

**Occurrence of alternariol and alternariol monomethyl ether in apple and  
tomato products and resistance to food processing**

by

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## ABSTRACT

# OCCURRENCE OF ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER IN APPLE AND TOMATO PRODUCTS AND RESISTANCE TO FOOD PROCESSING

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The objective of this study was to evaluate the occurrence of alternariol (AOH) and alternariol monomethyl ether (AME) in commercial foods as well as their resistance to food processing techniques. A survey was conducted on the presence of *Alternaria* mycotoxins in 116 apple and tomato products purchased from Canadian markets in the Spring of 2016. AOH and AME were found in 50% and 30% of these products respectively. The highest concentrations found were 75 µg/kg for AOH and 30 µg/kg for AME. In a second experiment tomato juice spiked with 80, 200, and 500 µg/kg of AOH and AME was subjected to high temperature and high pressure processing. A treatment of 121°C for 20 min reduced AOH content by 13.2% and AME by 15.3%. Processing at 600 MPa for 5 min reduced AOH by 24.9% and 12.8%.

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## Table of Contents

ABSTRACT.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Figures.....	vi
List of Tables.....	vii
List of Abbreviations.....	viii
Chapter 1: Introduction.....	1
Chapter 2: Literature review.....	4
2.1 Mycotoxins.....	5
2.2 Alternaria fungi.....	7
2.3 Alternaria mycotoxins.....	9
2.4 Toxicity.....	13
2.4.1 Tetramic derivatives (TeA).....	14
2.4.2 Dibenzopyrone derivatives (AOH, AME, ALT).....	14
2.4.3 Perylene derivatives (altertoxins).....	15
2.5 Regulation.....	15
2.6 Methods of analysis.....	16
2.6.1 Extraction methods.....	16
2.6.2 Chromatographic methods.....	17
2.7 Occurrence in food products.....	18
2.8 Factors affecting fungal growth/mycotoxin production.....	22
2.9 Mitigation of Alternaria toxins in food.....	24
2.10 Masked mycotoxins and co-occurrence of mycotoxins.....	29
2.11 Conclusion.....	30
Chapter 3: Occurrence of alternariol and alternariol monomethyl ether in apple and tomato products from the Canadian market.....	33
Abstract.....	34
3.1 Introduction.....	34
3.2 Materials and methods.....	36
3.2.1 Reagents.....	36
3.2.2 Preparation of standard solutions.....	37
3.2.3 Samples.....	37
3.2.4 Sample preparation.....	37
3.2.5 Mycotoxin extraction.....	38
3.2.6 HPLC conditions.....	38
3.2.7 Method validation.....	38
3.2.8 Exposure assessment.....	39
3.3 Results and discussion.....	40
3.3.1 Method development.....	40
3.3.2 Method validation.....	41
3.3.3 Occurrence.....	42
3.3.4 Exposure assessment.....	44
3.4 Conclusion.....	46

Chapter 4: Comparison of high temperature and high pressure processing treatments for the reduction of alternariol and alternariol monomethyl ether in tomato juice.....	48
Abstract.....	49
4.1 Introduction.....	49
4.2 Materials and methods.....	51
4.2.1 Reagents.....	51
4.2.2 Sample Preparation.....	52
4.2.3 Tomato juice characteristics.....	52
4.2.4 High temperature treatment.....	52
4.2.5 HHP treatment.....	53
4.2.6 Extraction of toxins.....	53
4.2.7 HPLC-DAD analysis.....	53
4.2.8 Data analysis.....	54
4.3 Results.....	54
4.3.1 Effect of temperature on mycotoxin reduction.....	54
4.3.2 Effect of pressure on mycotoxin reduction.....	56
4.3.3 Effect of initial concentration.....	58
4.3.4 Effect of trial.....	58
4.3.5 Effect of processing on pH and TPC.....	60
4.4 Discussion.....	61
4.5 Conclusion.....	64
Chapter 5: Conclusions.....	66
5.1 Conclusions.....	67
5.2 Recommendations.....	68
References.....	70

