HEAT STABILITY OF OCHRATOXIN A

A Thesis

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

Ochratoxin A (OTA) is a toxic and potentially carcinogenic fungal secondary metabolite found in a wide variety of agriculture commodities worldwide. It is stable under most food processing conditions. However, interaction of factors like temperature, moisture, pressure, and presence of food components affect its stability. Reduction of OTA was measured at different temperatures for variable heating times in aqueous buffer solutions. It was found that OTA stability was dependent on temperature, processing time, and pH. Greater than 90% reduction was achieved after 60 min of heating at 200°C in pH 7 and 10 while only 60% was lost in pH 4. The extrusion study examined effects of moisture, temperature, screw speed, and die size on OTA reduction. It showed that die size and screw speed had significant effect on reduction. The total reduction ranged 0-28% with maximum being achieved at 180°C, 250 rpm, 3mm and 20% moisture content.

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DEDICATION

This work is dedicated to my dad Guru Prasad Dahal and mom Kalyan Niraula Dahal, for their invaluable love and support to me. To my sisters, Shrijana and Sapana for always being there for me in my highs and lows. Without them this work would have not been possible.

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CHAPTER 1: Introduction

Mycotoxins are secondary metabolites of fungi, capable of having toxic effects in human and animals. The majority of the mycotoxins known are produced by some species of fungal genera: *Aspergillus, Pencillium,* and *Fusarium.* Some of the important mycotoxins are aflatoxins (B_1, B_2, G_1, and G_2), ochratoxin A (OTA), patulin, fumonisins (B_1 and B_2), zearalenone (ZEN), T-2 and HT- 2 toxins and deoxynivalenol (DON). Mycotoxins are produced in crops during their growth, harvest, drying and storage. The production of toxin depends on various factors such as moisture content, temperature, storage period, contamination rate, presence of insect, oxygen concentration, damage during harvesting, processing, and transportation (Lazzari, 1997; Scussel, 2002; Santos, 2002; Garcia *et al*., 2003; Scudamore, 2005). They are expected to be found in heterogeneous distribution in the same crop varying from their production year, places of cultivation or processing they undergo. As mycotoxins may not be removed completely during the processing, it is critical to prevent contamination in the field and storage by preventing the growth of the fungus. Mycotoxins have been a serious concern for trade of food commodities because of possible economic losses and potential health hazards.

Ochratoxin A (OTA) is one of the most important mycotoxins. It has a great public health and agro economic significance, due to the nephrotoxic, genotoxic, neurotoxic, immunotoxic, embryotoxic and teratogenic effects and its suspected carcinogenicity (JECFA, 2008). It was discovered in 1965 in South Africa. OTA was first isolated as a toxic metabolite of *Aspergillus ochraceus* from corn meal artificially inoculated with the fungus (Van der Merwe *et al*., 1965). In 1969, naturally occurring OTA was isolated from a commercial corn sample in the United States (Shotwell *et al*., 1969). Later, OTA was

recognized as a secondary metabolite of several *Aspergillus and Penicillium spp.* characterized by widespread occurrence and different behavior depending on the ecological niches, the products affected and the environment (Duarte *et al*., 2010). OTA has been documented as a global contaminant of a wide variety of commodities and staple food including cereal products, pulses, coffee, beer, grape juice, dried vine fruits and wine as well as cacao products, nuts and spices from all over the world (EFSA, 2006; JEFCA 47, 2001; Abrunhosa *et al.*, 2010; Scudamore *et al.*, 2003).

Chemical property

OTA, 7-(L-β-phenylalaninylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3, 4- dihydro-3R-methylisocoumarin, is a secondary metabolite produced by some toxigenic fungi (Xiao *et al*., 1995). It is a colorless crystalline compound having a melting point of 169ºC when crystallized with xylene and 90ºC when crystallized from benzene (Kuiper- Goodman and Scott, 1989). It is slightly soluble in water and completely soluble in organic polar solvents (Valenta, 1988). OTA is considered unstable to air and light (US department of Health and Human services, 2005) but its thermal stability is highly dependent on the matrix it is bound with. Raters and Mattissek (2008) found OTA to be stable up to 180ºC in some food matrices. Also, Tsubouchi *et al*. (1987) stated that, mode of contamination influences strength of heat stability in OTA.

There are three types of ochratoxin: ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC). OTA consists of a dihydro-isocoumarin moiety, a pentaketide synthesized through the acetate malonate pathway (Ferreira and Pitout, 1969), linked to phenylalanine through a carbonyl group (Steyn and Holzapfel, 1970) and a chlorine incorporated into the isocoumarin portion of the molecule (Wei *et al*., 1971) (Figure 1.1).

OTB is the de-chloro analogue (OTβ) of OTA and OTC is the ethyl ester of OTA. OTC is least common and least toxic of the three (Stormer, 1992). 4-R-hydroxyochratoxin A and 4-S-hydroxyochratoxin A are the hydroxylated form of OTA. The OTα and OTβ are products of hydrolysis of peptide bond of OTA and OTB, respectively, lacking phenylalanine moiety. OTA and its analogs such as ethylamide, D- phenylalanine, decarboxylated, O-methyl ether and methyl ester forms of OTA were synthesized and their crystalline structure was studied (Xiao *et al*., 1995) (Figure 1.1 and Table 1.1).

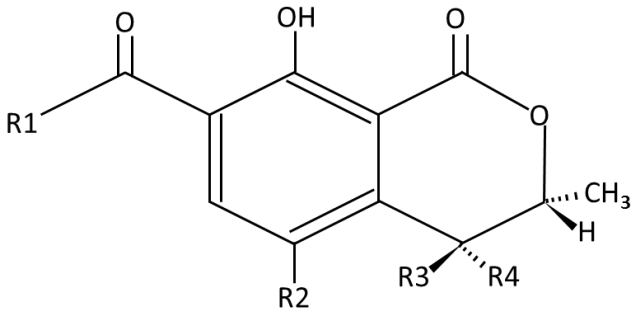

Figure 1.1. Chemical structures of ochratoxins and their major derivatives.

Table 1.1. R groups in the structure of ochratoxins (adapted from Li *et al*. 1997).

Common name	Abbreviation	R1	R2	R3	R4
Ochratoxin A	OTA	Phenylalanyl	Cl	H	H
Ochratoxin B	OTB	Phenylalanyl	H	H	H
Ochratoxin C	OTC	Phenylalanyl, ethyl ester	Cl	H	H
4-R-hydroxyochratoxin A	4-R-OTA-OH	Phenylalanyl	Cl	H	OH
4-S-hydroxyochratoxin A	4-S-OTA-OH	Phenylalanyl	Cl	OH	H
Ochratoxin α	OTα	OH	Cl	H	H
Ochratoxin β	OTβ	OH	H	H	H

Biosynthesis of OTA

Several studies have suggested about biosynthesis of OTA. The pathway is still unclear and involves some crucial steps whose order of the reactions is not yet well defined. Huff and Hamilton (1979) suggested a possible pathway for the biosynthesis of OTA in. First, mellein synthesis occurs by polyketide synthase, followed by carboxylation to form OTβ and transformation through a chloperoxidase to produce OTα. The second precursor, phenylalanine, is synthesized through the shikimic acid pathway. It undergoes esterification to produce phenylalanine ethyl ester. In the third step, linkage of these activated precursors takes place using synthetase and generates OTC, then go through de-esterification to produce OTA.

Harris and Mantle (2001) proposed a different pathway, in which mellein and OTC play no role in OTA biosynthesis, but leading from OTβ to OTα and then to OTA. Here, phenylalanine and OTα go through peptide synthase without phenylalanine ethyl ester and OTC intermediates. They also suggested alternative pathway where OTβ synthesizes OTB and follow chlorination to produce OTA. OTB may also be formed due to low chlorine concentration or to some extent by dechlorination of OTA.

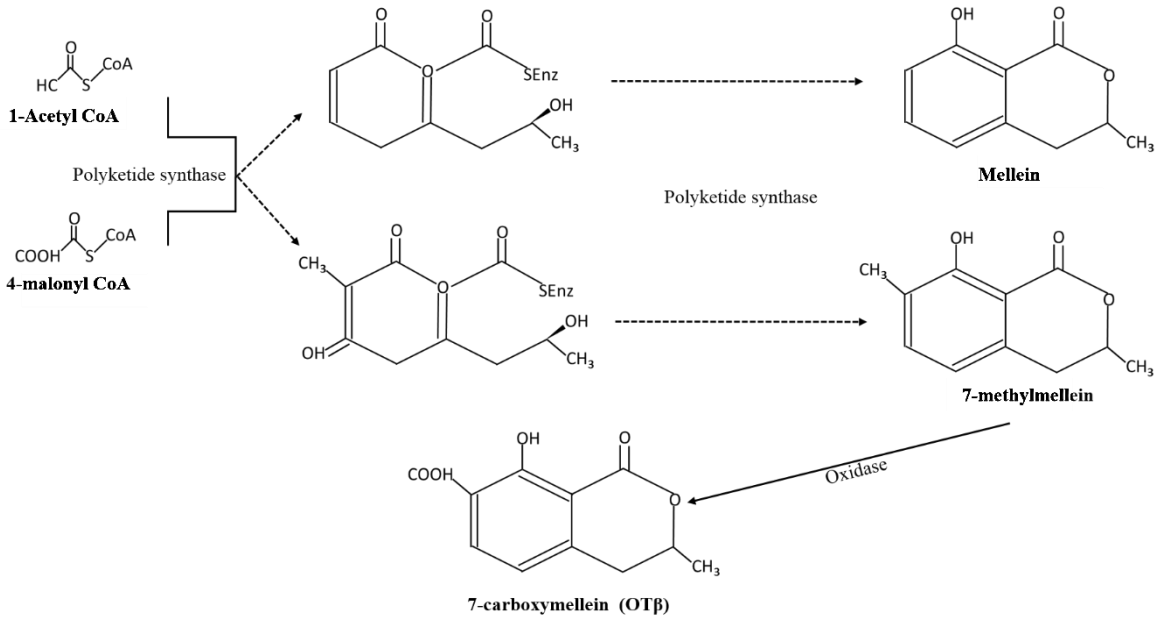

Figure 1.2. Synthesis of mellein precursor for ochratoxin A biosynthesis. Based on Huff and Hamilton (1979) and Harris and Mantle (2001).

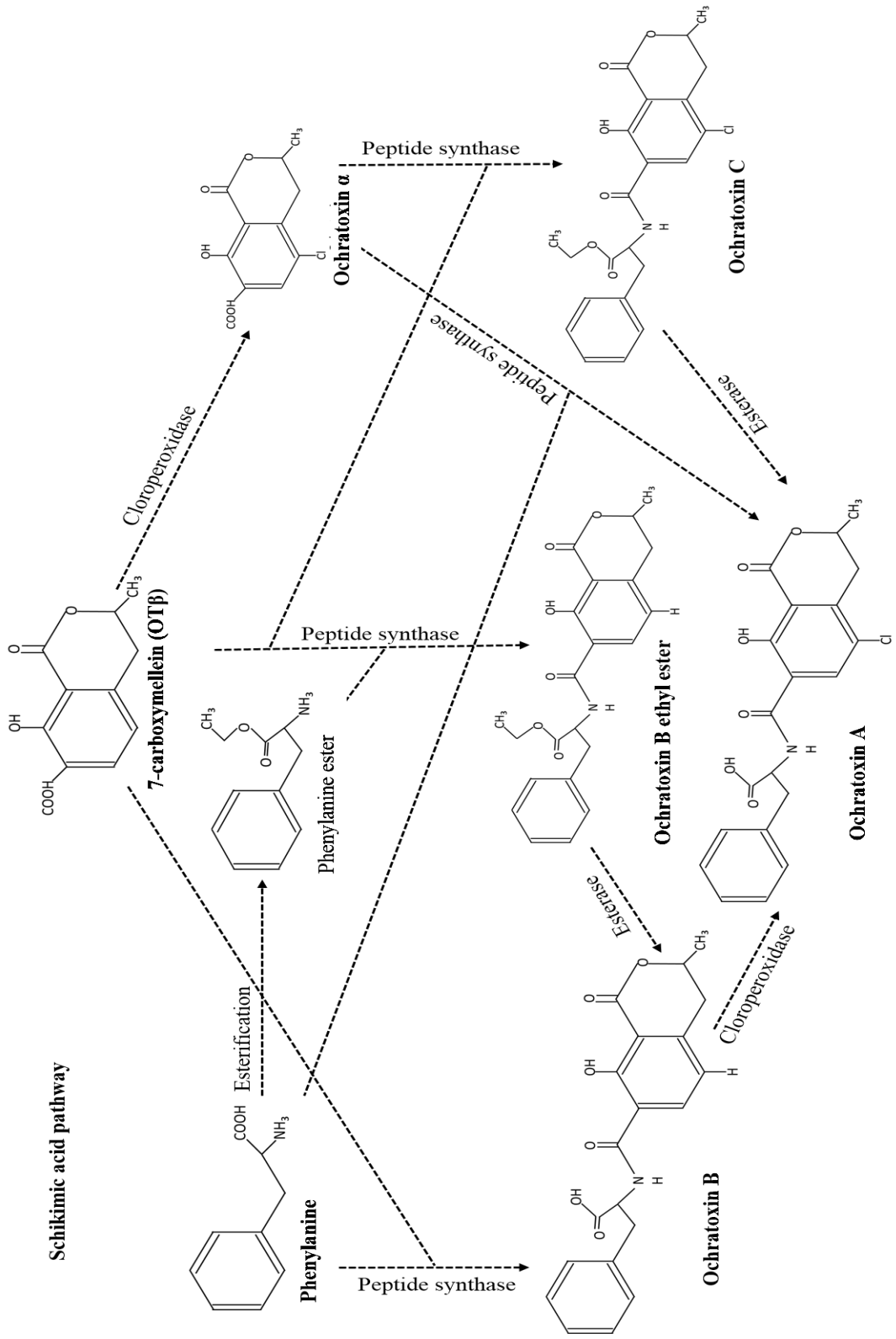

Figure 1.3. Hypothesis of ochratoxin A biosynthesis pathway based on Huff and Hamilton (1979) and Harris and Mantle (2001).

