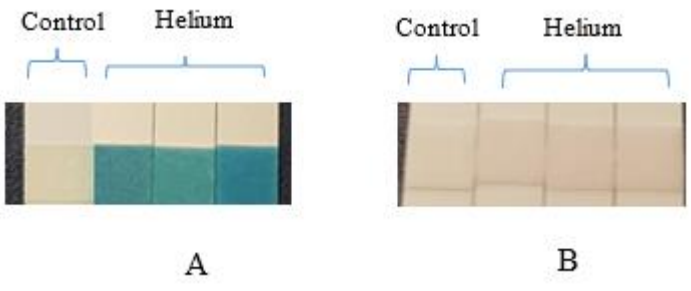


Fig 5. 14 Hydrogen peroxide (blue, A) and nitrite (pink, B) produced by 100% helium after 3 min treatment. The first strip is the control, and the next three strips are treated samples (triplicate).

The nitrogen-helium mixture used in the cold plasma reduced about 50% of the AFM1 which indicates that the reactive nitrogen species (RNS) were able to react with 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. These are likely to explain the significant reduction of AFM1 (Table 5.5) when air was used to mix with helium because RNS and ROS were generated by cold plasma using air (78% nitrogen-21% oxygen) as an operating gas

Table 5. 5 The residue of AFM1 by cold plasma after 3 min treatment with different gas types. Values with different capital letters are significantly different across the columns for various gas mixture concentrations. Values with different small letters are significantly different across the row for different types of gas at the same ratio ($P < 0.05$).

Mixture ratio (%), in Helium	AFM1 residue (µg/L)		
	Air	Oxygen	Nitrogen
Control	$9.81^{Aa} \pm 0.19$		
5	$4.14^{Bb} \pm 0.30$	$4.67^{Bbc} \pm 0.37$	$5.21^{Bc} \pm 0.41$
10	$3.21^{BCb} \pm 1.21$	$3.74^{Cbc} \pm 0.12$	$5.20^{Bc} \pm 0.45$
20	$1.90^{Cb} \pm 1.16$	$1.11^{Db} \pm 1.01$	$5.28^{Bc} \pm 0.75$

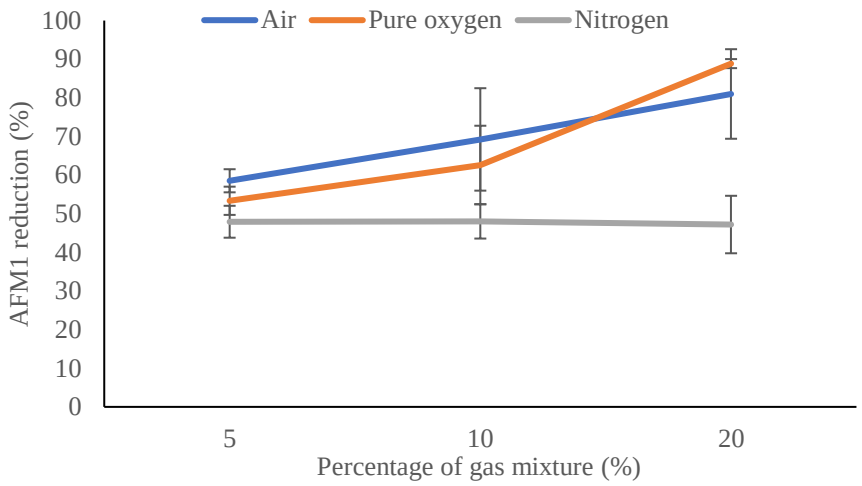


Fig 5. 15 Reduction of AFM1 solution by various operating gases.

A significant amount ($P < 0.05$) of AFM1 was degraded by air/helium and the pure oxygen/helium mixtures induced plasmas and the degradation increased by increasing the gas ratio, from about 50-90% with the gas mixture of 5-20% air or oxygen in helium, (Fig 5.15). 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 concentration 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 5-20% in helium) generated high levels of these reactive species (Fig 5.16, 5.17 and Table 5.6).

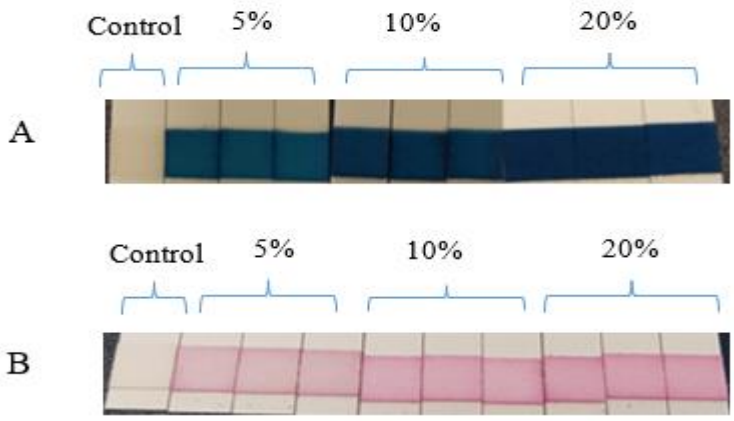


Fig 5. 16 Hydrogen peroxide (A) and nitrite (B) produced by 5, 10, 20% air mix after 3 min treatment. First left strip is the control, with the test strips in the following the order: 5, 10 and 20 % of air in helium (triplicates).

The increase in the concentration of reactive species (hydro peroxide and nitrite) in the sample increased the darkness of the strips. The concentration of hydrogen peroxide and nitrite in air induced plasma ranged from 30-100 ppm and < 5-10 mg/L (Fig 5.16), respectively, with the air in helium increasing from 5-20%. Air is mainly made up of nitrogen and oxygen so the increase in the air ratio in the gas mixture with helium, which led to the increase of oxygen and nitrogen in the gas mixture, enhanced the production of reactive species because these elements are the main ingredients producing ROS and RNS, via the following steps:

$$O_2 + e \rightarrow O + O$$

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

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

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

$$O + H_2O \rightarrow 2OH\cdot \qquad (*)$$

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

(Surowsky, Bußler, & Schluter, 2016)

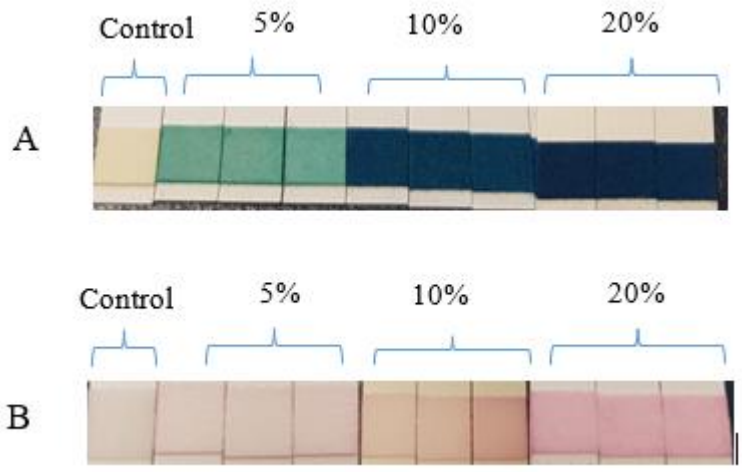


Fig 5. 17 Hydrogen peroxide (A) and nitrite (B) produced by 5, 10, 20% oxygen mix helium after 3 min treatment. First left strip is the control, the following strips represent 5, 10 and 20 % of pure oxygen in helium (triplicates).

Similarly, the increase of oxygen from 5-20% in helium increased the concentration of hydrogen peroxide and nitrite from 10-100 ppm and 1-5 mg/L, respectively, (Fig 5.17). 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 leading 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 (Björn Surowsky, Fröhling, Gottschalk, Schlüter, & Knorr, 2014). However, the amounts of nitrite induced by the oxygen /helium mixture tend to be lower than in the air/helium mixture which is probably due to the lack of nitrogen in the oxygen/helium gas mixture.

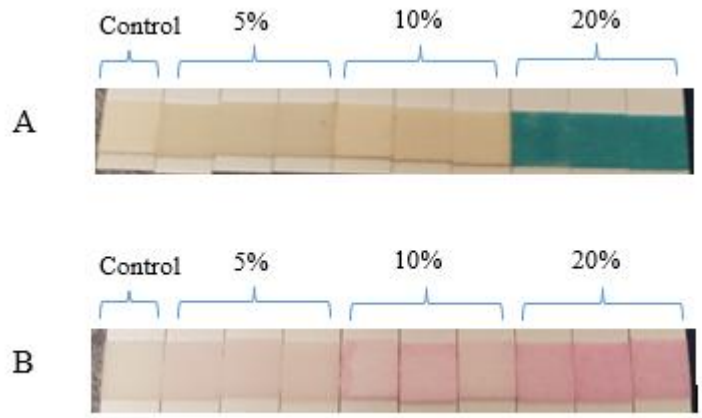


Fig 5. 18 Hydrogen peroxide (blue) and nitrite (pink) produced by 5, 10, 20% nitrogen in helium after 3 min treatment. First left strip is control, the following strips represent the order: 5, 10 and 20 % of air in the gas mixture (triplicates).