## List of Figures

Figure 2.1. Chemical structures Alternaria toxins, altenuene (ALT), alternariol (AOH), alternariol monomethyl ether (AME), altertoxin I (ATX-I), altertoxin II (ATX-II), altertoxin III (ATX-III), tenuazonic acid (TeA).....	12
Figure 3.1. Chemical structures of Alternaria toxins alternariol (AOH) and alternariol monomethyl ether (AME).....	35
Figure 3.2. HPLC-DAD chromatograms of 1: apple juice with no AOH or AME, 2: apple juice spiked with 15 µg/kg AOH and 15 µg/kg AME, 3: tomato juice with no AOH or AME, and 4: tomato juice spiked with 15 µg/kg AOH and 15 µg/kg AME. ....	
Figure 4.1. Reduction as a percent of initial concentration of AOH in tomato juice after high temperature processing. Abbreviations: T1 - 90 °C for 10 min, T2 - 90 °C for 20 min, T3 - 121 °C for 10 min, T4 - 121 °C for 20 min.....	55
Figure 4.2. Reduction as a percent of initial concentration of AME in tomato juice after high temperature processing. Abbreviations: T1 - 90 °C for 10 min, T2 - 90 °C for 20 min, T3 - 121 °C for 10 min, T4 - 121 °C for 20 min.....	55
Figure 4.3. Reduction as a percent of initial concentration of AOH in tomato juice after high pressure processing. Abbreviations: P1 – 300 MPa for 3 min, P2 – 300 MPa for 5 min, P3 – 600 MPa for 3 min, P4 – 600 MPa for 5 min.....	57
Figure 4.4. Reduction as a percent of initial concentration of AOH in tomato juice after high pressure processing. Abbreviations: P1 – 300 MPa for 3 min, P2 – 300 MPa for 5 min, P3 – 600 MPa for 3 min, P4 – 600 MPa for 5 min.....	57

## **List of Tables**

Table 2.1. Occurrence of Alternaria toxins in commercial food products. ....	21
Table 3.1. Performance characteristics for the method used for extraction and detection of AOH and AME. ....	42
Table 3.2. Occurrence of AOH and AME in apple and tomato products. ....	44
Table 3.3. Average daily intake of AOH and AME from apple and tomato products, based on the results of this study. TTC values were defined by EFSA (EFSA, 2011), and food consumption data is taken from Statistics Canada (Statistics Canada, 2002). ....	46
Table 4.1. Percent reduction of AOH and AME in tomato juice following processing by high pressure treatments. ....	59
Table 4.2. Percent reduction of AOH and AME in tomato juice following processing by high temperature treatments. ....	60
Table 4.3. Effect of high temperature and high pressure processing on tomato juice quality characteristics. ....	61

## List of Abbreviations

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AOH	Alternariol
AME	Alternariol monomethyl ether
ALT	Altenuene
TeA	Tenuazonic acid
TEN	Tentoxin
ATX-I	Altertoxin-I
ATX-II	Altertoxin-II
ATX-III	Altertoxin-III
AAL	<i>Alternaria alternata</i> f. sp. lycopersici toxins
LLE	Liquid-liquid extraction
SPE	Solid phase extraction
PVDF	Polyvinylidene fluoride
TLC	Thin layer chromatography
GC	Gas chromatography
LC	Liquid chromatography
HPLC	High performance liquid chromatography
UV	Ultra violet
FLD	Fluorescence detector
DAD	Diode array detector
UHPLC	Ultra high performance liquid chromatography
MS	Mass spectrometry
ELISA	Enzyme-linked immunosorbent assay
LOD	Limit of detection
LOQ	Limit of quantification
FAO	Food and Agriculture Organization of the United Nations
EFSA	European Food Safety Authority
EC	European Commission
HHP	High hydrostatic pressure
LD <sub>50</sub>	Lethal dose
GAE	Gallic acid equivalent
GMP	Good manufacturing practices
MPa	Megapascal
TTC	Threshold of toxicological concern

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# Chapter 1: Introduction

Mycotoxins are toxic secondary metabolites produced by fungi and are found in food and feedstuffs around the world. It has been estimated that up to 25% of the world's crops are affected by mycotoxins (Barug *et al.*, 2006). Due to the potential hazard they pose to human health, a number of mycotoxins are regulated in the food supply. These include patulin, ochratoxin A, deoxynivalenol, and the fumonisins (Barkai-Golan and Paster, 2008). As analytical methods continue to develop, more and more mycotoxins are being discovered (Kabak *et al.*, 2006). Thorough risk analysis and development of regulations takes time and a lot of data collection. As such many mycotoxins are not yet regulated.