Toxicity

Toxicity syndromes caused by the intake of mycotoxins are known as mycotoxicoses. The general pathway for ingestion in human and animals are through ingestion of contaminated food and feed from vegetal or animal origin. Partly, the exposure may be through the inhalation of contaminated dust. Until now, human mycotoxicoses have not been clearly defined. However, most animal mycotoxicoses have been experimentally proved (Smith *et al.*, 1995). OTA is classified as a possible human carcinogen (group 2B) by International Agency for Research on Cancer (IARC, 1993; Stormer, 1992; CAC, 2000), and OTA has been reported to have nephrotoxic, immunosuppressive, carcinogenic and teratogenic properties (Miraglia and Brera, 2002). Animal studies have shown oral LD_{50} values of 20mg/kg for young rats and 3.6 mg/kg for duck chicks (Pitt, 2000). Among all the organs, kidneys and liver are most effected by OTA. In animals, carrier mediated removal of toxin from blood reduces burden on organs however in human the elimination is slow, providing more time for damage to occur. This increases burden on the eliminating organs. Hence, specific toxic effects such as chronic nephropathy conditions (Pfohl-Leszkowicz and Manderville, 2007) and Balkan Endemic Nephropathy (BEN) (O'Brien and Dietrich, 2005) have been observed. Furthermore, an increased incidence of urinary tract tumors has also been reported due to OTA toxicity (Fink and Gremmels, 2005).

OTA also shows toxic effects at cellular level and effects several biological functions. It causes inhibition of ATP synthesis and enhances lipid peroxidation (Xiao *et al*., 1995). OTA inhibits phenylalanine-tRNA synthetase and thereby reduce protein synthesis (Creppy *et al*., 1980 and 1984). Other enzymes such as phosphoenolpyruvate carboxykinase (Meisner *et al*., 1983; Meisner and Krogh, 1986), succinate-cytochrome C

reductase and succinate dehydrogenase (Wei *et al.*, 1985) also get affected by exposure to OTA. OTA can also effect in the process of formation of DNA adducts. It can cause apoptosis and interference with cytoskeleton and inhibit mitochondrial activity (Stormer 1992; O'Brien and Dietrich, 2005). The inhibition of enzymes associated with proteins, DNA, and RNA synthesis, increased aneuploidy (an abnormal number of chromosomes) and subsequent tumor formation may be a possible pathway in development of carcinogenicity in affected individuals (Mossesso *et al.*, 2008).

Occurrence in food commodities

OTA is found in many plant based raw materials and processed food products (Pohland *et al*., 1992). It has been reported in cereals and cereal derived products (Duarte *et al*., 2009), corn (Magnoli *et al*., 2007), coffee (Lombaert *et al*., 2002), cocoa and cocoa products (Copetti *et al*., 2011), figs (Iamanaka *et al*., 2005), chili peppers (Thirumala-Devi *et al.*, 2000), liquorice (Majerus *et al*., 2000), grape juice and dried vine fruit (Majerus *et al*., 2001).

Studies by various authors indicated the occurrence of OTA in different food commodities. Recent survey in United States showed a very high incidence of OTA contamination in breakfast cereals involving oats, rice and wheat in the range of 0.10 and 7.43 µg/kg (Lee and Ryu, 2014). Similarly, study of cereal-based baby foods, breakfast cereals, and beers products in Spain showed mean OTA contamination ranging between 0.044-0.265 µg/kg (Araguas *et al*., 2005). In another study in Turkey, dried figs were found contaminated with detectable levels of OTA in the range of 0.12-15.31 µg/kg (Karbancioglu-Glurer and Heperkan, 2008). Chili samples including chili sauce, crushed chilli and powdered chili purchased from an open market in Pakistan were also found to be

contaminated with OTA (Iqbal *et al*., 2013). Another study in Brazil reported contamination of grape juice and with OTA (Rosa *et al*., 2003). Similarly, high level of contamination in concentrated feed for meat poultry was found in Venezuela (Figueroa *et al*., 2009).

Table 1.2. Occurrence of ochratoxin A in different food items.

Group	**Type of food involved**	**Levels(µg/kg)**	**References**
Cereals	Barley, wheat, maize, oat and their by-products	1-4	Speijers and Van egmond, 1993; Trucksess *et al*., 1999
	Corn	<1	Magnoli *et al*., 2006
	Breakfast cereals	0.10-7.43	Lee and Ryu, 2015
	Infant cereal	0.122-0.374	Kabak, 2009
Coffee	Green beans	0.20- 20.30	Vanesa and Ana, 2003
	Instant coffee	0.1-13.66	Lombaert *et al*., 2002
	Roasted coffee and brewed coffee	0.5-11.9	Tozlovanu and Pfohl-Leszkowicz A., 2010; Drunday and Pacin, 2013
Dried foods	Dried figs and black s ultana	>5	Iamanaka *et al.*, 2005
	Dried vine fruits	0.23-53.67	Magnoli *et al.*, 2003; MacDonald *et al.*, 1999
	Almonds, peanuts, pistachios	10-89	Zaied *et al.*, 2010
Alcohol drinks	Beer, wine	0.15-1.44	Araguas *et al.*, 2005; Bacaloni *et al.*, 2005

Spices	Black pepper, chili powder, cumin	0.46-98.2	Fatih and Bulent, 2012
	Red pepper	2.1-66.2	Fazekasa *et al.*, 2005
	Caraway, coriander, curcuma, black pepper, red pepper	203-290	Zaied *et al.*, 2010
Animal products	Meats, liver, sausages, cheeses and fermented meats	0.05-2.71	Jorgensen, 2005; Markov *et al.* 2013

Effect of processing on OTA

OTA is considered to be a relatively heat stable molecule. It is able to survive most food processing conditions to some extent. The processing may involve cooking, boiling, baking, frying, roasting, extruding, or fermenting. Presence and absence of water during processing has also found to show significant difference in reduction of OTA. Relatively few studies have been carried out on the reduction of OTA during processing. Parameters such as temperature, pH, pressure, and presence of other ingredients affect the stability of OTA. OTA is considered to be stable when heated under neutral conditions (Scudamore *et al.*, 2005). Under certain conditions of high temperature and acid or alkaline conditions or in the presence of enzymes, some chemical decomposition of OTA can occur. OTA in neutral condition is relatively stable during boiling processes (Jackson and Ciegler, 1978, Madsen *et al*., 1983). OTA is only partly destroyed during cooking and bread making process. Baking has been reported to reduce the toxin content by a low amount only. Physical treatment of raw food products has shown to bring some reduction but they are

basically redistributions of OTA rather than true loss in the final product (Bullerman and Bianchini, 2007).

There are conflicting reports about the heat stability of the OTA (Table 1.3). Moisture had an effect on OTA destruction in wheat; there was no change after 40-160 min of dry heat treatment at 100°C, whereas 50% reduction was obtained at same temperature after 120 min of humid heat treatment (Boudra *et al*., 1995). Similarly, autoclaving oatmeal with 50% water gave a 74% reduction of OTA, while autoclaving dry oatmeal or rice cereal gave greater losses of 86-87.5% (Trenk *et al*., 1971). Pressure-cooking beans at 115°C for 45 min in water, resulted in up to 84% reduction of OTA (Milanez and Leitao, 1996). Complete loss of OTA was observed in green coffee naturally contaminated with 0.4 μg/kg OTA while the green coffee artificially contaminated with 53.2 μg/kg OTA showed maximum reduction of 82.9% after roasting at 200 to 250°C (Suarez-Quiroz *et al.*, 2005). On the other hand, Perez de Obanos *et al*. (2005) reported 13-93% reduction of ochratoxin during roasting of coffee beans. Corn flaking has also been shown to reduce OTA concentration to some extent in the breakfast cereals (Aish *et al*., 2004). The combination of temperature, moisture content, screw speed, and residence time on the stability of OTA has been studied during extrusion processing but the maximum loss observed was only 40% of the initial concentration (Scudamore *et al*., 2004). It should be noted that disappearance of OTA does not necessarily mean absence or decrease in toxicity. The decomposition products may be formed and just as toxic as the parent molecule (Suarez-Quiroz *et al*., 2005).

Table 1.3. Effect of various processing methods on ochratoxin A.

Processing method	**Matrix**	**Temper-ature (°C)**	**Time (min)**	**% Reduction**	**References**
Cooking	Wheat	100	30	6	El-Banna and Scott, 1984
Roasting	Coffee	200	10-20	0-12	Tsubouchi *et al.*, 1987
Dry heating	Wheat	100	160	N/A	Boudra *et al,* 1995
Pressure cooking	Beans	115	45	84	Trenk *et al*., 1971
Wet heating	Wheat	100	120	50	Boudra *et al,* 1995
Wet autoclaving	Oatmeal	121	15	74	Milanez and Leitao, 1996
Dry autoclaving	Oatmeal/ rice cereal	121	15	86-87.5	Trenk *et al*., 1971
Roasting	Coffee	175	6	70	Santina *et al*., 2003
Roasting	Coffee	150	2.5	small	Perez *et al.*, 2005
Extrusion	Wheatmeal	119-136	30-40	11-39	Scudamore *et al.,* 2004
Heating	Liquorice	150	60	N/A	Ari no *et al*., 2007
Noodles	Wheat	100	7	13-24	Peng *et al.*, 2015
Baking	Wheat	200	40	64	Vidal *et al.,* 2015
Baking	Wheat	140	40	21	Vidal *et al.,* 2015

Regulatory limits for OTA

A number of countries particularly in Europe have established regulatory levels to monitor and control levels of OTA in various food commodities. Several scientific and socio-economic factors influence the establishment of limits and regulations for OTA including: availability of toxicological data, availability of data on the occurrence of mycotoxins in various commodities, knowledge of the distribution of mycotoxin concentrations within a lot, availability of analytical methods, legislation in countries with which trade contacts exist, and need for sufficient food supply. Risk assessment is an important factor to evaluate the probability of occurrence of possible hazards and provides basis for establishment of regulations (FAO, 2003).

There are many countries which do not imply regulations on OTA contaminations including United States. Recently, many countries have start to set up regulations but might not include all the food products contaminated with OTA. The FAO/WHO Joint Expert Committee on Food Additives (JEFCA) was first to establish a provisional tolerable daily intake (TDI) of 14 ng/kg body weight in 1995 (JECFA, 1995). Two years later, by 1997, eight countries established regulations for OTA in foods ranging from 1 to 50 ng/g (FAO, 1997). The Commission of European Communities (CEC), after adopting the scientific opinion of the European Food Safety Authority (EFSA), established a tolerable weekly intake (TWI) of OTA as 120 ng/kg bw in regulation 1881/2006 (CEC, 2006). Some of the regulatory limits that are set by European Union (EU) are listed below (Table 1.4) (CEC, 2006; Heydt *et al*., 2011).

Table 1.4. Regulatory limits set by European Union on ochratoxin A in different food commodities.

Food Matrix	**Limit (µg/kg)**
Unprocessed cereals	5.0
All products derived from unprocessed cereals	3.0
Dried vine fruit	10.0
Roasted coffee beans and ground coffee	5.0
Instant coffee	10.0
Wine	2.0
Grape Juice	2.0
Infant cereal based foods	0.5
Infant food for medical purposes	0.5
Cocoa and cocoa products	2.0

Detection of OTA

OTA in food can be detected using several analytical measurements. Validation of any analytical procedure in regards to their extraction efficiency, precision, sensitivity, specificity and robustness needs to be determined before its use. Recoveries range between 70%-120%.is considered ideal for extraction efficiency. Sensitivity or the lowest dilution of mycotoxin that can be detected by the method, repeatability, and reproducibility of method also determines selection of method for OTA analysis (Boque *et al.*, 2002).

Table 1.5. Major detection methods for OTA (Lee and Ryu, 2015).

Detection method	Selectivity and sensitivity	Advantages
High-performance liquid chromatography (HPLC)	High	Low detection limit, possible quantification of multiple mycotoxin in single analysis.
Liquid chromatography coupled with mass spectrometry (LC/MS)	Very high	Very low detection limit, possible to quantify multiple mycotoxins in a single analysis, identify and characterize mycotoxin metabolites or degradation products.
Enzyme-linked immunosorbent assay (ELISA)	Good	Rapid analysis, not requiring clean-up or concentration step, ease of application, relatively low cost, portable, not requiring complex equipment and special training.

The most popular method used for detection of OTA concentrations in various samples are chromatographic techniques. Being a derivative of isocoumarinic acid linked to L-phenylalanine, OTA displays optical activity and fluorescence properties. This makes OTA highly favorable for chromatographic analysis (Dall'Asta *et al.*, 2004). Quantification and identification of OTA through high performance liquid chromatography (HPLC) linked with fluorescence (FLD) or diode array detector (DAD) and liquid chromatography - mass spectrometry (LC-MS/MS) have been reported. They have high accuracy and reproducibility (Monaci and Palmisano, 2004; Turner *et al*., 2009; Hayat et al., 2012). Alternative biochemical methods such as ELISA, fluorescence immunoassays, electrochemical immune

sensors, and immune tests can also be used for OTA detection. They usually require no sample clean-up or simple extraction process such as filtration and dilution. These methods can be used for parallel analysis of multiple samples allowing rapid screening (Turner *et al*., 2009). Besides these, aptamer based method such as colorimetric assays, fluorescence assays, electrochemical aptasensors, luminescent aptasensors, and strip assays have been shown to be successful as analytical tool for OTA quantification in food (Mairal *et al.*, 2007). Some of the widely used detection methods and their advantages as listed in Table 1.5.

Objectives

The objectives of this study were; (i) to determine thermal stability of OTA in an aqueous buffered model system at acidic, neutral, and basic pH levels and (ii) to optimize processing conditions during extrusion to obtain maximum possible reduction of OTA using spiked oat flakes. The central hypothesis of the study was that the concentration of OTA will be significantly reduced by high heating temperature and extrusion processing. Since, different food processing methods are reported to give variable effects on OTA reduction, the heating of pure OTA in buffered model system is expected to provide clear interaction between processing time and temperature on reduction without interference by any food matrixes. Similarly, the goal of extrusion study was to maximize the reduction of OTA. Extrusion processing utilizes higher pressures, temperatures and shear forces. The optimization of process variables was supposed to provide greater OTA reductions.