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 – 3 ppm (Fig 5.18). 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·) (***) which are produced by the dissociation of water from the electron collision (reaction **) instead of the combination of the reactions (*) (in session 5.3.2.2), (**) and (***).

$$e + H_2O \rightarrow H\cdot + OH\cdot + e \qquad (**)\ \text{(Whitehead, 2016)}$$

$$2OH\cdot \rightarrow H_2O_2 \qquad (***)$$

Similarly, the concentration of nitrite rose from 1-5 mg/L responding to 5-20% nitrogen in helium gas, however, the generation of RNS in the nitrogen/helium is 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.

Table 5. 6 Ozone concentration (ppm) induced by various gas mixture (5-20% air/pure oxygen/nitrogen in helium) after 3 min treatment, values with different letters are significantly different ($P < 0.05$).

Gas ratio (%) in helium	Ozone concentration (ppm)		
	Air	Oxygen	Nitrogen
5	$24.4^{b} \pm 3.1$	$560^{c} \pm 90$	$4.43^{a} \pm 1.56$
10	$25.73^{b} \pm 3.82$	$365^{c} \pm 152$	$5.57^{a} \pm 0.9$
20	$19.47^{b} \pm 8.43$	$410^{c} \pm 186$	$4.43^{a} \pm 2.02$

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 5.6). 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 is 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, at 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 5.6).

This is probably due to the distribution of the input energy for ionization and dissociation of the operation gas molecules during cold plasma treatment. The first ionization energy of oxygen is 13.6181 eV while the nitrogen's one is 14.5341 eV which indicates that the strength of the bond between nitrogen molecules is stronger than the oxygen. The ionization and dissociation of oxygen occurs at a lower level of engergy compared to nitrogen (Whitehead, 2016). Therefore, most of the input energy transferred oxygen into ozone in the pure oxygen induced plasma while it consumed more engergy to dissociate and ionize nitrogen in air induced plasma. That may lead to the ozone concentration genenrated in air being much lesser than in in pure oxygen plasma.

The absence of oxygen probably led to the low level of ozone generated by nitrogen/helium gas. However, the insignificant ($P > 0.05$) difference in the amount of ozone was observed in all three gases (nitrogen/helium, air/helium and oxygen/helium) at three different ratios (5, 10 and 20%). The possible explanation is due to the difference in the gas ratio which was insufficient to make a significant change in the ozone generation.

The difference in AFM1 reduction by air and pure oxygen induced plasma were 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 mechanism for this remains unknown but the explanation is likely to be the chemistry of the plasma that is driven by the operating gases. For instance, at the ratio of 20% in the gas mixture, the amount of oxygen in pure oxygen mixture was five time more than in air leading to a higher level of ozone induced by the pure oxygen/helium plasma than air/helium. However, the concentration of hydrogen peroxide and nitrite was 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. These indicated that ozone, hydrogen peroxide and nitrite all play an important role in reducing AFM1. In addition, the reduction of AFM1 by nitrogen induced plasma was not different (Table 5.15) for the various gas ratios. The reduction of AFM1 was approximately 50% at different gas mixtures ranging from 5-20% nitrogen/helium. At 5% nitrogen/helium, plasma reduced AFM1 by about 50% like air/helium and pure oxygen/helium, although 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-live reactive species which are produced during the treatment and disappeared after switching off the power, also play a key role in degrading AFM1 by cold plasma together with the long-live species including ozone, hydrogen peroxide and nitrite.

5.3.2.3 Effect of treatment time on degradation of AFM1 by cold plasma

The effect of treatment time on AFM1 reduction by air/helium (10/90%) induced plasma was significant (Fig 5.19). 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.

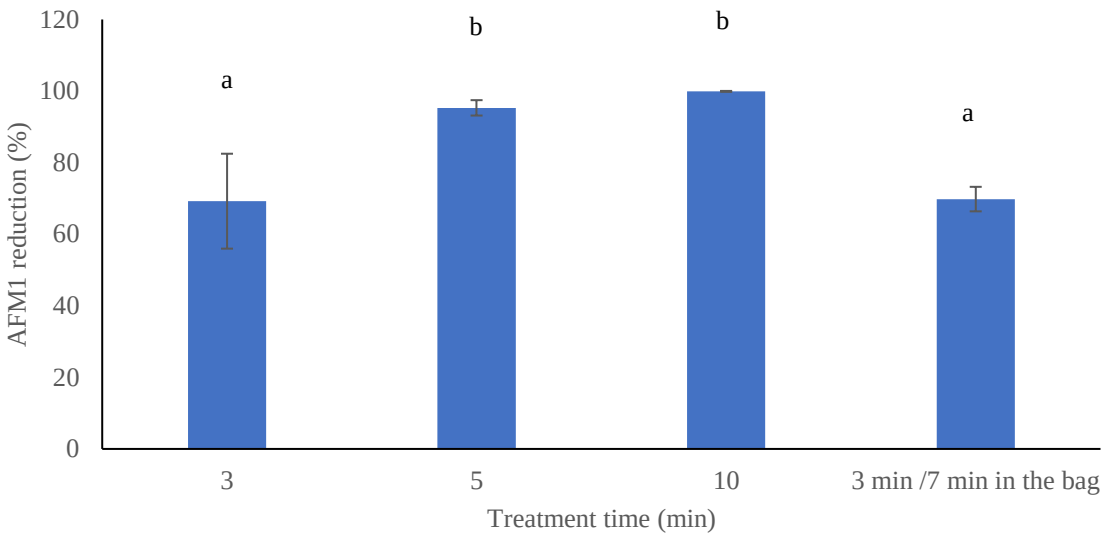


Fig 5. 19 The reduction of AFM1 (10 µg/L) by air/helium (10/90%) induced plasma at different treatment time. Values with different letters are significantly different ($P < 0.05$).

To evaluate the effect of short-lived and long-lived reactive species (particularly ozone - O_3), an experiment was caried out to compare the AFM1 reduction in the 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 leaking of O_3. No significant ($P < 0.05$) difference between these samples was found (Fig 5.19). This indicated that the other 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 and 10 min (with 7 min without power) 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-live species in the degradation of AFM1 by cold plasma.

An improvement in the cold plasma efficacy caused by the increase in treatment time was also found in previous studies where it was used to reduce *Escherichia coli* and *Listeria monocytogenes* in pork loins, *E. coli* in milk, *Enterococcus faecalis*, *E. coli*, *L. monocytogenes*, and *S.* typhimurium in milk (Chang & Chen, 2016; Gurol, Ekinci, Aslan, & Korachi, 2012; Kim, Yong, Park, Choe, & Jo, 2013; Kim, Yong, Park, Kim, Choe, & Jo, 2015). However, the efficacy can vary due to the different set-up of the cold plasma generator, the sample substrate and the targeted subjects (microorganisms, toxin, etc.). In the present trial, the increase in the concentration of reactive species (short and long-lived ones) over time is the most likely reason for the improvement in AFM1 reduction. The concentration of hydrogen peroxide and nitrite increased from 10->100 ppm and 0.1-0.3 g/L, respectively, indicated by the increasing darkness of the indicators after 3, 5 and 10 min treatment (Fig 5.20).

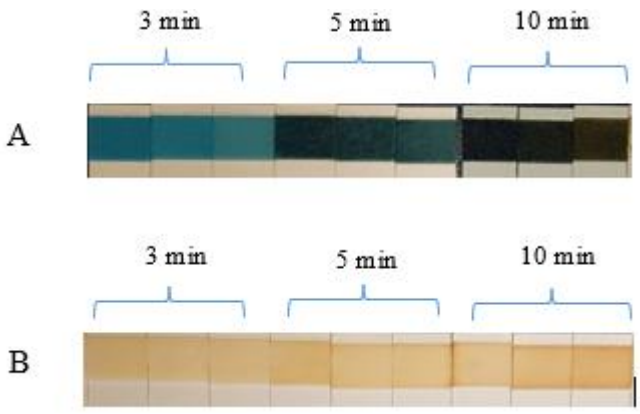


Fig 5. 20 Hydrogen peroxide (A) and nitrite (B) produced in water/acetonitrile (85/15) during 3, 5, 10 min treatment (from left to right triplicates) in 10% air in helium.