Included in the list of toxins that are currently unregulated are the *Alternaria* mycotoxins (EFSA, 2011). *Alternaria* is a genus of fungi in which a number of species are able to produce upwards of 70 phytotoxins of which many have been designated as mycotoxins (Ostry, 2008a). Species of these fungi are widespread in the environment and cause disease in several key agricultural crops such as cereal grains, oil seeds, tomatoes, citrus fruits, and apples (Logrieco *et al.*, 2009a). They are of concern to food producers and processors due to the economic impact caused by crop decay as well as the potential health concern from consumption. The most important of the mycotoxins produced by *Alternaria* spp. in terms of their relevance to food safety are alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), and the altertoxins (ATX-I, ATX-II, ATX-III) (Scott, 2004). *Alternaria* toxins are regarded as having relatively low acute toxicity as compared to other mycotoxins (Pero *et al.*, 1973b). There is cause for concern due to evidence of liver and kidney damage in animals as a result of *Alternaria* toxin consumption and it has been reported that alternariol monomethyl ether and alternariol may be factors in human oesophageal cancer (An *et al.*, 1989; Liu *et al.*, 1992).

Occurrence of these toxins has been observed in a few raw and processed commercial products including cereal grains, oils, grapes, tomatoes, and apples (EFSA, 2011). Some assessments have been made in the past by scientific bodies concerning the risk of these toxins but insufficient toxicological and exposure data has prevented accurate analysis (Barkai-Golan and Paster, 2008; BfR, 2003; EFSA, 2011). Due to the potential impact of the *Alternaria* mycotoxins on human health and economic losses they should be viewed as a potential threat. More research is required to set specific guidelines, particularly more information is required on the prevalence of the mycotoxins in commercial products and their stability to processing.

The objective of this study was to provide more information regarding the potential risk of *Alternaria* mycotoxins to human health. To this end the probability of significant exposure to the toxins alternariol and alternariol monomethyl ether was studied by investigating their presence in commercial food products and their resistance to processing techniques.

## **Chapter 2: Literature review**

## 2.1 Mycotoxins

Mycotoxins are a category of secondary metabolite produced by a wide variety of filamentous fungi (Logrieco, 2010; Richard, 2007). While many fungi are capable of producing mycotoxins, the most important genera of the mycotoxin producing fungi are *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium* (Kabak *et al.*, 2006). There are hundreds of different mycotoxins that have been identified and characterized thus far and this number is expected to increase as more advanced analytical techniques are developed (Kabak *et al.*, 2006). There is no set pairing of a specific fungus to a specific mycotoxin. Instead, some fungi are able to produce more than one toxin and some mycotoxins can be produced by more than one fungus (Sakuda and Kimura, 2010). Similar to other secondary metabolites, mycotoxins are typically produced after the exponential growth phase of the fungi during the idiophase (D’Mello and Macdonald, 1997; Demain, 1986; Hussein and Brasel, 2001). Their production is regulated by a complex, not fully understood set of mechanisms, though it is commonly induced by nutrient limitations in the growth medium.

Mycotoxins are not necessary for fungi to grow, develop or reproduce and their purpose is not fully known, though it is theorized that they function as a defence mechanism against other microbial species (Fox and Howlett, 2008). This theory is substantiated by the antimicrobial effect of many of the toxins (Bräse *et al.*, 2013). Another possibility is that mycotoxins serve to weaken the receiving host as a means to increase proliferation of the fungi through improvement of their immediate environment (Yang *et al.*, 2014).

Mycotoxins are common contaminants of food and feedstuffs. The Food and Agricultural Organization (FAO) of the United Nations has estimated that as much as 25% of the world’s

crops are contaminated with mycotoxins (Kabak *et al.*, 2006). Contamination by mycotoxins has been reported in a large number of food products, both raw and processed, in numerous geographical locations (Barug *et al.*, 2006; CAST, 2003; Dashti and AL-Hamli, 2010; Meister, 2004; Soriano and Dragacci, 2004; Yuan *et al.*, 2010). Commonly affected crops include wheat, barley, apples, tomatoes, maize, and grapes. Infection by fungi and subsequent production of mycotoxins can occur in the field, during storage, and/or during processing (Weidenborner, 2011; Yang *et al.*, 2014).