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CHAPTER 2: Heat stability of ochratoxin A in an aqueous buffered model system

Abstract

Ochratoxin A (OTA) represents one of the most widespread mycotoxins in agricultural commodities in the world and considered as a possible human carcinogen with its potent nephrotoxicity. OTA is stable under most food processing conditions, however, higher temperature treatment may reduce OTA content in foods. Since OTA can be found in processed products destined for both human and animal consumption, factors affecting its stability or reduction during thermal processes are investigated. Reduction of OTA was measured during variable heating times (up to 60 min) at different temperatures (100, 125, 150, 175, and 200°C) in aqueous buffer solutions at different pH values (pH 4, 7, and 10). Quantification of OTA was carried out with high performance liquid chromatography-fluorescence detection (HPLC-FLD). The results showed that the rate and extent of OTA reduction or decomposition were dependent on pH, processing time and temperature; greater than 90% of OTA reductions were achieved at 200°C for all treatments except pH 4. After processing under alkaline condition (pH 10) at 100°C for 60 min, about 50% of OTA was lost, while 60 min heating under neutral and acidic conditions at 100°C did not show significant reduction of OTA.

Keywords: Ochratoxin A (OTA); heat stability; pH; HPLC-FLD.

Introduction

Ochratoxins are fungal secondary metabolites produced by several species of *Aspergillus* and *Penicillium* (Stormer, 1992). It consists of a dihydroisocoumarin moiety linked through its 7-carboxyl group by its amino bond to L-phenylalanine (Studer-Rohr, 1995). The OTA is chlorinated, which is unusual for natural products (Figure 3.1) (Stormer, 1992). OTA has been a significant public health concern due to its range of toxicity such as immunotoxic (Alvarez, 2004), teratogenic (Vesela, 1983), nephrotoxic (Luhe, 2003), neurotoxic (Schaaf, 2002), and carcinogenic (Lioi, 2004) effects. Because of its widespread occurrence on a large variety of agricultural commodities and the potential health risks, mainly toward humans, OTA has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993). Given the known human exposure and the abundance of toxicological data from animal studies, the European Union Scientific Committee has recommended the OTA levels below to 5 ng/kg of body weight per day (Sweeney *et al.*, 2000 and Hayat *et al.*, 2012).

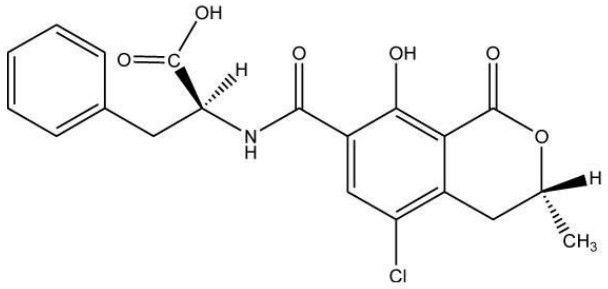

Figure 2.1. Chemical structure of ochratoxin A (OTA).

OTA is a naturally occurring mycotoxin and is found in a wide variety of agricultural commodities worldwide ranging from cereal grains like barley, corn, wheat, rye, and oats to dried fruits to wine and green coffee beans (Leong *et al.*, 2004; Pitt *et al.*, 1997; Gallaz and

Staddler, 1976; Petzinger and Ziegler, 1995). It has been frequently reported in other plant products such as beans, nuts and spices (Nyugen and Ryu, 2014). Recent study has shown high prevalence of OTA in thermally processed breakfast cereals items (Lee and Ryu, 2015). It has also been detected in roasted coffee beans (Micco *et al.,* 1989; Tsubouchi *et al.,* 1987).

The OTA has a melting point of 169°C and is almost stable when heated to a temperature of 200°C (Redgwell *et al*., 2002*;* Scott, 1984). It is considered to be a relatively stable compound when heated under neutral conditions but under certain conditions of high temperature and acid or alkaline conditions or in the presence of enzymes, some chemical decomposition can occur (Scudamore, 2005; Categnagro *et al*., 2006). The particularity of OTA is due to its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule.

Different food processing techniques have been used to study the thermal stability of OTA and the results are conflicting. Moisture had an effect on OTA destruction in wheat; there was no change after 40-160 min of dry heat treatment at 100°C, whereas 50% reduction was obtained at same temperature after 120 min of humid heat treatment (Boudra *et al.,* 1995). Similarly, autoclaving oatmeal with 50% water gave a 74% reduction of OTA, while autoclaving dry oatmeal or rice cereal gave greater losses of 86-88% (Trenk *et al.,* 1971). Pressure-cooking beans at 115°C for 45 min in water, resulted in up to 84% reduction of OTA (Milanez and Leitao, 1996). Complete loss of OTA was observed in green coffee naturally contaminated with 0.4 µg/kg OTA while the green coffee artificially contaminated with 53.2 µg/kg OTA showed maximum reduction of 83% after roasting at 200 to 250°C.

OTA may be found in food products destined for both human and animal consumption. Therefore, better understanding of its stability in thermal processes is necessary. The objective of this study was to determine the thermal stability of OTA in an aqueous buffered model system at acidic, neutral, and basic pH levels. This is the first systematic study of the thermal stability of OTA in the absence of a food matrix. Therefore, possibilities such as binding with food matrixes or analytical problems were eliminated to estimate the true losses of OTA during the heat processing.

Materials and methods

Chemicals and reagents

Teorell and Stenhagen's citrate-phosphate-borate buffer was prepared and adjusted to the three-pH levels of 4, 7, and 10 (Sober, 1968). One batch of buffer solution was made for each pH level and replication. Stock solution of OTA was prepared by dissolving purified OTA (Sigma Aldrich Co., St. Louis, MO, USA) in methanol and then adding the mixture to buffer solution to achieve a final concentration of 100 ng/ml. HPLC grade acetic acid (99.5%) (EMD Chemicals Ind., Darmstadt, Germany), methanol, water, and acetonitrile (Macron Chemicals, Center Valley, PA, USA) were used for analysis. All reagents were of analytical grade.

Processing of the model system

A 1 ml reaction vial (Wheaton, Millville, NJ, USA) filled with 1 ml of the Teorell and Stenhagen's citrate-phosphate-borate buffer solution containing OTA was placed in an aluminum heating block and heated to processing temperatures of 100, 125, 150, 175 and 200°C using an electric heating mantle. The actual temperature of the reaction mixture during the process was monitored by the thermometer placed on the heating block. The

aluminum heating block was wrapped with fiberglass insulation tape (Fisher Scientific Company LLC., Pittsburgh, PA, USA) to prevent heat loss during processing. The stirring speed was 100 rpm in all the experiments. Samples were collected at 10 min intervals for a total period of 60 min after the desired processing temperature was reached. After the heat treatment, the sample vials were immediately placed in a water bath maintained at 50°C for 5 min and then cooled to room temperature for 5 min. The reaction mixture was transferred to 2 mL Agilent vials (Agilent technologies, Palo Alto, CA, USA) and then stored at 10°C until analysis.

Detection of OTA by HPLC-FLD

The concentrations of OTA in the buffer solution before and after processing were determined by high-performance liquid chromatography (HPLC) as previously described (Ryu and Nyugen, 2013). Samples were injected directly into the HPLC without further cleanup.

The HPLC system (Agilent 1260 Infinity HPLC system series, Palo Alto, CA, USA) was equipped with a quaternary pump (DEAB 705541), auto sampler (DEACC 14016), port column switching valves (DEACN 15339), and a fluorescence detector (DEABO 02622). The chromatographic separation was performed on a reversed-phase Hypersil GOLD C18 column (3×100 mm, particle size 1.9 μm, Thermo Scientific, Waltham, MA, USA) with the isocratic elution using 50% acetonitrile and 50% water containing acetic acid 1% at flow rate of 0.4 ml/min. The column temperature was kept at room temperature (25°C). OTA was detected at 334 nm and 460 nm wavelengths for excitation and emission, respectively. Injection volume was 10 μl for the analysis

Calculation of kinetics

The rate of destruction of OTA (R_Z) was expressed by the equation:

$R_Z = d(C_R)/dt = -kC_R$

where, C_R is the remaining concentration of OTA (μg/ml) at time t (min) and k is the reaction rate constant (min^{-1}). An equation to describe the first-order reaction was obtained by integration,

$ln\ (C_R) = ln\ (C_0) - kt$

where, C_0 and C_R refer to the concentration (μg/ml) of initial and the remaining OTA after processing time t (min), respectively. The initial concentration (C_0) was taken to be the concentration of OTA when the desired processing temperature was reached. Processing time was plotted with respect to the $ln\ (C_R)$, and the reaction constant was calculated from the slope of the rate law equation. The half-life was calculated from the rate law equation by allowing C_R to equal 0.5 C_0.

Statistical analysis

The entire experiment was replicated three times, and the data obtained from this experiment were analyzed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, USA). The differences among the groups were evaluated by the one-way analysis of variance and Tukey's multiple range tests. All data are reported as means ± standard deviation (SD).

Results and discussions

Effect of pH, temperature, and time on reduction of OTA

Reduction of OTA during thermal processing depended on the pH of the solution (Figure 2.2 and 2.3). At processing temperatures less than 200°C, loss of OTA was most rapid and extensive at pH 10, followed by that at pH 7 and 4. DI water and pH 7 followed

similar reduction patterns. Complete reduction of OTA was not achieved at any pH after 60 min of processing in any processing temperatures. Overall, the OTA was least stable at pH 10 and the most stable at pH 4. Studies about other mycotoxins show that the thermal stability of mycotxins is high under alkaline conditions while this study presents opposite results about the OTA (Ryu *et al.*, 2003; Jackson *et al.*, 1996; Jackson *et al.*, 1996a); the reduction was very high and rapid in pH 10 compared to pH 4 or 7 indicating alkaline condition favors OTA reduction.

Figure 2.2 and 2.3 also demonstrates that the rate of reduction of OTA was highly dependent on temperature. In general, the extent of reduction increased with processing temperature and time. Regardless of pH, no significant losses in OTA occurred during processing at 100°C and 1-38% of OTA was lost at 125°C for pH 4, 7, and DI water. In comparison, reduction of OTA reached up to 74% for pH 7 and 84% for pH 10 after 60 min of processing at 150°C. At temperature of 175°C, 16 - 86% of OTA was lost after the 60 min processing. Except pH 4, more than 90% of OTA reduction was achieved at the end of 200°C for all pH variations. The pH 10 started showing greater than 80% reduction at all processing temperatures applied. This instability can be related to the finding by Ingrid *et al.* (2003) which suggests the formation of open ring structure of OTA under alkaline condition, leading to underestimation of OTA.

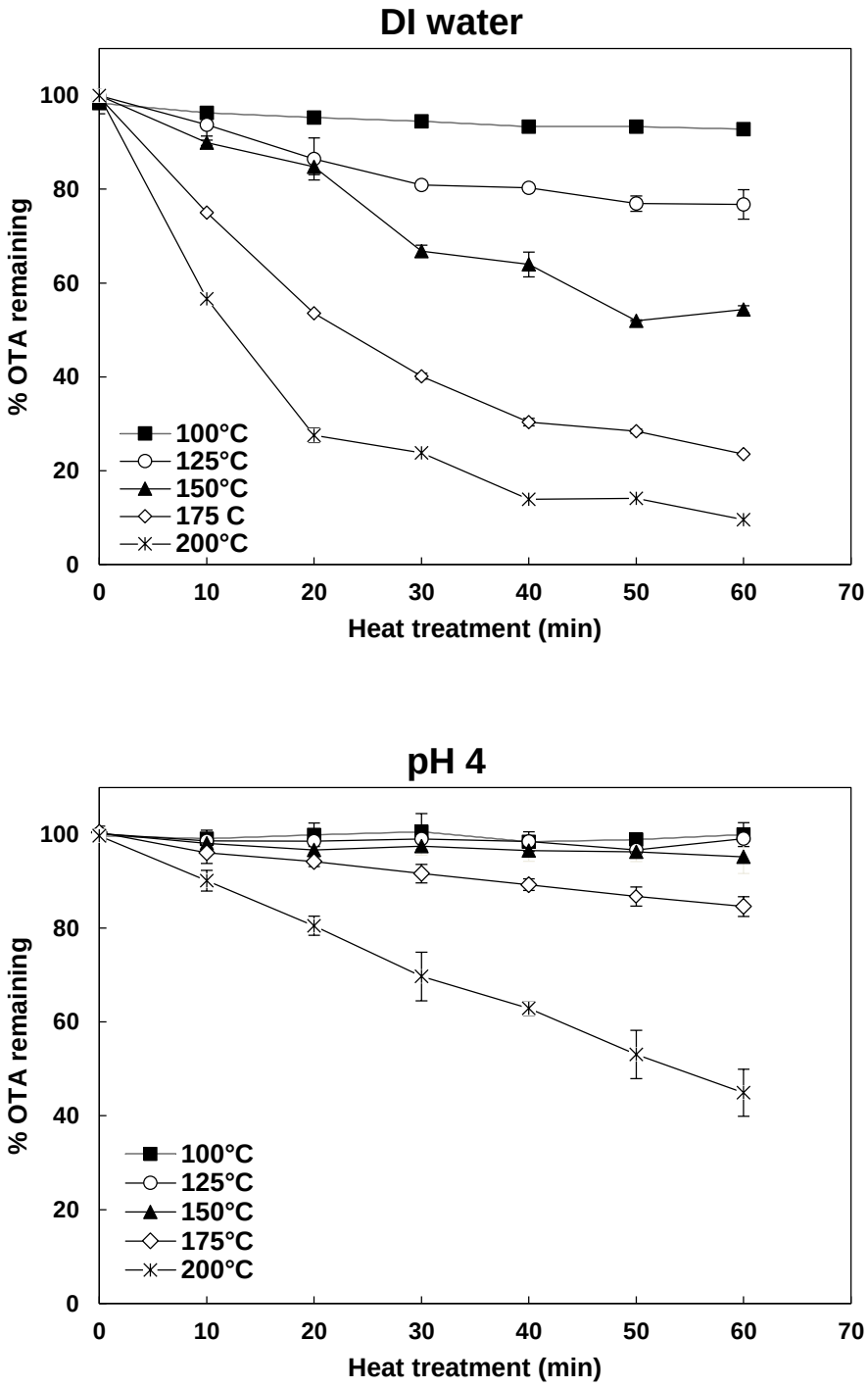

Figure 2.2. Effect of DI water, pH 4, temperature, and time on reduction of OTA. Each point indicates average of three replicates for DI water and six replicates for pH 4. Error bar indicates standard deviation from mean.