However, the ozone concentration at different treatment times tended to be unchanged (Table 5.7). The same 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, Stroshine, Ileleji, & Keener, 2017). This is probably because

the ozonation process by plasma treatment is quick so that the saturation of ozone is achieved after a short time and remains stable regardless of the treatment time.

Table 5. 7 Ozone concentration (ppm) induced by 10% air in helium at different treatment times. Values with different letters are significantly different ($P < 0.05$).

Treatment time (min)	Ozone concentration (ppm)
0	$0.4^{a} \pm 0.1$
3	$25.73^{b} \pm 3.82$
5	$23.4^{b} \pm 7.83$
10	$20.43^{b} \pm 6.98$

5.3.3.4 The reduction of AFM1 in skim milk by air induced cold plasma and the effect of different milk components

The reduction of AFM1 in skim milk by air/helium (10/90) induced plasma at different treatment times, is shown in Fig 5.21, ranging from 13.63% to 32.89% after 3, 5 and 10 min. The increase in the treatment time improved the AFM1 degradation which is likely due to the increase in the concentration of reactive species (H_2O_2 and NO_2) over time. The concentration of hydrogen peroxide and nitrite induced by the 10% air in helium plasma ranged from 3 -< 100 ppm and 0.1-2 g/L, respectively, indicated by the increasing darkness of the indicators after 3, 5 and 10 min (Fig 5.22). Increasing the reactive species concentration by longer treatment time resulted in the increase in the reduction of AFM1 in milk. However, the change in ozone level over time was

insignificant (P > 0.05) which again is probably explained by the quick induction of ozone reaching saturated concentration and remaining unchanged (Table 5.8).

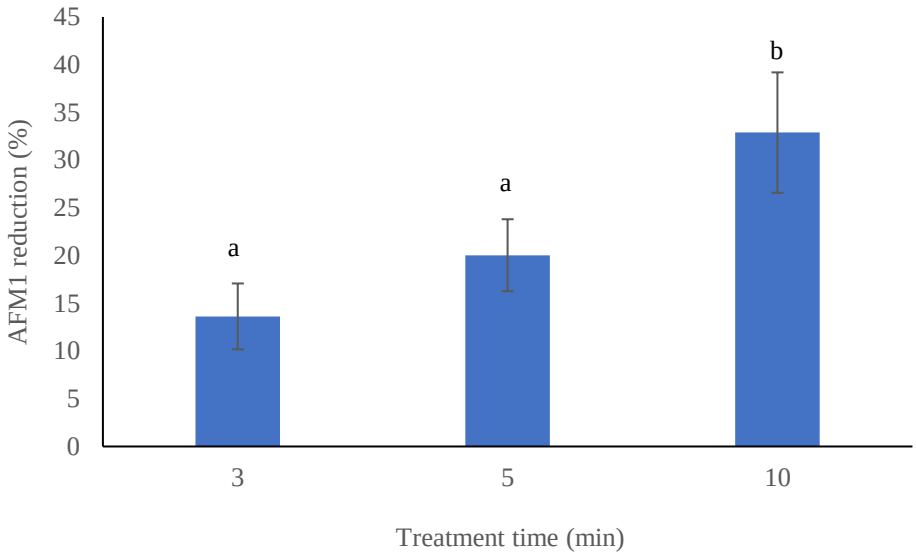


Fig 5. 21 The reduction (%) of AFM1 in skim milk by air/helium (10/90%) induced plasma at various treatment time. Values with different letters are significantly different (P < 0.05).

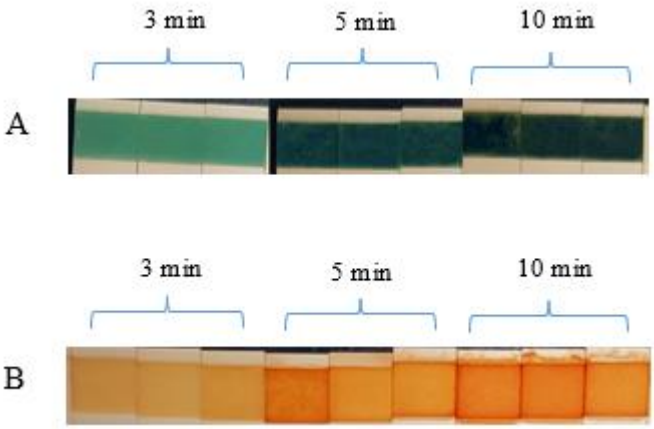


Fig 5. 22 Hydrogen peroxide (A - blue, green) and nitrite (B - orange) produced in milk during 3, 5, 10 min treatment (from left to right triplicates) by 10% air/helium mixture.

Table 5. 8 Ozone concentration (ppm) in skim milk induced by 10% air in helium at different treatment times. Values with different letters are significantly different ($P < 0.05$).

Treatment time (min)	Ozone concentration (ppm)
0	$0.4^{a} \pm 0.1$
3	$29.23^{b} \pm 5.19$
5	$20.17^{b} \pm 13.32$
10	$20.6^{b} \pm 12.93$

The efficacy of cold plasma on the AFM1 reduction in skim milk was much lower than in water/acetonitrile (85/15) although the nitrite concentration in milk was much higher than in water/acetonitrile (85/15). This lower efficacy is likely due to either the lower level of hydrogen peroxide or the low penetration of the reactive species into milk sample.

To investigate which components in milk, prevents the cold plasma from reducing AFM1, three different components of milk (lactose, casein and whey protein) contaminated with AFM1 were treated with cold plasma. No significant ($P > 0.05$) difference in AFM1 degradation was found between skim milk, casein and, whey protein (approximately 15%) while AFM1 was reduced by 68.34% in lactose, closer to the reduction of AMF1 in water/acetonitrile (85/15) solution (Fig 5.23). This is

probably due to either the transparency of these fluids or the blocking of the reaction between reactive species and AFM1 molecules by the milk protein. The dissolving of lactose into water did not change the transparency of the solution (water) but the solutions of casein and whey protein were less transparent compared with lactose or water (became hazy solutions, Fig 5.24).

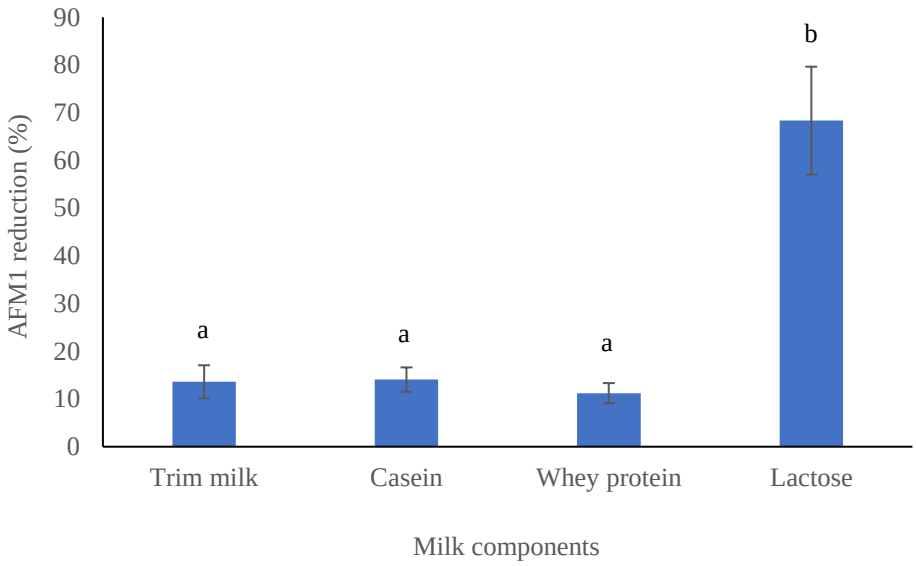


Fig 5. 23 The reduction of AFM1 in skim milk and other milk components after 3 min treated by air/helium induced cold plasma.

Fig 5. 24 Casein (2.7%), lactose (5%) and whey (0.7%) protein solutions.

5.3.3.5 Identification of degradation products of AFM1 after cold plasma treatment

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 original AFM1 peak area were taken forward for structure elucidation (peaks1, 2, 3 and 4, Fig 5.25) (smaller peaks had large variations between triplicates).