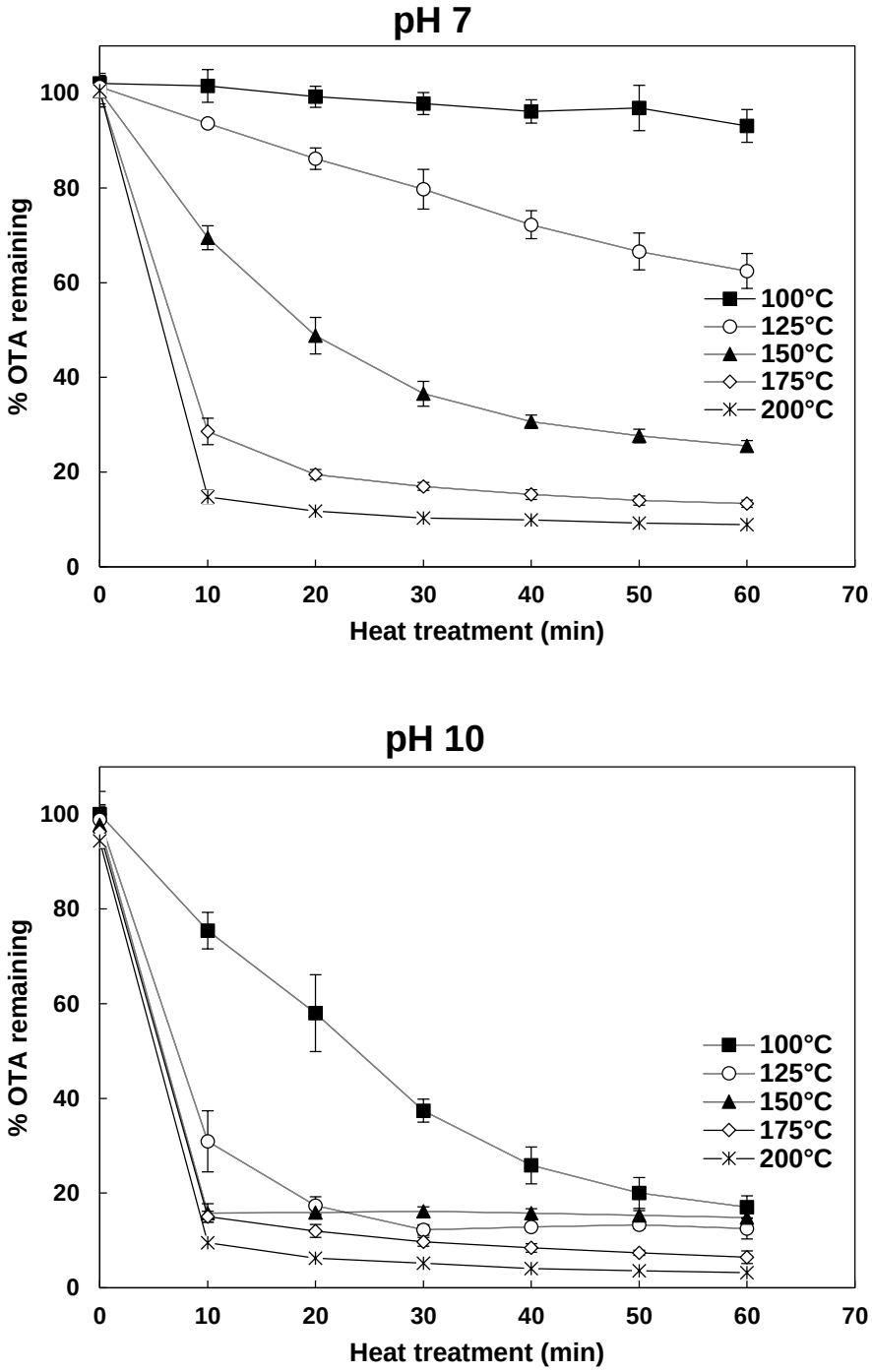

Figure 2.3. Effect of pH7, pH 10, temperature, and time on reduction of OTA. Each point indicates average of three replicates. Error bar indicates standard deviation from mean.

In general, the results shown in this study are in agreement with the previous results of studies on thermal stabilities of OTA. No complete reduction was achieved for all pH variations and at all processing temperatures, there was a slight percentage of OTA remaining after 60 min processing time. For all pH variations, OTA did not appear to be sufficiently degraded until the processing temperature reached 125°C. El-Banna and Scott (1984) observed 6% loss of OTA during cooking of wheat at 100°C for 30 min which is comparable to results from pH 4, 7 and DI water. Greater than 83% loss of OTA was observed in roasting coffee at 200-250°C (Suarez-Quiroz, 2005) which is similar to results for 200°C of heat treatment in pH 7 and 10 for 60 min. Only slight degradation of OTA in pure form was achieved at heat treatment of 180°C for 60 min (Raters and Mattissek, 2008) which is the case for most of our treatments where 100, 125, and 150°C have not been able to show higher reduction of initial OTA concentrations. Muller (1982) showed that OTA is only partially degraded at normal conditions of cooking which is supported by our data.

When compared to other mycotoxins such as aflatoxin and fumonisin, it can be said that OTA is fairly heat stable toxin. According to Raters and Matissek (2008), heat treatment temperature of 180°C led to 100% degradation of aflatoxin B_1 but only slight loss of the OTA was observed in its pure form. Similar study was done for the heat stability of fumonisin B_2 in different pH models (pH 4, 7, and 10). It showed that after 60 min of processing at 200°C, all fumonisin B_2 was decomposed at each pH levels (Jackson *et al., 1996)*. Another similar study by Jackson *et al.*(1996a) showed complete reduction of fumonisin B_1 when heat treated at temperatures greater than 175°C in pH 4, 7, and 10 after 60 min The study done with zearalenone also showed complete reduction of zearalenone after 60 min of processing at 200 and 225°C (Ryu *et al.*, 2003). All these studies showed

that rate of reduction of mycotoxin increased with processing time and temperature which is comparable to the OTA thermal stability study.

Kinetics of OTA reduction

The reduction of OTA in buffers (pH 4, 7, and 10) and DI water heated at 100, 125, 150, 175, and 200°C followed first-order reaction. Linear correlation coefficients (*k*) shown in Table 3.1 demonstrate the straight-line relationship between processing time and the natural log of the fraction of OTA remaining. The Table 2.1 summarizes the kinetic data for the reduction of OTA at temperatures 100-200°C. Half-lives ($t_{1/2}$) and first-order reaction constants indicate that the greatest reduction of OTA occurred at 200°C. The half-life achieved at 200°C of pH 7 is comparable to half-life achieved at 150, 175, and 200°C in pH 10. The half-life for 100°C at pH 4 was highest of all treatments. In general, the reduction of OTA was fastest at pH 10 and slowest at pH 4.

These data confirm that OTA is very heat-stable, especially at acidic pH. In general, the loss of OTA was more rapid and extensive under alkaline conditions than at neutral or acidic pH, but temperature and processing time were more critical factors that affected the reduction of OTA. The processing temperatures are chosen in the range of 100 to 200°C to imitate the conditions used in general food processing. These results suggest that little or no change in OTA content would be expected when foods are heated at boiling or retort temperatures, i.e., 100-125°C. However, substantial reduction of OTA may be achieved in food processes such as baking, frying i.e., >150-250, but complete elimination is not possible in the given range. It should also be noted that although loss of OTA was shown by HPLC, this might not mean detoxification. More research is needed to fully assess the

effects of thermal processes in reducing the biological activity of OTA and to prove loss of toxicity.

Table 2.1. Reaction rate constants (*k*), half-lives ($t_{1/2}$) and linear correlation coefficients (R^2) of ochratoxin A processed at 100, 125, 150, 175 and 200ºC in aqueous buffers of pH 4, 7, and 10.

Treatments	**Processing temperature**	***K* (min^{-1})[a]**	**$t_{1/2}$ (min)[b]**	***R*^{2}**
DI water	100°C	0.001	753	0.92
	125°C	0.005	154	0.92
	150°C	0.011	61	0.95
	175°C	0.024	28	0.97
	200°C	0.037	18	0.94
pH 4	100°C	0.000	17,200	0.01
	125°C	0.000	2680	0.28
	150°C	0.001	1021	0.81
	175°C	0.003	251	0.99
	200°C	0.013	53	0.99
pH 7	100°C	0.001	464	0.93
	125°C	0.008	84	1.00
	150°C	0.023	30	0.93
	175°C	0.090	8	0.91
	200°C	0.200	3	0.92
pH 10	100°C	0.031	22	0.99
	125°C	0.090	8	0.99
	150°C	0.198	3	0.98
	175°C	0.200	3	0.83
	200°C	0.225	3	0.90

Acknowledgements

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CHAPTER 3: Reduction of ochratoxin A in oat flakes by twin screw extrusion

Abstract

Ochratoxin A (OTA) is one of the most important mycotoxin due to its observed toxicity and potential carginogenicity. It occurs in a wide range of agricultural commodities worldwide with cereals reported to have highest incidence of contamination. In this study, heat stability of OTA during extrusion of artificially contaminated oat flakes was examined using twin screw extruder and quantified by high performance liquid chromatography-fluorescence detection (HPLC-FLD). Central composite design was used for experimental design. Factors examined were moisture content (20, 25, and 30%), temperature (140, 160, and 180ºC), screw speed (150, 200, and 250 rpm) and die size (1.5, 2, and 3 mm). The initial concentration of OTA in the artificially contaminated oat flakes was found at a mean concentration of 112 μg/kg as measured by HPLC. The reductions of OTA upon extrusion processing ranged of 0-28%. OTA reduction was significantly affected by screw speed and die size ($p < 0.005$).

Keywords: Ochratoxin A (OTA); extrusion; heat stability; reduction; HPLC-FLD.

Introduction

OTA is one of the most important mycotoxins produced by certain species of toxigenic *Aspergillus* and *Penicillium* (Stormer, 1992*).* It is found in the wide range of foods such as cereals, coffee, spices, wine, dried vine fruits, meat, and cheese (Magnoli *et al*., 2006; Joosten *et al*., 2001; Fatih and Bulent 2012; Rufino *et al.*, 2007; Iamanaka *et al.*, 2005; Jorgensen, 2005; Dall'Asta *et al*., 2008). OTA has been detected in wide variety of processed cereals along with infant cereals, instant coffee and roasted coffee beans at a level up to 5.67 µg/kg (Lee and Ryu, 2015; Kabak B., 2009; Lombaert *et al*., 2002; Tozlovanu and Pfohl-Leszkowicz, 2010).

OTA is reported to cause immunotoxic, nephrotoxic, teratogenic and carginogenic effects (Alvarez *et al., 2004;* Luhe *et al., 2003; Vesela* et al., *1983; Lioi et al., 2004).* It is classified as a possible human carcinogen (group 2B) by International Agency for Research on Cancer (IARC, 1993, JEFCA, 2001). Studies suggest correlation between occurrence of Balkan Endemic Nephropathy (BEN) and OTA (O'Brien and Dietrich, 2005). Currently few countries, particularly in Europe have established regulatory limits to monitor and control the levels of OTA in various food commodities. The Commission of European Communities established a tolerable weekly intake (TWI) of OTA as 120 ng/kg bw. Similarly, the European Union has set the maximum level for cereals to be regulated between 3-5 µg/kg and infant cereals to be 0.5 µg/kg (CEC, 2006; Heydt et al., 2011).

Oat is an important cereal grain from its nutritional point of view. It is a significant source of carbohydrate, protein, lipid and dietary fiber particularly β- glucans, which are beneficial to human health. They are primarily used for animal feeds and human consumption as oat meal or oat flakes for breakfast cereals, baby foods and so on (YiFang,

2013). Recent study by Lee and Ryu (2015) showed a high incidence of contamination of OTA in oat based breakfast cereals in U.S.

Extrusion cooking is a high-temperature, short-time process in which moistened, expansive food materials are cooked in a tube by a combination of moisture, pressure, temperature and mechanical shear. (Havck & Huber, 1989; Castells et al., 2005). It is used for the manufacture of snacks, breakfast cereals, texturized proteins, flat breads, animal feeds and others. During extrusion, the temperature of barrel can be as high as 200ºC. Significant physico-chemical transformations occur in a very short cooking time of less than one minute. This causes breakage of chemical bonds in the polymer and formation of free radicals (Harper and Clark, 1979; Guy, 2001). Contaminant such as OTA might be subjected to both high temperature and chemical reactions by free radical mechanisms leading reduction. When oats are extruded, it can cause gelatinization of starch, some denaturation of protein and minute changes in the dietary fiber (Guy, 2001). The proteins coagulate and form gel when exposed to high temperatures but the dietary fiber may retain their macrostructure undergoing only small amount of breakdown during extrusion. (Webster and Wood, 2011; Guy, 2001). When present in high amount, like in oats, dietary fibre are known to disrupt the starch matrix and absorb water, reducing the expansion capacity of the starch melt. The lipid content of oats are about 7%. This is higher than in most other cereal grains (Decker *et al.* 2013). The lipids in oats can create a lubrication effect, which reduces the shear force created inside the barrel of the extruder (Camire, 2000). Extrusion cooking does not aim to decontaminate mycotoxins but can lead in reduction of mycotoxin levels in cereals.

Little work has been published about effect of different food processing methods on OTA. OTA's resistance to thermal processes during processing of products such as cereals, coffee, cocoa, wine, and beer have been reported. They indicate contradiction. Thermal processes can give different effects, according to the processing time, moisture conditions, and temperature reached. OTA is generally stable to the level of heat utilized in ordinary cooking. Baking wheat flour at 140°C for 40 min gave only 21% OTA reduction while baking at 200 gave 64% reduction. Copetti *et al.* (2013) reported 93.6% loss of OTA present in cocoa beans during the chocolate making process that involved heat treatment of the beans at 110-140°C for about 30 min (Kamphuis, 2009). On the other hand, because of the high temperatures used for coffee roasting, a higher percentage of destruction is observed. A study on coffee beans reported that roasting at 200 to 250°C gave 82.9% reduction while another study showed only 0-12% reduction during roasting at 200°C for 10-20 min (Suarez-Quiroz *et al.*, 2005; Tsubouchi *et al., 1987).* Boudra *et al*. (1995) showed that moisture had significant effect on OTA reduction. They reported partial decomposition of OTA in wheat after 120 min of heating at 100°C in the presence of water. No change was observed when wheat was dry heated under same condition up to 160 min. Contradicting, 74% OTA reduction was achieved during autoclaving oatmeal in humid condition and greater than 86-87.5% was achieved with dry autoclaving (Trenk *et al*., 1971). Few studies about effects of extrusion on OTA suggest, maximum 86% reduction of initial concentration can be achieved with the harshest extrusion conditions. Study carried by Castells *et al.* (2006) reported that extrusion cooking could reduce 17-86% OTA in extruded barley meal. Similarly, study on whole wheat grain by Scudamore *et al*. (2004) reported

that maximum loss observed was no greater than 40% and that a higher temperature, a long contact time and high moisture content were related to a bigger OTA reduction.

Extrusion is one of the novel technologies in the area of food processing and cereals are one of the best material to be used in extrusion processing because of their high starch content. They are also the highest contaminated food products with OTA (Duarte *et al.*, 2010). The combination of higher temperature, pressure and other varying factors propose a higher stress to the heat stable OTA leading greater chances of reduction. The objective of this study was to determine the effect of moisture, screw speed, temperature and die size on the stability of OTA and determine optimum conditions to achieve maximum reduction. Information about the behavior of OTA during such processing is important in assessing and reducing consumer risk. They might assist in developing strategies for reducing consumer exposure to this mycotoxin.

Materials and methods

Chemicals and reagents

Purified standards of OTA produced by *Petromyces albertensis* was purchased from Sigma-Aldrich (St. Louis, Mo, USA). HPLC grade acetic acid (99.5%) (Fischer Scientific, Pittsburgh, PA, USA), methanol, acetonitrile, water (Macron Chemicals, Center Valley, PA, USA), and Phosphate-Buffered Saline (PBS) tablets (Sigma-Aldrich, St. Louis, MO, USA) were used for analysis. Ochra-Test immunoaffinity columns (IAC) were purchased from (VICAM, Watertown, MA, USA).

Raw materials

Oat flakes was obtained from Grain Millers Inc. (Eugene, OR, USA). The flakes were evaluated for moisture and equilibrated at three moisture levels: 20, 25, and 30% ± 0.5

(db) by mixing each flakes with the appropriate amount of water and then storing overnight at 4°C in an airtight container similar to a procedure by Wang *et al*. (1991). The OTA was mixed with the water during the time of moisture adjustment to spike the oat flakes, giving final concentration of 100 µg OTA / kg oats (db) in the samples.

Extruder and processing conditions

Extrusion was performed using a 20 mm co-rotating twin screw extruder (Model# TSE 20/40, 7.5 HP, CW Brabender, S. Hackensack, NJ, USA). The overall length of the extruder barrel was 400 mm and had a length to diameter (L/D) ratio of 20:1. The barrel had 4 individual heating zones plus a feed zone. The temperature profile of the extruder was kept at 140, 160, and 180°C with the feed zone being constant at ~50°C. The temperature of the subsequent compression, metering and die zones were changed according to experimental design. Rod-shaped dies with a diameter of 1.5, 2, and 3 mm were used. The extruder screw-speed was varied at 150, 200, and 250 rpm for all treatments. The feed rate of the flakes was maintained at ~4 kg/h with a volumetric twin screw feeder (Allen Bradley, Oak Brook, IL, USA). Only non-mixing screws were used in the screw profile.

The moisture equilibrated samples were fed into the extruder. Extrusion was carried out maintaining full feed, ensuring that the flights and feed port were full throughout the extrusion runs. Extrudates were collected when system stabilized, with constant pressure at the die, constant torque, and constant output flow rate. Continuous data from the extruder, including zone temperature, die pressure, and motor torque, were recorded using Data Acquisition System for ATR and Intelli-Torque (CW Brabender, S. Hackensack, NJ, USA) with one data point being taken every 20 s. The extruder was not stopped in between runs but cleaned with wet milled corn having 20% moisture (wb) before loading new sample.

One kg of oat flakes was run through the extruder for each experimental unit, and ~500 g of extrudate sample was collected after achieving steady state flow in the extruder. Extrudate samples were dried in a convection oven (VWR International, LLC, PA, USA and Thermo Scientific, Germany) at 40°C for 12-24 h yielding an average moisture content below 10% (wb) and then stored in air-tight plastic bags at -18°C until analyzed.

Analysis of extruded products

Samples were extracted and cleaned according to the Rahman and Jinap (2010) with some modifications. The extruded samples were ground to a fine consistency using a laboratory grinder (Intertek, Torrington, CT, USA). Twenty five g of ground sample was extracted with 100 mL of acetonitrile/ water (80:20, v/v) by shaking for 30 min using a wrist action shaker. The extract was then filtered through Whatman No. 4 filter paper and 10 mL of the filtrate was diluted with 40 mL of Phosphate Buffered Saline (PBS). A total of 10 mL of the diluted extract was passed through immune affinity columns (IAC) (OchraTestwb, VICAM, Milford, MA, USA) at a flow rate of about 2-3 mL per min. The column was washed with 10 mL of PBS followed by 10 mL of water and OTA was eluted with 3 mL of methanol into a vial at a flow rate of one drop/s. Then the eluate was evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was re-dissolved in 500 mL methanol/water (50:50, v/v) and injected into the HPLC.

HPLC-FLD system

The HPLC system (Agilent 1260 Infinity HPLC system series, Palo Alto, CA, USA) is equipped with a quaternary pump (DEAB 705541), auto sampler (DEACC 14016), port column switching valves (DEACN 15339), and fluorescence detector (DEABO 02622). The chromatographic separation was performed on reversed-phase Hypersil GOLD C18

column (3×100 mm, particle size 1.9 µm) (Thermo Scientific, Waltham, MA, USA) with the isocratic elution using 50% acetonitrile and 50% water containing acetic acid 1% at flow rate of 0.4 ml/min. The column temperature was kept at room temperature (25°C). OTA was detected at 334 nm and 460 nm wavelengths for excitation and emission, respectively. Injection volume was 10 µl for the analysis.

Experimental design and statistical analysis.

Central composite design was used to design trials for extruded products. The initial operating conditions were optimized through preliminary trials and multiple screw profile modifications. Preliminary optimization was identified by finding the operable range of the extruder. Through these preliminary studies, the independent variables considered were feed moisture (20, 25, and 30%), screw speed (150, 200, and 250 rpm), die size (1.5, 2, and 3 mm) and temperature (140, 160, and 180°C). All other extrusion processing variables were kept constant. The average of data points in the 10 min of steady state conditions were then used to calculate the different parameters for the process responses.

The data was analyzed for ANOVA analysis using response surface analysis in Minitab 17.0 (Minitab Inc., State College, PA, USA).

Process responses

Specific Mechanical Energy (SME) was calculated according to Godavarti and Karwe (1997) as following.

$$SME = \frac{(total\ torque - friction\ torque) x\ screw\ speed\ x\ rated\ motor\ power}{(maximum\ torque\ x\ maximum\ screw\ speed\ x\ mass\ flow\ rate)}$$

The units for torque, screw speed, rated motor power and mass flow rate were Nm, revolution per minute (rpm), kW, and kg/s respectively. SME was expressed as kJ/kg.

Results and discussions

Extrusion of oat flakes was a stable process and a range of conditions was examined to determine which factors affect the stability of OTA. High temperatures and low moisture were considered to be harsher in comparison to the normal operation of the extruder and not necessarily relate to loss of OTA. It was expected to produce free radicals in the polymer while low temperatures combined with greater water activity tends to hydrate starch polymers in oats and encourage aqueous reactions (Guy, 2001). The detailed operating parameters used for the experimental design is given in Table 3.1.

HPLC method used for quantitating OTA in extruded oat flakes resulted in good recoveries. The recovery of OTA when spiked at 100 μg/kg from non-extruded and extruded oat flakes was 95-100% and the sensitivity of the method was 0.5 μg/kg. HPLC chromatograms presented in Figure 4.1 indicate that clear separation of OTA occurred with the method used.

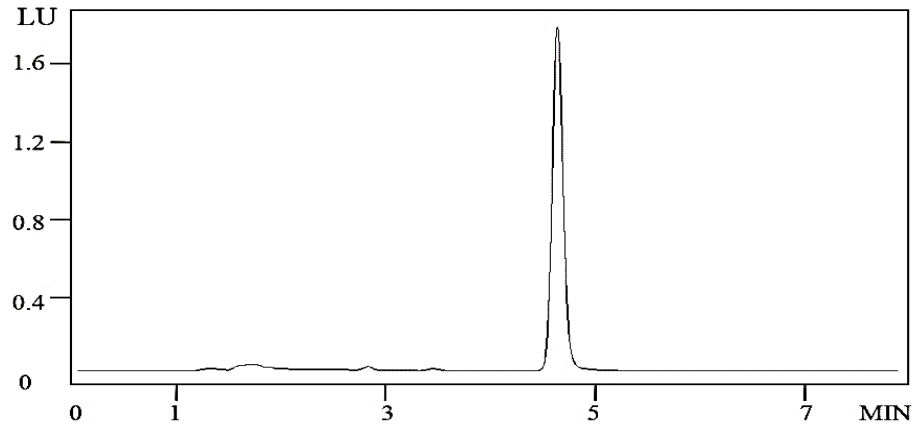

Figure 3.1. HPLC chromatogram of spiked oat flakes after extrusion at 180°C, 250 rpm, 3mm, and 20% moisture.

Effect of temperature, moisture, screw speed, and die size on reduction

The stability of OTA in oat flakes was significantly affected by the extrusion cooking parameters. Percent reduction in OTA, based on the recovery from spiked oat flakes, ranged 0-28% depending on moisture, temperature, screw speed and die size. ANOVA analysis indicated that the effects of both screw speed ($P < 0.021$) and die size ($P < 0.006$) were significant to the amount of OTA recovered. Recovery decreased as die size and screw speed increased (Figure 3.3, 3.4). Highest reduction obtained was 27.6% from 250 rpm screw speed, 180°C temperature, 3 mm die size, and 20% moisture. The use of optimizing tool for response surface analysis in Minitab 17.0 predicted maximum 27.6% of reduction could be achieved using combination of 221 rpm screw speed, 162°C temperature, 3 mm die size, and 30% moisture.

When the mean values of individual factors were compared with total mean reduction, it could be observed that 160ºC was more efficient than 140 and 180ºC. Castells *et al.* (2005) also observed this pattern when extruding spiked barley meal at same temperatures. Also, the mean reduction increased with increasing screw speed, die size, and moisture content (Figure 3.2). The contour plot and response surface plot indicates higher reduction is achieved at highest die size (3mm) and screw speed (250 rpm) used (Figure 3.3, 3.4).

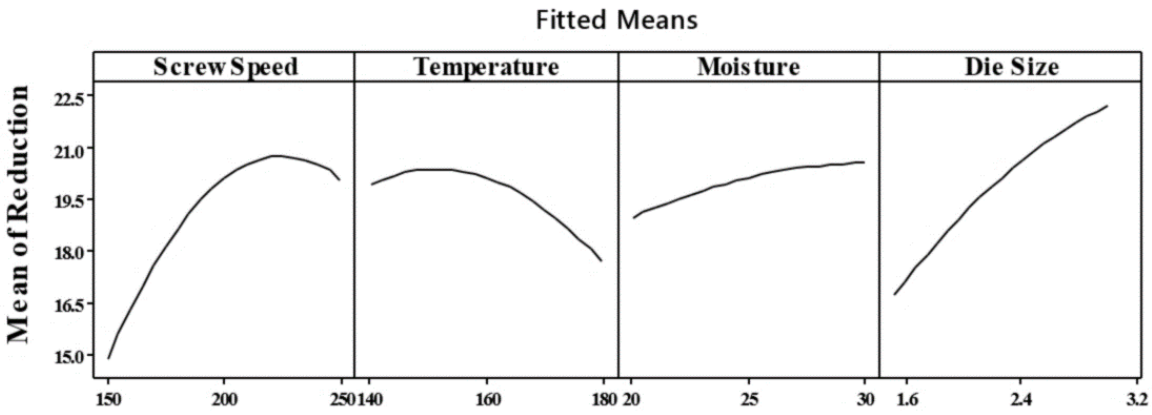

Figure 3.2. Individual effect of moisture, temperature, screw speed and die size on the reduction of ochratoxin A.