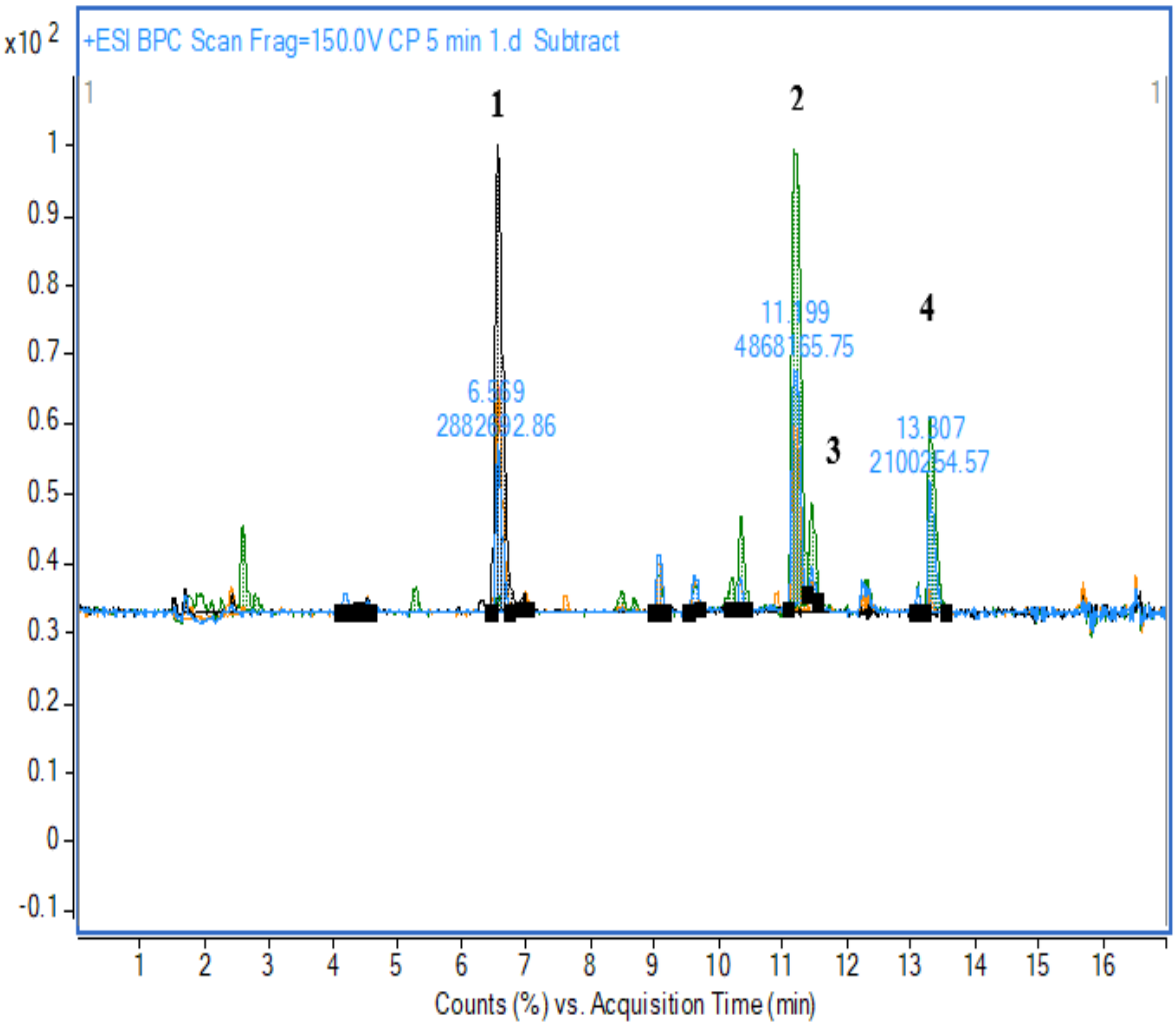


Fig 5. 25 Overlay of the base peak chromatograms (MS1) of the air/helium (10/90%) cold plasma treated samples (0 min - black, 3 min - green, 5 min - blue and 10 min - orange).

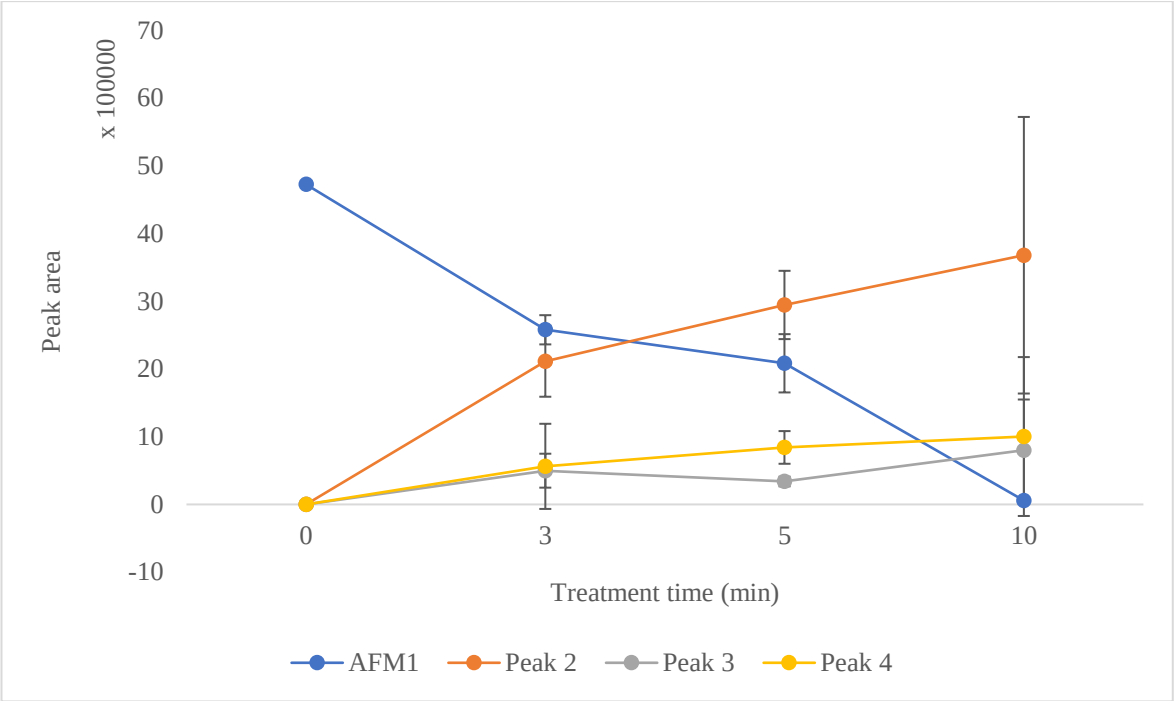


Fig 5. 26 Relative change in the increase of degradation products responding to the reduction of AFM1 over treatment time (error bars represent standard deviation of triplicate).

Note: The peak areas of AFM1 (peak 1), peak 2 and peak 4 were divided by 2 to have the same scale as peak 3.

Peaks 2, 3 and 4 were the degradation products of AFM1 by cold plasma so they were only produced after treatment. Before the treatment at 0 min, the area of peaks 2, 3 and 4 were zero and the AFM1 peak area was highest. When the treatment time increased, the AFM1 started reducing with an increase in the production of peaks 2, 3 and 4. The production of peaks 2, 3 and 4 varied from each other leading to the difference in their peak areas. The difference in the sensitivity to the detection method of these compounds may also explain the differences in the peak areas. The change in the peak areas of the degraded AFM1 over treatment time is shown in Fig 5.26. AFM1 reduced

gradually over time; from approximately 40% after 3 minutes of treatment, to about 60% after 5 minutes of treatment, and almost 100% after 10 minutes of cold plasma treatment. Meanwhile, the formation of degradation products increased gradually for all three products.

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 5.9). The formula with the highest score (closest to 100%) are listed below.

Table 5. 9 Possible formula of the degradation products of AFM1 after cold plasma treatment.

No	RT	Proposed formula	Ion mass (m/z)	Diff (ppm)	DBE	Score (%)
1	6.573	$C_{17}H_{12}O_7$ (AFM1)	329.0666	4.19	12	95.91
2	11.2	$C_{17}H_{22}O_6$	323.1463	-2.25	7	96.69
3	11.5	$C_{17}H_{22}O_6$	323.1463	-2.25	7	96.69
4	13.311	$C_{17}H_{10}O_7$	327.1565	>5	13	< 90

Note: The m/z of $[M+H]^+$. DBE: double bond equivalents. Diff: difference between observed and theoretical mass.

The confirmation of the proposed structures of peaks 2 and 3

The structures of peaks 2 and 3 were proposed by fragmentation. The selected peaks/ions were fragmented (MS/MS) to form the structure. The results for peak 2 are shown in Figs 5.27 and 5.28).

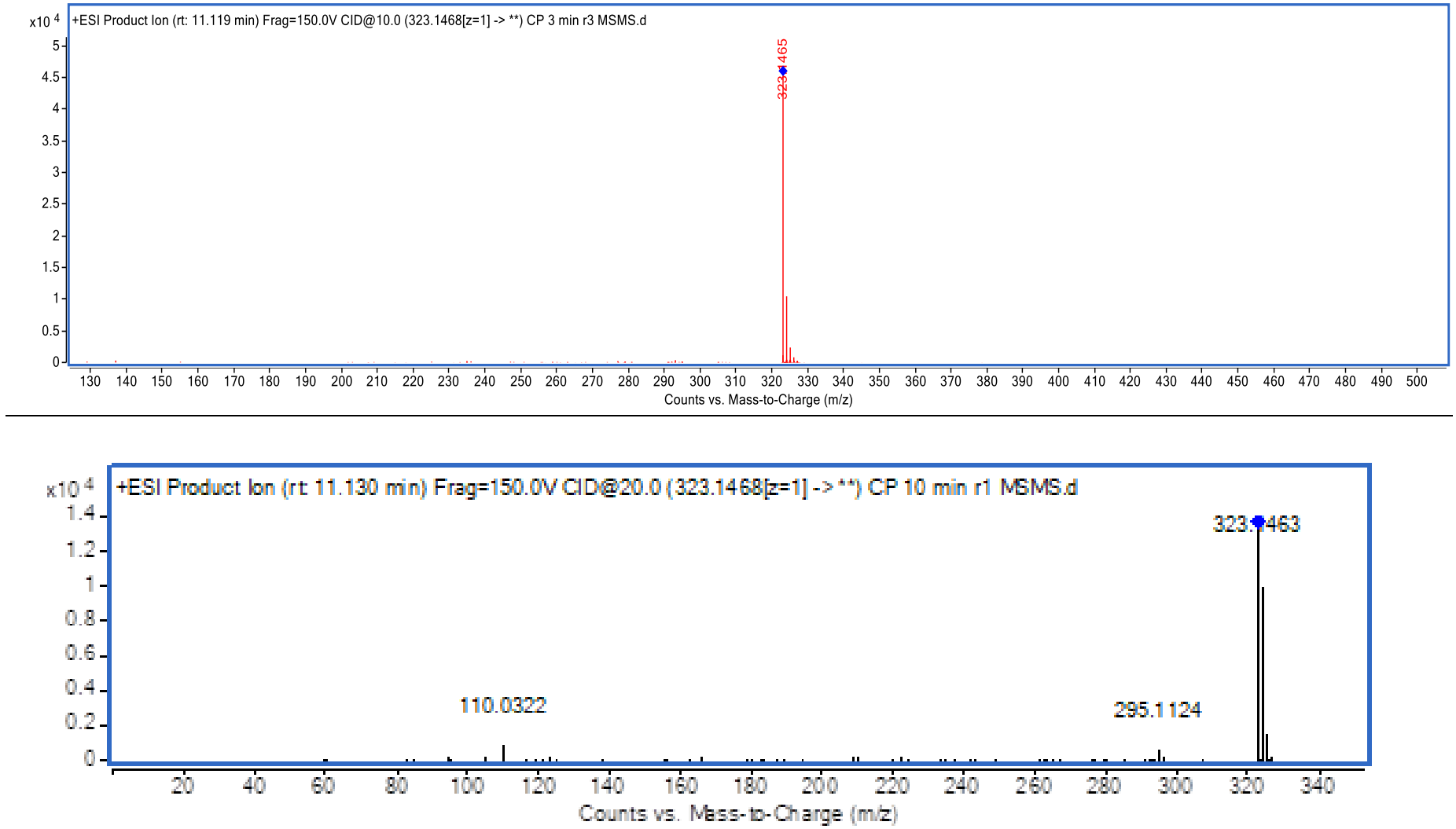


Fig 5. 27 MSMS spectra of ion 323.1463 (CID at 10 and 20).

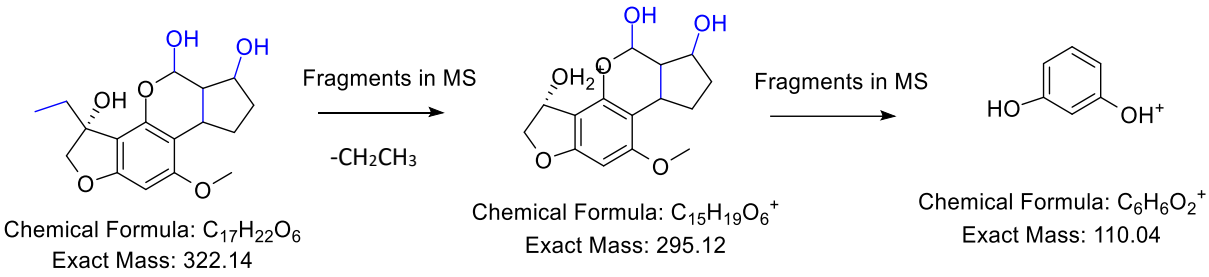


Fig 5. 28 Potential fragmentation pathway of ion 323.1463.

The precursor ion - 322.14 was broken down to ion 295.15, with a 27 Dalton difference, which indicated that the precursor ion lost the - CH_2CH_3 fragment and it was likely to happen at the C9-C9a bond of the molecule. This bond tends to be less stable and broken easily because the - OH (hydroxyl) group in C9a and - CO (ketone) in the C6a which attracts more electrons due to the high electronegativity of oxygen leading to the bond's break with collision.

Similarly, peak 3 structure was proposed by MS/MS fragmentation (Figs 5.29 and 5.30)