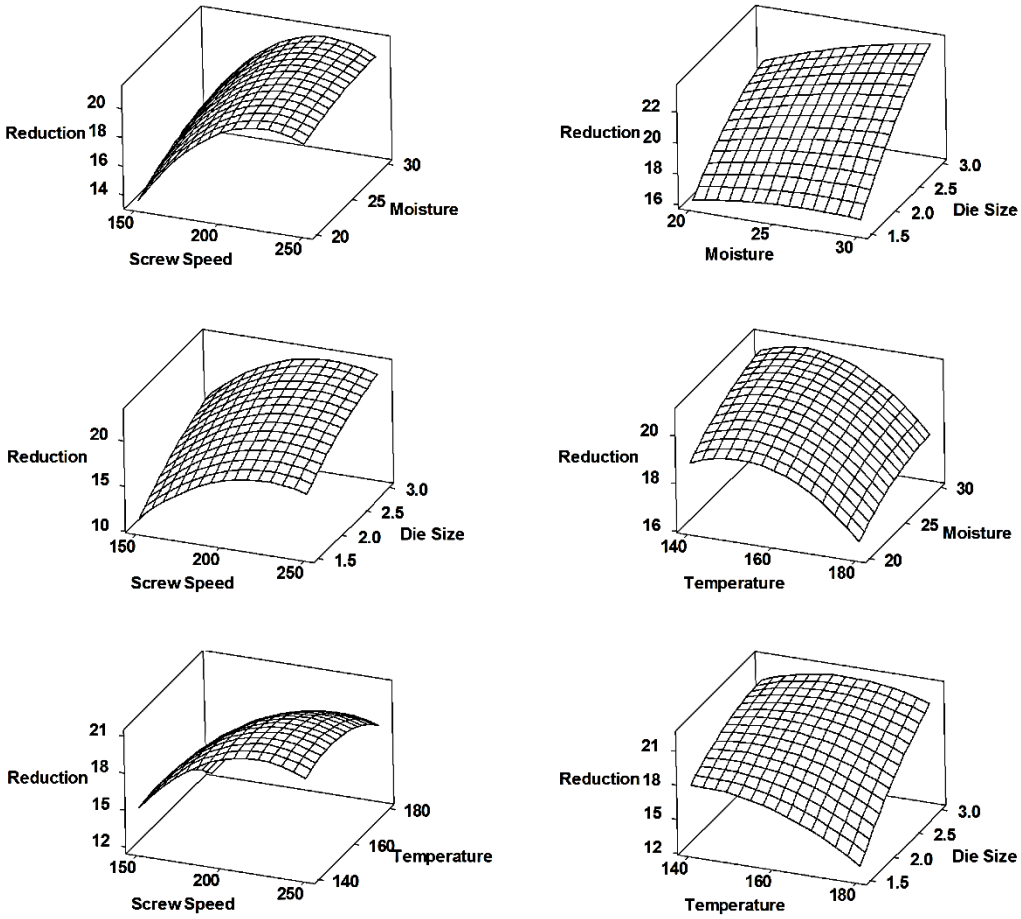

Figure 3.3. Response surface plots of cohratoxin A reduction (%) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

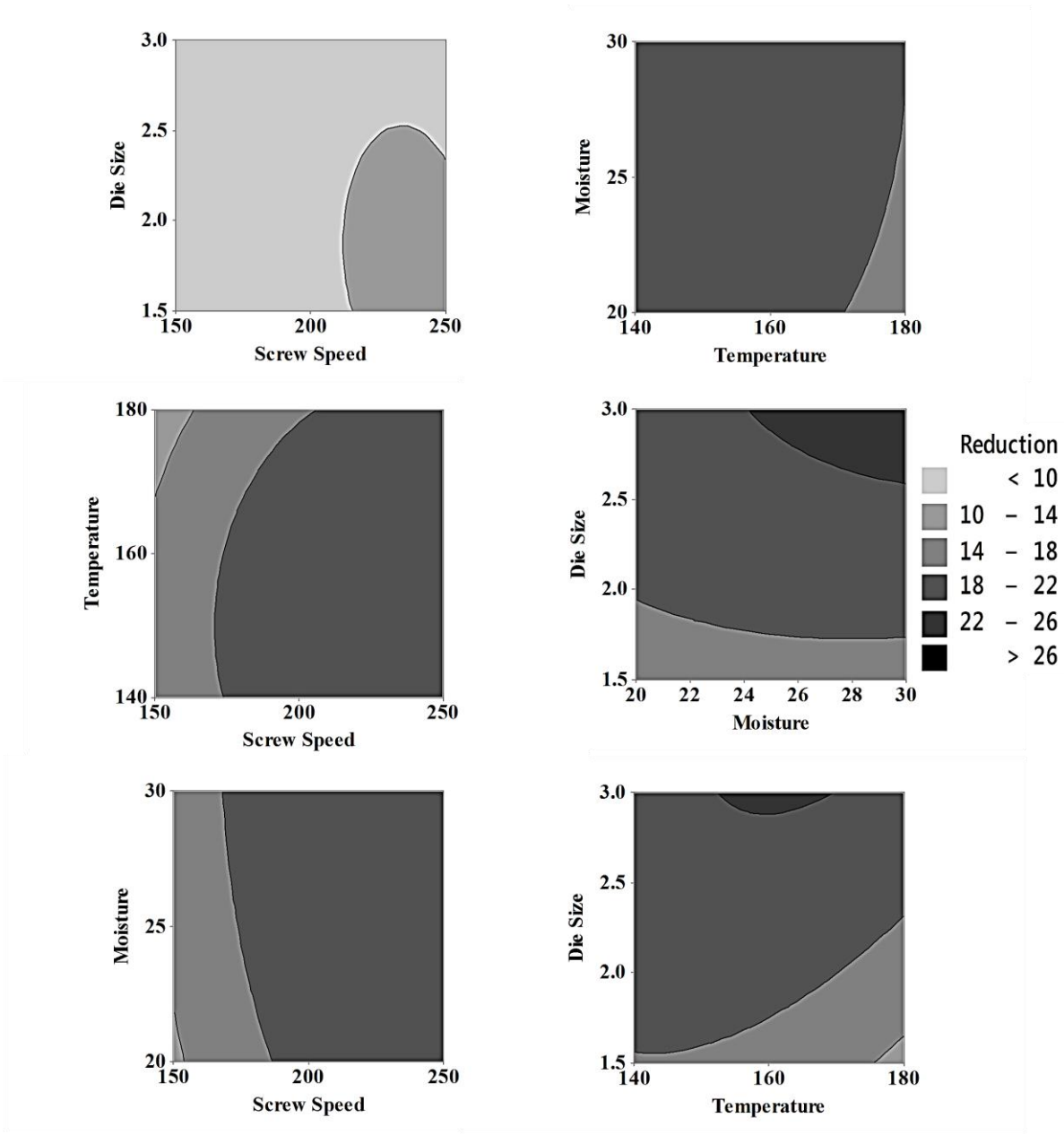

Figure 3.4. Contour plots of ochratoxin A reduction (%) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

OTA thermal stability has been studied by several authors with contradicting results about reductions, majority in agreement that OTA cannot be completely removed even under the harshest conditions used during processing. OTA has been detected after treatment at 250ºC during coffee roasting (Suarez-Quiroz *et al.*, 2005). This temperature is particularly very high and not commonly applied in general food processing. In our experiment, OTA remains to be fairly heat stable with only 27.6% reduction at maximum. Inconsistent results was observed with temperatures applied in combination of process variables used. Similarly, OTA presented a complex relationship with moisture. The highest reduction was obtained at 180ºC and 20% moisture followed by 160 and 140ºC at 25 and 30% moisture respectively. The results are in agreement with Castells *et al. (2005*) and Scudamore *et al*. (2004), who reported non-uniform OTA loss with varying temperature and moisture during extrusion of barley meal and whole wheat grains respectively. Scudamore *et al.* also reported that keeping the moisture constant gave greater OTA reductions at higher temperatures. Similarly, Castells *et al.* (2005) reported that 17-86% OTA was reduced during their extrusion. In our experiment, the range did not exceed 0-28% and was comparable to 0-40% reported by Scudamore *et al.* (2004).

Screw speed of extruder is related with exposure of raw material to stress. Lower screw speed provides longer exposure to heat. Similarly, lower die size is comparable to higher pressure stress during processing (Ludewig, 1990). Both are expected to give greater reductions of OTA. However, our results showed significantly greater reduction of OTA by higher die size and screw speed throughout the extrusion

The results are different from those of Katta *et al*. (1999), Castelo *et al*. (2001), and Scudamore *et al*. (2004), which suggested more efficient removal of mycotoxins at lower

screw speed. Meister (2001) on other hand concluded that the highest fumonisin reduction was reached using high screw speed along with other process variables suggesting possibility of greater OTA reduction with higher screw speed in combination to other processing factors. Also, higher screw speed correlates to higher specific mechanical energy (SME) (Figure 3.5). The SME is the amount of mechanical energy dissipated as heat inside the material. It indicates extent of molecular breakdown or degradation, a material undergoes during extrusion process (Mercier *et al*., 1989). This explains greater reduction of OTA in our experiment.

In the overall extrusion, minimum residence time of extruder ranged from 30-41 sec. Surging was observed when smaller die size was used and the product was coming out faster and curlier. This surging brings pressure fluctuation in the system leading to non-uniform treatment (Luker, 2016; Giles *et al.*, 2004). However, when bigger die size was used, the product was coming out straight, slowly and consistently. This slow and consistent release of product in the extruder can be attributed to more uniform treatment of oat flakes and comparatively higher residence time which also correlates to longer exposure to stress in the raw material, explaining higher OTA reduction. Besides that, combinations of other process variable might contribute such pattern in our experiment (Figure 3.7).

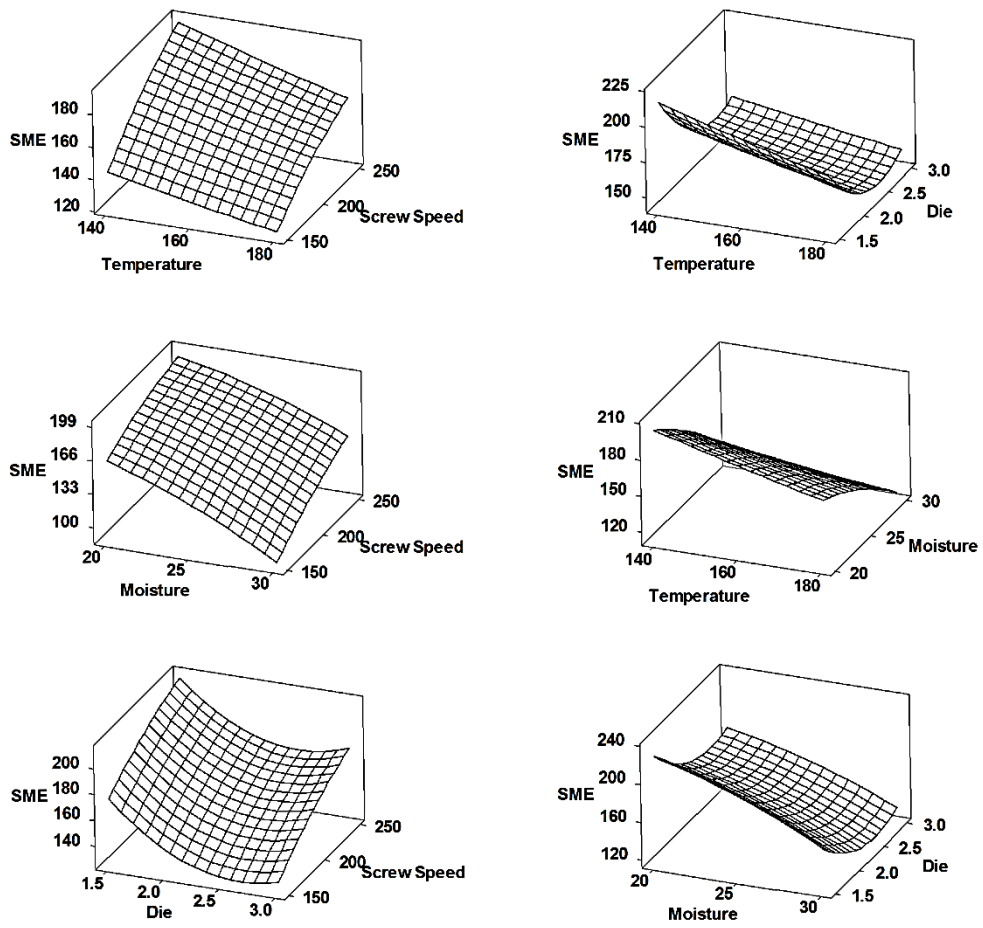

Figure 3.5. Response surface plots of specific mechanical energy (SME) (kJ/kg) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

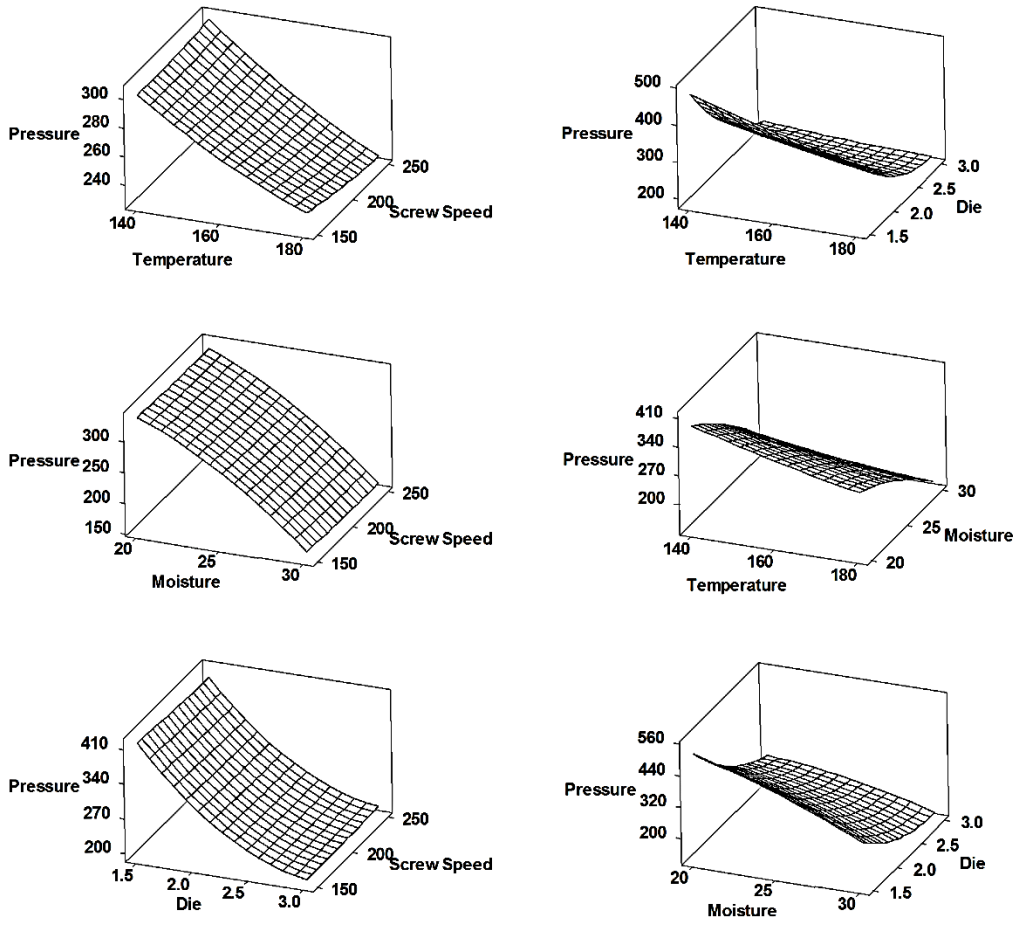

Figure 3.6. Response surface plots of extruder pressure (MPa) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