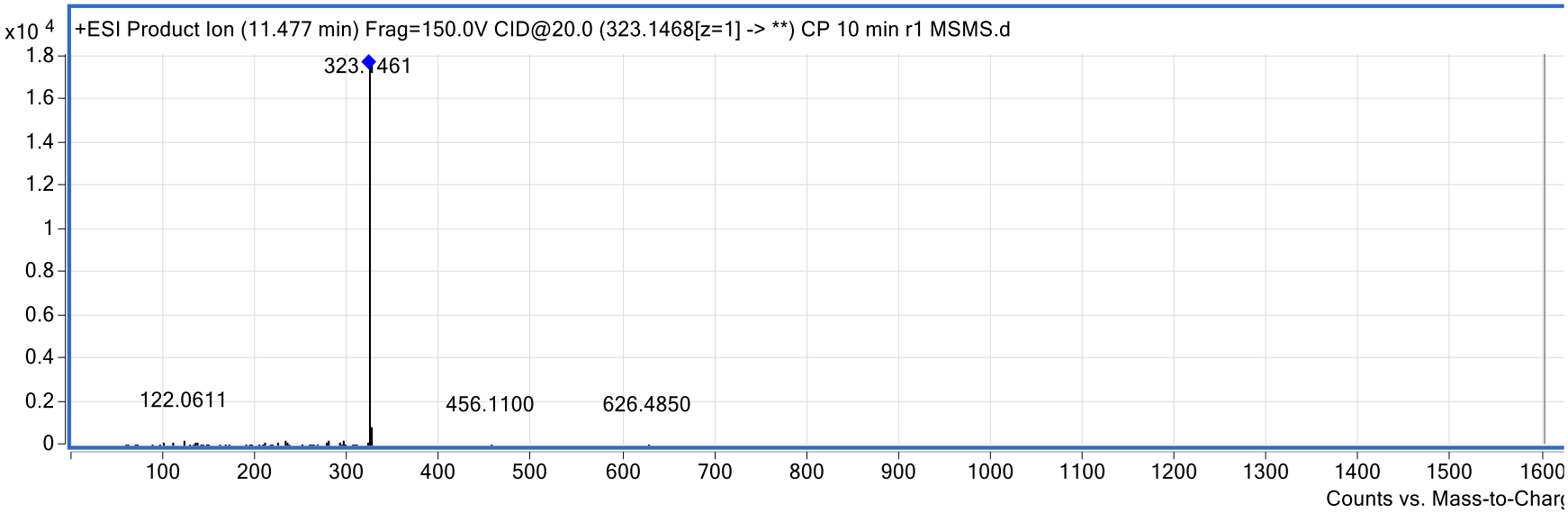


Fig 5. 29 MS/MS spectra of ion 323.1463

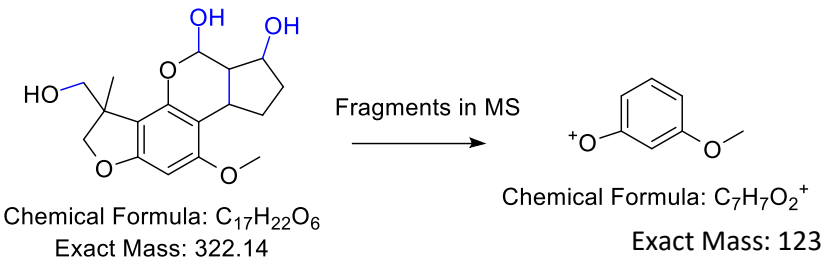
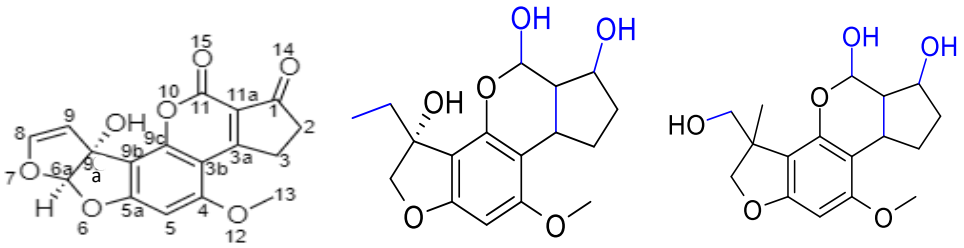


Fig 5. 30 Structure of MS/MS fragment of ion 323.1463.

Proposed structures that matched the selected formulas are shown in Fig 5.30.



Peak 1, AFM1 Peak 2 Peak 3

Fig 5. 31 The possible structures of the two degradants.

The structures of the possible compounds were proposed based on the findings from the literature for aflatoxins degradants using various types of treatment. 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. The oxidation of the AFM1 molecule under UV treatment occurred at C8=C9 (Stanley, Patras, Pendyala, Vergne, & Bansode, 2020) or became aflatoxin M2a (Yousef & Marth, 1987) in which the –OH group attached to either C8 or C9 of the double bond. The double bond C8=C9 of the furan ring and the lactone ring of the AFB1 molecule were destroyed after many

treatments including UV, treatment with plant extracts, ozone, gamma ray radiation and ultrasound (Iram et al., 2015; Liu, Li, Liu, & Bian, 2019; Luo et al., 2014; McKenzie et al., 1998; Wang et al., 2011) and reacted with reactive species in cold plasma (Shi et al., 2017).

In addition, the retention time of the compounds was used as one of the parameters to confirm the proposed structure. The polarity of the compound is one of the factors affecting the retention time of the compound. In reverse-phase chromatography, the retention time of compounds generally increases with a decrease in polarity (Zuvela, Liu, Wong, & Baczek, 2020). LogP of a compound is also an indication of the retention time of that compound in reverse-phase chromatography. The compound with a lower logP value is supposed to elute faster compared with the higher logP ones. In this study, the logP of peaks 2 and 3 were higher than AFM1 and that explained the shorter retention time of AFM1 (Table 5.10). Peaks 2 and 3 are isomers as they both have the same chemical formula and mass but different retention times and a small increase in the retention time of peak 3 compared with peak 2 was observed although the logP value of peak 3 was slightly lower. The explanation is likely due to the stereochemistry and other intramolecular bonding affecting the retention time, and the interaction between these compounds with the C18 of the stationary phase. The polarity of the mobile phase would also be one of the explanations. The retention of peak 3 was slightly longer than peak 2 which was probably due to the stronger association of peak 3 to the stationary phase than peak 2. The difference in the structure of these isomers leading to the difference in the stereochemistry and their interaction with the stationary phase of the HPLC are likely responsible for the slightly longer retention of peak 3 compared with peak 2.

Table 5. 10 LogP value of AFM1 and the degradation products.

Compound	Peak 1 (AFM1)	Peak 2	Peak 3	Peak 4
LogP value	0.9	1.33	1.02	1.29
Retention (min)	6.57	11.2	11.5	13.3

Note: logP is the partition coefficient of the solute or the lipophilicity determination of the neutral form of ionizable compounds.

The proposed structure of peak 4 (t_r at 13.3 min)

The proposed peak structure was based on the ions observed in the MS/MS spectra (Fig 5.32) and the literature. The bond between C2-C3 becoming a double bond in the degradation product of aflatoxin B1 was reported in the previous studies where different treatments, (UV light 200-300 nm, *Ocimum basilicum*, *Cassia fistula* and *Corymbia citriodora* aqueous extracts treatment) were used to degrade aflatoxin B1 in water (Iram et al., 2015; Iram, Anjum, Iqbal, Ghaffar, Abbas, & Khan, 2016; Patras, Julakanti, Yannam, Bansode, Burns, & Vergne, 2017). The C2-C3 bond was attacked by the treatments while the remaining part of the aflatoxin B1 molecule was unchanged so it was assumed that aflatoxin M1 behaved the same way in this study because they both are similar in structure. Although the logP value of the proposed structure (1.29, t_r at 13.3 min) was unable to be used to compare its retention time with peaks 2 (1.33, t_r at 11.2 min) and peak 3 (1.02, t_r at 11.5 min) this was probably due to the retention of this compound in the column leading to an increase in its retention time. The difference between observed and theoretical mass was more than 5 ppm but the MS/MS fragments tended to match (Figs 5.33 and 5.34).

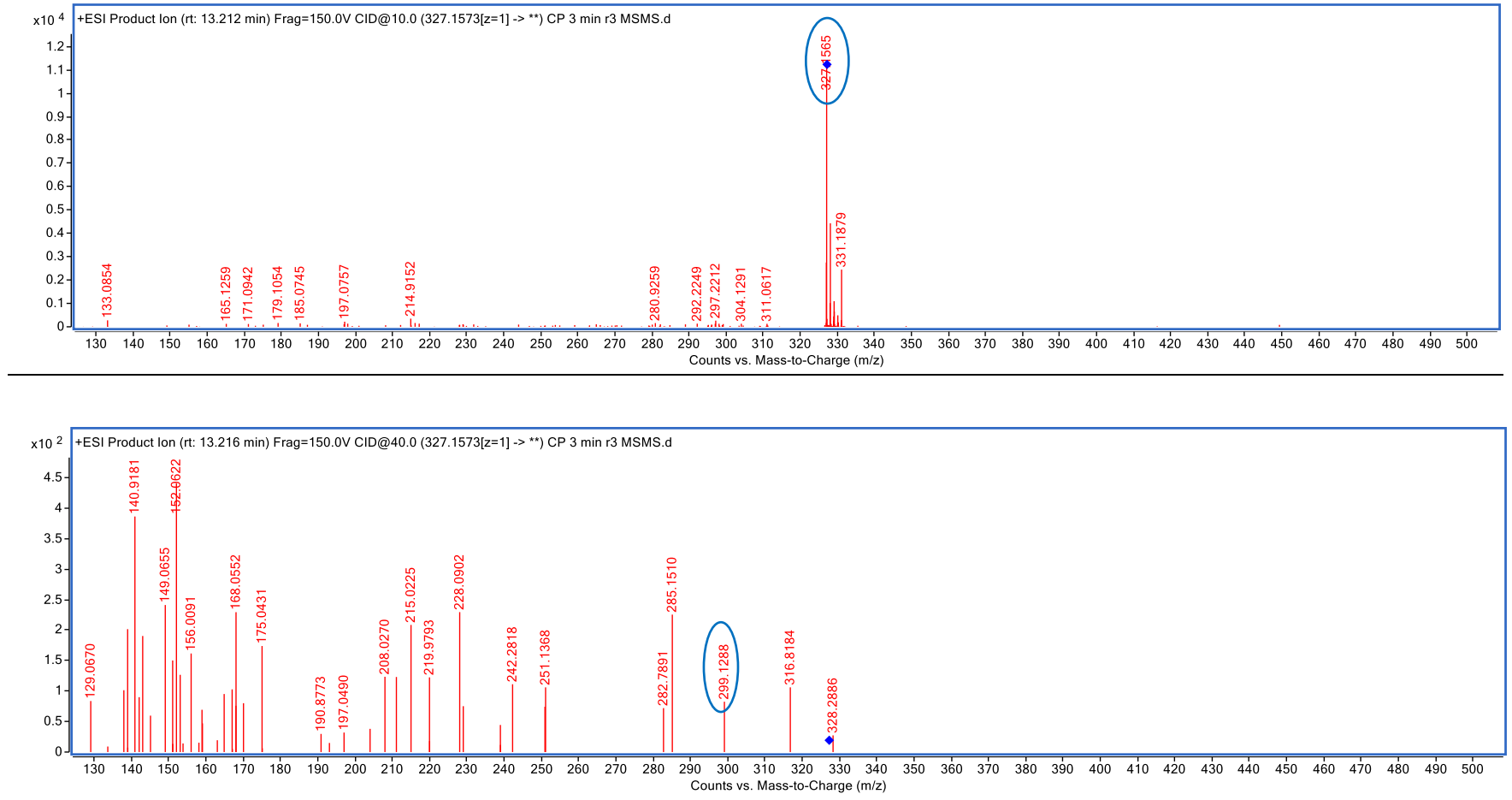


Fig 5. 32 MSMS fragmentation of ion 327.1565 at CID 10V and 40V.

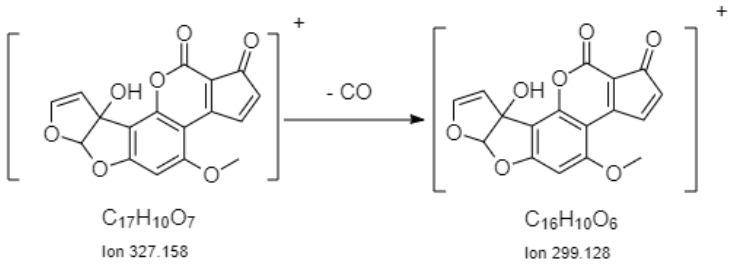


Fig 5. 33 Structure of the MSMS fragment of ion 327.1565.

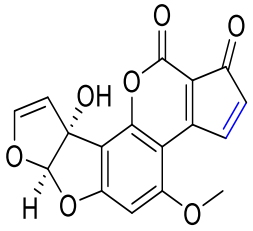


Fig 5. 34 Proposed structure of peak 4.

However, a second analysing method, such as proton nuclear magnetic resonance is required for confirmation of the proposed structure.

5.3.3 Conclusions – DBD Study

A small scale DBD (37kV) cold plasma system was built to explore the effect of different operating gases, various ratios of operating gases and the influence of milk components on AFM1 reduction. The degradation products of AFM1 after treatment were also determined. 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 these reactive species associated with the improvement in the efficacy of cold plasma on AFM1 reduction. In addition, the higher concentrations of the reactive species caused by the increase in the treatment time was associated with significant increases in AFM1 reduction with 70 and almost 100% reduction after 3 and 10 min treatment, respectively. The reduction of AFM1 tended to be less in the milk samples compared with water. The effects of the milk protein and the transparency of the solutions were probably responsible for the decrease in the efficacy of cold plasma on the reduction of milk samples. The structures of three different degradants of AFM1 after cold plasma treatment were elucidated although one of them needs further analysis (proton nuclear magnetic resonance) for confirmation.

5.4 Overall Chapter Summary and Conclusions

HVACP can degrade AFM1 in skim milk with more than 60% reduction during 20 min treatment which reduced it to below MRL (0.5 µg/L) from the average level of 1 µg/L. A slight change in pH and insignificant ($P > 0.05$) change in milk colour were observed after treatment. The factors that increased the concentration of reactive species induced in HVACP including voltage, volume of sample, treatment time and operating gases enhance the reduction of AMF1 in skim milk.

A DBD cold plasma system (cold plasma) with smaller capacity of the voltage generator compared with the HVACP and built in our lab, showed several challenges but produced reliable results once these were overcome and is recommended for future studies. This system was used to continue exploring the application of cold plasma in AFM1 reduction although the cold plasma chemistry might be varied due to the small

difference (low capacity of voltage generator and small gap between two electrodes) in the DPD system compared with the HVACP. Approximately 90% of AFM1 is degraded by cold plasma after 3 min treatment, however, the reduction of AFM1 in skim milk is much less which was probably due to either the effect of milk protein or the decrease in the exposure of AFM1 molecules and reactive species in cold plasma caused by the haziness of the milk samples. A significant ($P > 0.05$) reduction of AFM1 in skim milk was achieved by HVACP indicating that using the voltage generator with higher capacity may probably be able to overcome this obstacle by improving the efficacy of cold plasma on AFM1 reduction in milk.

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 believed to be due to the differences in the concentrations of the reactive species. The degradation products of AFM1 after cold plasma treatment were identified. 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 however, the toxicity tests are needed to confirm.

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CHAPTER 6. SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

6.1 Thesis overview

The aim of this study was to explore methods to degrade AFM1 in (skim) milk where traditional preventative approaches to AFM1 contamination are not practical.

Feed contaminated with aflatoxins consumed by dairy cattle leads to the contamination of milk with aflatoxin M1. Aflatoxin B1 and M1 have been classified as group one carcinogens, therefore, consuming food contaminated with these aflatoxins poses a risk to human health. The preferred approach to reduce the food safety issue with AFM1 in milk is to prevent cows consuming mouldy feed containing aflatoxin. However, this is not always practical, especially in warm humid countries. The alternative approach is to eliminate aflatoxin M1 from the milk.

Techniques have been proposed to reduce AFM1 in milk but many of these involve a long treatment time that may affect the milk quality, have low efficacy or are reversible (such as absorption methods). Two approaches that potentially overcome some of these limitations are narrow wavelength UVC and cold plasma treatment and these were investigated in this study.

UVC equipment at Massey University (School of Food and Advanced Technology) was used to investigate the reduction of AFM1 in skim milk and the effects of treatment time (min), depth of sample (mm), contamination level (μg/L), stirring, temperature and fat content in milk. The colour and pH of milk samples were measured to evaluate the effect of the treatment on these values. The identification of the degradation products of AFM1 after UVC treatment was also included.

Cold plasma treatment was investigated in a preliminary study carried out at Iowa State University (USA) by using High Voltage Atmospheric Cold Plasma (HVACP) to

explore its efficacy on the reduction of AFM1 in (skim) milk at different treatment times (5, 10 and 20 min), two different operating gases (air and MA65 - 65% O_2, 30% CO_2, 5% N_2) and at three voltages (60, 70 and 80 kV), using direct and indirect treatment. Other factors investigated included sample volume and different AFM1 contamination levels (0.1; 1 and 50 µg/L). The colour and the pH of the milk samples after treatment were monitored.

Another investigation focussed on operating gases, the influence of milk components and the degradation products following AFM1 treatment was done using a lower voltage cold plasma system built at Massey University. This cold plasma unit consisted of similar components to the HVACP including two aluminium electrodes and a sealed reaction bag filled by operating gas connecting to high voltage generator, however, the compacity of the high voltage generator was 40 kV instead of 120 kV of the HVACP system.

6.2 Key findings and recommendations corresponding to the objectives

6.2.1 Objective 1:

Investigation of the reduction of AFM1 in skim milk by UVC (254 nm) treatment and the influencing factors

- Findings

UVC treatment reduced AFM1 spiked into skim milk by approximately 50-70% during 20 min treatment with no change to the pH of the milk samples but with a noticeable change in colour. This demonstrated the feasibility of using UVC to reduce AFM1 in milk to below the MRL (0.5 µg/L) from an average commination concentration of 1 µg/L. Increasing the treatment time and reducing the depth of the

sample improved the degradation of AFM1 in skim milk believed to be due to an improvement in the penetration of UVC into milk samples. Shortening the treatment time reduced the change in milk colour. The initial level of AFM1 and the milkfat did not affect the efficacy of UVC on AFM1 degradation.

- Recommendations

A small volume of milk sample (5 mL) was used in this study for AFM1 reduction which showed the potential of the method. Higher volumes of milk need to be tested using commercial units designed for industrial milk processing such as the SurePure Turbulator (http://surepure.net/index.html) designed to increase the exposure of milk to UVC. This system has been used for microbial control in milk but not for AFM1 reduction. This system may avoid the change in colour seen in the present trial and permit further trials on the sensory of milk samples treated with UVC and explore any effect on the milk components, in particular protein and fat.

6.2.2 Objective 2:

Identification of the degradation products of AFM1 post UVC treatment

- Findings

The decrease in AFM1 (in solution) following UVC treatment was monitored, along with the degradation products, which reached maximum levels after 5 min of UVC treatment and were at undetectable level after 20 min. The invisibility of degradation products after 20 min UVC treatment could be explained by the change in the AFM1 to other compounds that were either too small to be detected or different in characteristics which cannot be detected by the methods used. The structure of the compound with maximum intensity at 5 min was elucidated. The double bond of the

furan ring in the AFM1 molecules was oxidized forming the degradation product under UVC treatment, which reduces the toxicity of AFM1. Because the furan ring structure of aflatoxin molecules is believed to be responsible for the toxicity of the toxin, once it's destroyed this will mitigate the toxicity.