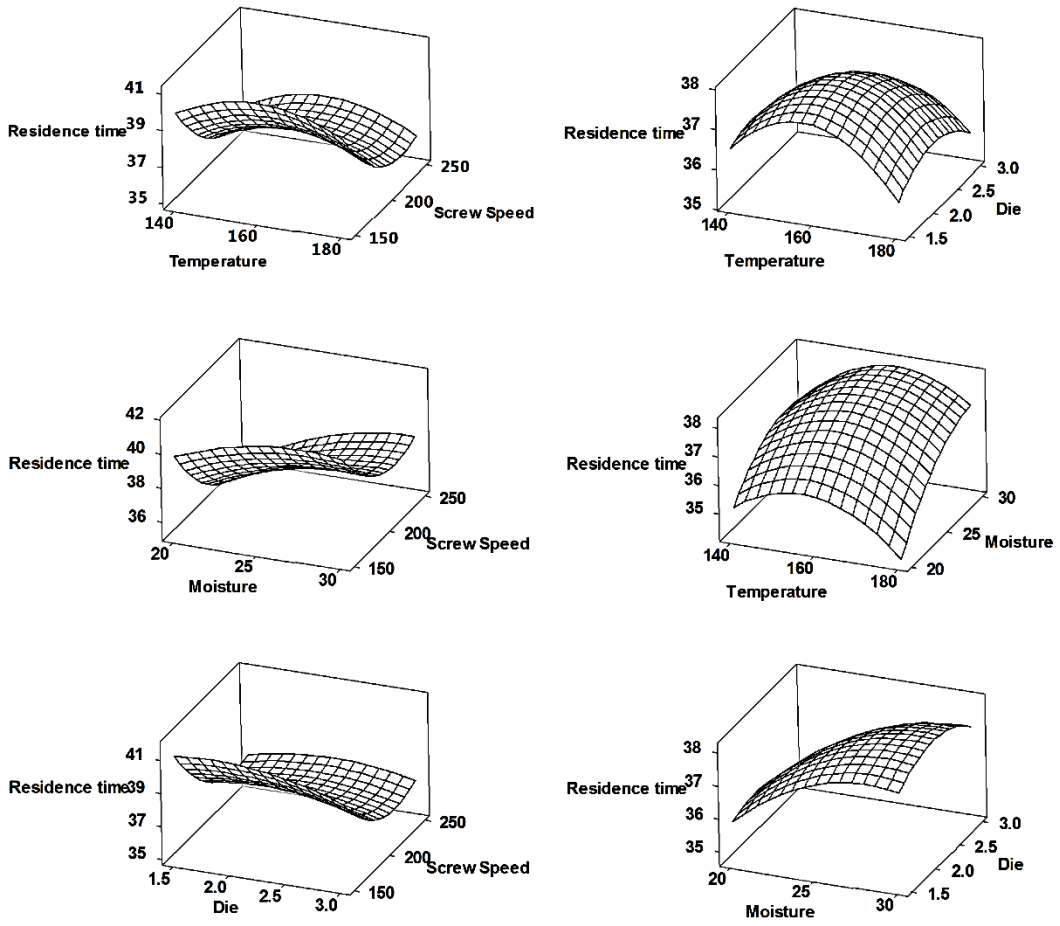

Figure 3.7. Response surface plots of minimum residence time (sec) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

Color analysis

The *L*, *a*, and *b* values indicated that the overall color of the product did not change considerably at the different extrusion parameters tested (Table 3.1). The lightness (*L*) of extrudates was significantly affected by moisture and interaction of screw speed between temperature and moisture while the yellowness (*b*) of the extrudate was significantly affected by moisture only. Mild yellowing of the product was observed at all conditions. Decrease in moisture increased lightness of extrudate (figure 4.8 and 4.9). Ilo *et al*. (1999) also observed same effect of moisture along with increasing barrel temperature when extruding maize grits. This fact could be ascribed to the processing conditions used in extrusion cooking (high temperature and low water content) which favor Maillard condensation of amino groups with reducing sugars, leading to the formation of color compounds (Berset, 1989). Color changes in extrusion cooking can be used as a visual indicator to assess the process intensity concerning chemical changes or nutritional loss in food.

Overall, most of the studies on mycotoxins suggest higher reductions at higher temperatures. Aflatoxin B_1, fumonisin B_1, fumonisn B_2, and zearalenone reported complete reduction at 180, 175, 200, and 200ºC wheat heated in aqueous buffered solutions (Raters and Matissek 2008; Jackson *et al.*, 1996a*;* Jackson *et al.*, 1996; Ryu *et al.*, 2003). Similarly, 51-95% aflatoxin, 44-66% fumonisin $B_{2,}$ 34-95 % fumonisin $B_{1,}$ and 66 -83% zearalenone reduction have been reported during extrusion (Castell *et al*., 2006; Meister, 2001; Katta *et al.*,1999; Ryu *et al.*, 1999) This indicates the capacity of OTA to sustain high temperature stresses. It may be concluded that concluded that OTA is stable during extrusion and only 28% of OTA could be reduced within the entire experimental conditions used. In general,

the reduction was greater at highest die size and screw speed used. The effect of temperature and moisture showed complex relationship. Further study on these variables are needed to determine true effect on OTA reduction during extrusion.

Extrusion cooking involves physical and chemical steps that may reduce mycotoxins in contaminated commodities. It is grabbing increased attention worldwide because of its advantages over traditional cooking methods. Perhaps, more research after addition of other food components or additives is needed. Such studies should assist in suggesting modifications to operating procedures that will minimize the exposure of consumers to OTA. It should also be noted that although loss of OTA was shown by HPLC, this might not mean detoxification. More research is needed to fully examine the effects of thermal processing in minimizing the biological activity of OTA and to justify loss of toxicity.

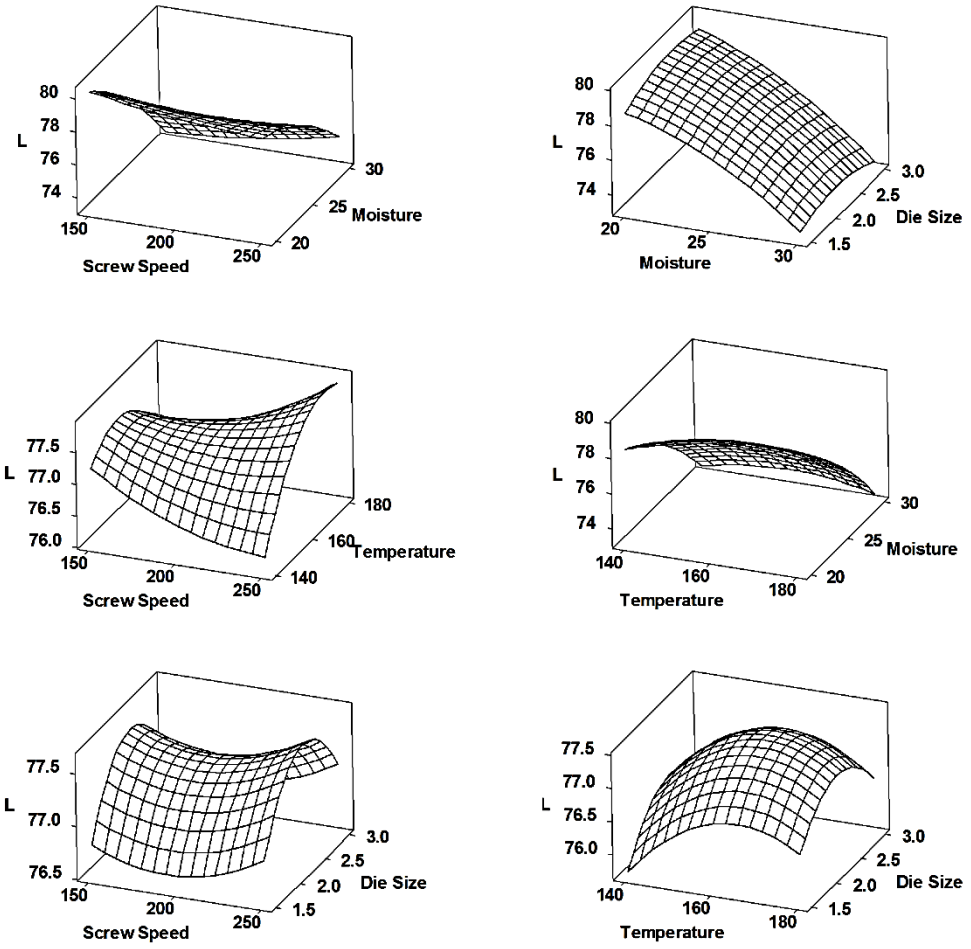

Figure 3.8. Response plots of lightness (*L*) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

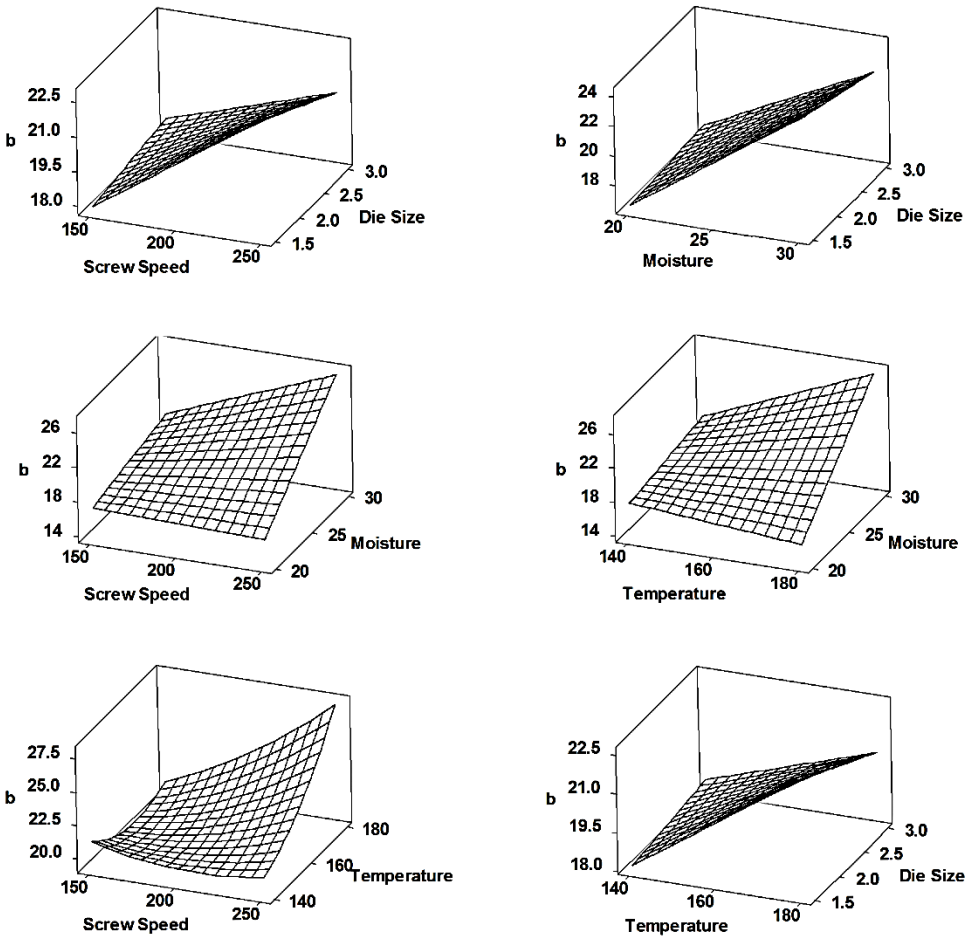

Figure 3.9. Response plots of yellowness (*b*) as a function of oat moisture (%), process temperature (°C), screw speed (rpm), and die size (mm).

Table 3.1. Central composite design used in extrusion cooking of oat flakes and their color parameters.