- Recommendations

Toxicity tests including the Ames test, MTT test or brine shrimp are recommended to confirm the mitigation of toxicity of the degradant after treatment.

6.2.3 Objective 3:

Building the cold plasma induced unit

- Findings

A DBD cold plasma system was built from basic equipment. This is running reliably after many modifications which were required to meet NZ Electrical/Safety Standards and the study's objectives.

- Recommendations

The current unit is working at the limits of the capability of the system. Future work would benefit by a higher voltage generator, like the one used in the trials at Iowa State University.

6.2.4 Objective 4:

Investigation of the reduction of AFM1 in milk by cold plasma and influencing factors

6.2.4.1 Using HVACP

- Findings

HVACP treatment of skim milk resulted in a 60-80% in AFM1 reduction after 20 min treatment using air and MA65 as operating gases. HVACP can reduce the AFM1 in skim milk to below the MRL (0.5 µg/L) from the average of contamination level of 1 µg/L. The change in milk colour and change in pH were small and regarded as insignificant. The reduction of AFM1 in milk was improved by factors that were able to increase the concentration of reactive species (ozone and NOx) generated in cold plasma as well as the exposure to the reactive species. These were achieved by altering the voltage, volume of sample, treatment time, treatment mode and different operating gases. The contamination level of AFM1 in milk had an insignificant effect on the efficacy of HVACP in that the percentage reduction was similar at all levels of contamination.

- Recommendations

The volatile fatty acids of milk samples after HVACP treatment should be profiled to evaluate the effect of treatment on milk quality and sensory properties. In addition, an investigation into the effect of treatment on milk protein is also recommended.

6.2.4.2 Using the DBD cold plasma system

- Findings

Cold plasma induced by the DBD system reduced AFM1 in water by 50-90% after 3 min treatment with a range of gas mixtures from 5-20% of air, oxygen and nitrogen in helium. The concentration of the reactive species (ozone, hydrogen peroxide and nitrite) in cold plasma increased in response to the increase of nitrogen and oxygen in the gas mixture, which was associated with an improvement in the AFM1 reduction.

Increasing in the treatment time was shown to have a significant influence on AFM1 reduction by cold plasma and was associated with an increase in the concentrations of reactive species. The reduction of AFM1 in milk was low compared with AFM1 in water, which may be explained by the effect of milk protein or the opacity of the milk leading to the decrease in the exposure of AFM1 to the reactive species.

- Recommendation

A voltage generator with higher capacity can be used to overcome the effect of milk protein and the opacity of milk resulting in an increase in the efficacy of cold plasma on the reduction of AFM1 in milk.

6.2.5 Objective 5:

Identification of AFM1 degradation products by after cold plasma treatment

- Findings

The furan ring in the AFM1 molecules was destroyed by cold plasma treatment forming isomers as the degradation products with the – OH group at different positions in the carbon skeleton. The structure of these isomers was confirmed by MS/MS fragmentation for two degradation products. The damage of the furan ring in the structure of AFM1 is expected to decrease the toxicity of the sample after treatment as the furan ring is one of the main factors responsible for the toxicity of aflatoxins. The structure of the third degradation product was proposed with hydration happening at C2-C3 of the AFM1 molecule.

- Recommendations

A toxicity test is recommended to evaluate the toxicity of the AFM1 and degradation products after treatment. In addition, another analysis technique such as Proton nuclear

magnetic resonance is suggested to confirm the proposed structure of the third degradation product.

Appendices

Appendix A. Seven (7) milk samples contaminated at 0.05 µg/L were analysed on another day to evaluate within-lab repeatability (precision) by using a one-way analysis of variance (ANOVA).

No	12 - Dec	13 - Dec
1	0.0317	0.0446
2	0.0302	0.0365
3	0.0384	0.0412
4	0.0383	0.0356
5	0.0409	0.0367
6	0.0355	0.0343
7	0.0325	0.0414
Mean	0.0354	0.0386
repeatability limit (r), S_r, mg/kg	0.0040	0.0038
Repeatability, RSD_r, %	11.3526	9.8191
Repeatability limit (r), r, mg/kg	0.0112	0.0106

No	AFM1, µg/L	AFM1, µg/L
1	0.0317	0.0446
2	0.0302	0.0365
3	0.0384	0.0412
4	0.0383	0.0356
5	0.0409	0.0367
6	0.0355	0.0343
7	0.0325	0.0414
Sr	0.00	
SL	0.00	
SR	0.006	
RSD R	4.113041358	
R	0.017039508	

Anova: Single

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	7	0.24754	0.035362857	1.61172E-05
Column 2	7	0.27031	0.038615714	1.43771E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.70338E-05	1	3.70338E-05	2.42889956	0.145087034	4.747225336
Within Groups	0.000182966	12	1.52471E-05			
Total	0.000219999	13				
Total	151253.0034	11				

R: Reproducibility

RSD_R: Relative standard deviation for reproducibility

S_R: Standard deviation of reproducibility

RSD_r: Relative standard deviation for repeatability

r: Repeatability limit

S_r: Standard deviation of repeatability

S_L: the average of all test results and whose standard deviation

Appendix B

MS Instrument Parameters/ MS Source Parameters	
Acquisition mode	AutoMS2
Mass range MS1 (m/z)	100-3000
Mass range MSMS (m/z)	50-3000
Scan rate (spectra/sec)	4.00
MS Abs. threshold	200
MS/MS Abs. threshold	5
Use fixed collision energies (V)	20, 10, 40
Gas Temp (°C)	325
Gas Flow (L/min)	10
Nebulizer (psig)	35
Sheath Gas Temp (°C)	350
Sheath Gas Flow (L/min)	11
Capillary Voltage (VCap, V)	4000
Nozzle Voltage (V)	500
Fragmentor (V)	150
Skimmer1 (V)	65
OctopoleRFPeak	750

Appendix C

Auto MS/MS Preferred/Excluded ions

Mass	Delta Mass (ppm)	Charge	Type	Retention Time (min)	Delta Ret. Time (min)	Isolation Width	Collision Energy
443.3388	1000	1	Preferred	0.51	0.2	Medium (~4 amu)	30
448.2397	1000	1	Preferred	6.2	0.3	Medium (~4 amu)	20
553.4591	1000	1	Preferred	7.5	0.3	Medium (~4 amu)	45
1199.7733	1000	1	Preferred	6.4	0.3	Medium (~4 amu)	60
647.4588	1000	1	Preferred	8.7	0.3	Medium (~4 amu)	40
452.2655	1000	1	Preferred	6.2	0.3	Medium (~4 amu)	20
481.4	1000	1	Preferred	2.4	0.2	Medium (~4 amu)	25
1521.97148	100	1	Exclude	0		Medium (~4 amu)	
2421.91399	100	1	Exclude	0		Medium (~4 amu)	
322.048121	100	1	Exclude	0		Medium (~4 amu)	
121.050873	100	1	Exclude	0		Medium (~4 amu)	
1221.99064	100	1	Exclude	0		Medium (~4 amu)	
338.34	100	1	Exclude	0		Medium (~4 amu)	
381.3	100	1	Exclude	0		Medium (~4 amu)	
353.27	100	1	Exclude	0		Medium (~4 amu)	
282.28	100	1	Exclude	0		Medium (~4 amu)	
288.28	100	1	Exclude	0		Medium (~4 amu)	
329.16	100	1	Exclude	0		Medium (~4 amu)	

Appendix D

DRC 16 forms

DRC 16

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UNIVERSITY OF NEW ZEALAND

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Thu Nguyen
Name/title of Primary Supervisor:	Steve Flint
In which chapter is the manuscript /published work:	Chapter 2

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Nguyen, T., Flint, S., & Palmer, J. (2020). Control of aflatoxin M1 in milk by novel methods: A review. Food Chem, 311, 125984. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/31855773. doi: 10.1016/j.foodchem.2019.125984

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Thu Nguyen Digitally signed by Thu Nguyen Date: 2021.12.01 08:56:54 +1300
Date:	01-Dec-2021
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2021.12.01 09:05:05 +1300
Date:	1-Dec-2021

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Candidate's Signature:	Thu Nguyen Digitally signed by Thu Nguyen Date: 2021.12.01 08:56:54 +13'00'
Date:	01-Dec-2021
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2021.12.02 13:33:01 +13'00'
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○ The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Thu Nguyen Digitally signed by Thu Nguyen Date: 2021.12.01 08:56:54 +13'00'
Date:	01-Dec-2021
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2021.12.02 13:54:36 +13'00'
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