Screw Speed (rpm)	**Temperature (ºC)**	**Moisture (%)**	**Die Size (mm)**	***L***	***a***	***b***	**ΔE***
150	140	20	1.5	78.53	2.29	17.87	7.65
250	140	20	1.5	77.56	2.38	18.53	8.82
150	180	20	1.5	79.53	2.16	17.34	6.55
250	180	20	1.5	78.63	2.50	17.75	7.51
150	140	30	1.5	72.04	3.47	21.19	10.69
250	140	30	1.5	72.48	3.39	21.01	10.22
150	180	30	1.5	73.08	3.35	20.91	9.67
250	180	30	1.5	74.03	3.21	20.88	8.88
150	160	25	1.5	76.66	2.63	18.85	8.28
250	160	25	1.5	76.30	2.90	19.48	8.99
200	140	25	1.5	75.23	2.79	19.44	9.78
200	180	25	1.5	77.78	2.64	18.46	7.18
200	160	20	1.5	79.25	2.39	17.84	7.12
200	160	30	1.5	72.19	3.55	21.37	10.68
200	160	25	1.5	76.13	2.75	19.10	8.86
200	160	25	1.5	76.98	2.77	18.85	8.05
200	160	25	1.5	76.61	2.79	19.04	8.46
200	160	25	1.5	75.83	3.00	19.38	9.29
200	160	25	1.5	76.66	2.85	19.07	8.44
200	160	25	1.5	76.36	2.85	19.04	8.65

Screw Speed (rpm)	Temperature (ºC)	Moisture (%)	Die Size (mm)	*L*	*a*	*b*	ΔE
150	140	20	2	79.50	2.28	17.48	6.68
250	140	20	2	77.87	2.60	18.27	8.43
150	180	20	2	79.17	2.31	17.47	6.91
250	180	20	2	78.00	2.63	17.86	8.07
150	140	30	2	74.54	3.09	20.31	8.12
250	140	30	2	75.20	2.96	20.15	7.49
150	180	30	2	69.93	4.14	21.90	12.93
250	180	30	2	75.05	75.05	75.05	94.26
150	160	25	2	77.71	2.56	18.44	7.21
250	160	25	2	77.37	2.71	18.74	7.68
200	140	25	2	75.69	2.99	19.47	9.46
200	180	25	2	77.68	2.69	18.60	7.35
200	160	20	2	78.43	1.94	17.66	7.57
200	160	30	2	75.80	2.73	19.70	6.73
200	160	25	2	76.92	2.77	18.99	8.19
200	160	25	2	77.47	2.61	18.47	7.42
200	160	25	2	77.08	2.69	18.98	8.05
200	160	25	2	77.00	2.75	19.07	8.18
200	160	25	2	76.94	2.76	18.77	8.03
200	160	25	2	76.73	2.79	18.93	8.29
150	140	20	3	79.17	2.57	18.03	7.33

Screw Speed (rpm)	Temperature (ºC)	Moisture (%)	Die Size (mm)	*L*	*a*	*b*	ΔE
250	140	20	3	77.71	2.58	18.09	8.43
150	180	20	3	79.09	2.44	17.53	7.02
250	180	20	3	79.14	2.42	17.60	7.03
150	140	30	3	73.53	3.33	20.50	9.06
250	140	30	3	71.59	3.55	21.17	11.07
150	180	30	3	70.30	3.83	21.87	12.56
250	180	30	3	74.29	3.08	20.47	8.41
150	160	25	3	77.72	2.65	18.37	7.17
250	160	25	3	76.94	2.81	18.97	8.16
200	140	25	3	74.36	3.38	20.47	11.16
200	180	25	3	77.06	2.75	18.99	8.08
200	160	20	3	78.83	2.46	17.57	7.24
200	160	30	3	72.63	3.47	20.89	10.04
200	160	25	3	77.63	2.66	18.53	7.34
200	160	25	3	76.93	2.80	18.88	8.11
200	160	25	3	77.17	2.77	18.83	7.89
200	160	25	3	77.44	2.73	18.75	7.64
200	160	25	3	77.35	2.75	18.86	7.78
200	160	25	3	77.36	2.70	18.63	7.61

$* \Delta E = \sqrt{((\text{L sample} - \text{L standard})^2 + (\text{a sample} - \text{a standard})^2 + (\text{b sample} - \text{b standard})^2}$

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CHAPTER 4: Conclusions

Food safety is one of the most essential exigencies in the world market. Food producers and governments are responsible to assure the quality and safety of food stuff. An important aspect of food safety is the control of mycotoxins in food products and the possibility of elimination or reduction to acceptable levels. The food and agriculture organization (FAO) has estimated that at least 25% of the world cereals is contaminated with mycotoxins, one of them being the OTA. OTA has also been detected worldwide in a large variety of widely consumed agricultural commodities such as coffee, beer and dried foods. Several animal studies done in rats, pigs and duck chicks have proved the toxicity of OTA. Toxic effect on kidneys and liver along with immunotoxic, neurotoxic and teratogenic effects have been reported. Furthermore, International Association for Cancer Research (IARC) classified OTA to be possible human carcinogen (group 2B).

The present study focused on the heat stability of OTA. In general, food processing is considered as one of the best methods to reduce mycotoxins in contaminated foods. Previous studies done on the major mycotoxins such as aflatoxin, fumonisin, zearalenone, and deoxynivalenol have shown that mycotoxins are sensitive to high temperatures. They can be completely reduced after processing for certain periods of time. However, on the other hand, very few studies focused on OTA indicate that OTA is very heat stable and can survive in high temperature for long period of time. The first part of the thesis studied heat stability of OTA in an aqueous buffered model system. It was found that OTA was sensitive to heating temperatures and processing time. Ninety percent of reduction was achieved at 200ºC after 60 min but no complete reduction was achieved within experimental temperatures used. The extent of reduction was very rapid in pH 10 followed by de-ionized

(DI) water, pH 7, and pH 4. This study indicated possibility of higher OTA reduction in alkaline conditions. Pure OTA standard was used for this study to remove interference of food matrix in reduction efficiency. Further study with food matrix may provide greater picture about heat stability of OTA.

The second part of the thesis emphasized on the reduction of OTA in artificially contaminated oat flakes during extrusion processing. Consumption of oats has increased worldwide due to increasing awareness of health and wellbeing while studies have found beneficial effects of oats including reduced risk of coronary heart diseases. Many food products with oats either as a major or minor ingredient are released continually in the market. However, incidences of OTA contamination have been particularly high in cereals and their derived products. Recent survey in U.S. showed high concentrations of OTA were detected in oat based cereal. Extrusion processing is one of the novel technologies in the area of food processing. It utilizes high temperature, pressure, and shear forces in the cooking process which is believed to be effective in reducing toxin levels present in raw materials. The results of our study showed that OTA is mostly stable during extrusion. Effect of factors such as screw speed, die size, temperature, and moisture was studied to optimize conditions to give highest reduction. It was found that only screw speed and die size affect the reduction significantly. The overall reduction was 0-28%. Such a low reduction was not expected and might be because of very short time of processing being 31-41 sec.

The results of overall study provided basis for further confirmation to heat stable nature of OTA. Further study implying alkaline conditions, more variations in the extrusion processing and use of different food matrixes are required to maximize reduction. Research

on the degradation products OTA and their toxicity after heat processing in future might provide more insights in estimating true losses after processing.

APPENDICES

Appendix 1: Statistical analysis of variance of screw speed, die size, temperature, and moisture on the reduction of ochratoxin A during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	626.20	62.620	1.73	0.100
Linear	4	565.74	141.436	3.91	0.008
Screw Speed	1	204.64	204.637	5.66	0.021
Temperature	1	38.33	38.333	1.06	0.308
Moisture	1	19.21	19.208	0.53	0.469
Die Size	1	303.57	303.565	8.40	0.006
2-Way Interaction	6	46.23	7.704	0.21	0.971
Screw Speed*Temperature	1	3.62	3.619	0.10	0.753
Screw Speed*Moisture	1	1.72	1.717	0.05	0.828
Screw Speed*Die Size	1	0.38	0.380	0.01	0.919
Temperature*Moisture	1	0.08	0.079	0.00	0.963
Temperature*Die Size	1	33.20	33.197	0.92	0.343
Moisture*Die Size	1	7.23	7.234	0.20	0.657
Error	49	1770.61	36.135		
Lack-of-Fit	34	1669.34	49.098	7.27	0.000
Pure Error	15	101.27	6.751		
Total	59	2396.81			

Appendix 2: Statistical analysis of variance of screw speed, die size, temperature, and moisture on the specific mechanical energy (SME) during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	63641.0	6364.1	24.07	0.000
Linear	4	59965.7	14991.4	56.69	0.000
Temperature	1	4769.0	4769.0	18.03	0.000
Moisture	1	29042.8	29042.8	109.82	0.000
Die	1	12413.0	12413.0	46.94	0.000
Screw Speed	1	13740.9	13740.9	51.96	0.000
2-Way Interaction	6	2148.9	358.1	1.35	0.252
Temperature*Moisture	1	644.9	644.9	2.44	0.125
Temperature*Die	1	253.0	253.0	0.96	0.333
Temperature*Screw Speed	1	145.2	145.2	0.55	0.462
Moisture*Die	1	248.0	248.0	0.94	0.338
Moisture*Screw Speed	1	732.7	732.7	2.77	0.102
Die*Screw Speed	1	125.2	125.2	0.47	0.495
Error	49	12958.1	264.5		
Lack-of-Fit	34	12247.4	360.2	7.60	0.000
Pure Error	15	710.6	47.4		
Total	59	76599.1			

Appendix 3: Statistical analysis of variance of screw speed, die size, temperature, and moisture on the pressure during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	764004	76400	85.96	0.000
Linear	4	701336	175334	197.27	0.000
Temperature	1	36647	36647	41.23	0.000
Moisture	1	224425	224425	252.50	0.000
Die	1	440174	440174	495.23	0.000
Screw Speed	1	91	91	0.10	0.751
2-Way Interaction	6	34817	5803	6.53	0.000
Temperature*Moisture	1	4668	4668	5.25	0.026
Temperature*Die	1	11846	11846	13.33	0.001
Temperature*Screw Speed	1	263	263	0.30	0.589
Moisture*Die	1	17683	17683	19.90	0.000
Moisture*Screw Speed	1	0	0	0.00	0.982
Die*Screw Speed	1	357	357	0.40	0.529
Error	49	43552	889		
Lack-of-Fit	34	40420	1189	5.69	0.000
Pure Error	15	3132	209		
Total	59	807556			

Appendix 4: Statistical analysis of variance of screw speed, die size, temperature, and moisture on the residence time during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	149.120	14.9120	6.19	0.000
Linear	4	132.300	33.0751	13.73	0.000
Temperature	1	0.126	0.1262	0.05	0.820
Moisture	1	33.580	33.5798	13.94	0.000
Die	1	5.703	5.7032	2.37	0.130
Screw Speed	1	92.891	92.8910	38.57	0.000
2-Way Interaction	6	12.116	2.0194	0.84	0.546
Temperature*Moisture	1	3.542	3.5420	1.47	0.231
Temperature*Die	1	1.626	1.6259	0.68	0.415
Temperature*Screw Speed	1	0.133	0.1325	0.06	0.816
Moisture*Die	1	1.190	1.1895	0.49	0.486
Moisture*Screw Speed	1	3.335	3.3351	1.38	0.245
Die*Screw Speed	1	2.291	2.2913	0.95	0.334
Error	49	118.018	2.4085		
Lack-of-Fit	34	101.475	2.9846	2.71	0.021
Pure Error	15	16.542	1.1028		
Total	59	267.138			

Appendix 5: Statistical analysis of variance of screw speed, die size, temperature, and moisture on lightness of extrudate during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	254.878	25.488	21.37	0.000
Linear	4	234.881	58.720	49.24	0.000
Screw Speed	1	0.094	0.094	0.08	0.780
Temperature	1	1.812	1.812	1.52	0.224
Moisture	1	232.863	232.863	195.26	0.000
Die Size	1	0.112	0.112	0.09	0.761
2-Way Interaction	6	19.025	3.171	2.66	0.026
Screw Speed*Temperature	1	6.984	6.984	5.86	0.019
Screw Speed*Moisture	1	9.745	9.745	8.17	0.006
Screw Speed*Die Size	1	0.000	0.000	0.00	0.999
Temperature*Moisture	1	1.460	1.460	1.22	0.274
Temperature*Die Size	1	0.161	0.161	0.13	0.715
Moisture*Die Size	1	0.675	0.675	0.57	0.455
Error	49	58.437	1.193		
Lack-of-Fit	34	57.000	1.676	17.50	0.000
Pure Error	15	1.437	0.096		
Total	59	313.315			

Appendix 6: Statistical analysis of variance of screw speed, die size, temperature, and moisture on yellowness of extrudate during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	910.44	91.044	1.98	0.056
Linear	4	495.68	123.921	2.70	0.041
Screw Speed	1	94.79	94.795	2.07	0.157
Temperature	1	77.97	77.966	1.70	0.199
Moisture	1	318.51	318.507	6.94	0.011
Die Size	1	4.42	4.417	0.10	0.758
2-Way Interaction	6	379.42	63.236	1.38	0.242
Screw Speed*Temperature	1	107.32	107.315	2.34	0.133
Screw Speed*Moisture	1	102.80	102.796	2.24	0.141
Screw Speed*Die Size	1	8.69	8.695	0.19	0.665
Temperature*Moisture	1	147.36	147.362	3.21	0.079
Temperature*Die Size	1	6.79	6.789	0.15	0.702
Moisture*Die Size	1	6.46	6.460	0.14	0.709
Error	49	2248.57	45.889		
Lack-of-Fit	34	2248.08	66.120	2045.72	0.000
Pure Error	15	0.48	0.032		
Total	59	3159.01			

Appendix 7: Statistical analysis of variance of screw speed, die size, temperature, and moisture on redness of extrudate during extrusion.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	1261.18	126.118	1.59	0.137
Linear	4	545.17	136.291	1.72	0.160
Screw Speed	1	155.81	155.814	1.97	0.167
Temperature	1	155.25	155.253	1.96	0.168
Moisture	1	229.06	229.062	2.89	0.095
Die Size	1	5.04	5.036	0.06	0.802
2-Way Interaction	6	666.31	111.052	1.40	0.233
Screw Speed*Temperature	1	205.51	205.511	2.60	0.114
Screw Speed*Moisture	1	198.20	198.203	2.50	0.120
Screw Speed*Die Size	1	13.53	13.533	0.17	0.681
Temperature*Moisture	1	222.71	222.711	2.81	0.100
Temperature*Die Size	1	13.58	13.576	0.17	0.681
Moisture*Die Size	1	12.78	12.779	0.16	0.690
Error	49	3878.76	79.158		
Lack-of-Fit	34	3878.68	114.079	22461.37	0.000
Pure Error	15	0.08	0.005		
Total	59	5139.9			