Mycotoxins vary significantly in their chemical structure and physiochemical properties. It is difficult to make any accurate generalization about their chemistry. Their primary similarities are their small molecular weight, their production by fungi and their toxicity against animals and/or humans (Rai and Varma, 2010). As a result of the chemical diversity of mycotoxins, the individual toxicities are quite varied. Consumption of mycotoxins, or mycotoxin contaminated food/feed has been shown to cause teratogenic, oestrogenic, carcinogenic, neurotoxic, immunosuppressive, and mutagenic effects among others (Barug *et al.*, 2006; Moss, 1998; Root and Barrett, 2005). The extent of the toxic effects is dependent on a number of factors including intake level, duration of exposure, the mechanism of action, and characteristics of the individual including sex, age, and health (Hussein and Brasel, 2001).

Due to the known occurrence and toxicity of mycotoxins in foods and feeds, regulations have been established in a number of countries to control these toxins. On a global scale, at least 99 countries had some form of regulation concerning mycotoxins in 2003 (FAO, 2004). Some of the mycotoxins under regulatory scrutiny include aflatoxins, ochratoxin A, patulin, deoxynivalenol, and zearalenone.

Mycotoxins have negative effects on crops, animals, and humans and can cause significant economic losses and serious illness (Zain, 2011). Research has been conducted on methods to prevent fungal contamination of produce and to mitigate the presence of mycotoxins (Barkai-Golan and Paster, 2008). Despite these efforts mycotoxins still persist in our food and feed products. *Alternaria* mycotoxins are a group of mycotoxins produced primarily by the fungal genus *Alternaria*. The *Alternaria* mycotoxins have not yet received the same attention as some of the other mycotoxins. As a result, there are still many areas that require further investigation. There is not enough toxicological data to accurately describe the risk to humans or animals and the rate of occurrence is also unclear. This review will examine all available literature on this set of mycotoxins as an emerging threat to food safety.

## **2.2 *Alternaria* fungi**

The fungal genus *Alternaria* was described in 1816 by Nees von Esenbeck with the species *Alternaria tenuis* (Nees von Esenbeck, 1816). They are black molds that belong to the Deuteromycota division of fungi (Bottalico and Logrieco, 1998). Molds in the *Alternaria* species can be pathogens and saprophytes (Ostry, 2008a). They are able to contaminate and live on both live and decaying plant matter. Some species of *Alternaria* are plant pathogens, known to cause disease and decay in crops (Oviedo *et al.*, 2009). Other species are known for their postharvest pathogenicity and their ability to produce toxic secondary metabolites. *Alternaria* spp. are widely distributed in soil as a common component of its microflora and found almost ubiquitously in the air throughout the world (Barkai-Golan and Paster, 2008; Gregory, 1973). *Alternaria* spp. are able to grow in both humid and semi-arid regions and are able to infect plants growing in the field as well as during harvest and post-harvest stages (EFSA, 2004; Logrieco *et*

*al.*, 2009a). This genus is known to be able to infect more than 4000 different plants and ranks 10<sup>th</sup> among fungal genera in the number of different hosts that it can infect (Farr *et al.*, 1989; Thomma, 2003). *Alternaria* spp, are the primary fungi known to contaminate the cereal crops wheat, sorghum, and barley (Desphande, 2002). Beyond these cereal crops, *Alternaria* fungi are also known to occur in tomatoes, apples, citrus fruits, olives, sunflower seeds, and rape seeds (Barkai-Golan and Paster, 2008).

The most common species is *Alternaria alternata* (Barkai-Golan and Paster, 2008). It contains host-specific pathogen strains, that infect ripening plants, and saprophytic strains capable of spoiling crops postharvest or in storage (Logrieco *et al.* 2009). It is considered one of, if not the most important of the *Alternaria* species because it has a number of variants which produce host-specific toxins and causes diseases in many types of plants. For example, it is responsible for, brown necrotic lesions on foliage, blight on pistachios, black pit disease on potatoes, and brown spot in citrus fruit (Aradhya *et al.*, 2001; Droby *et al.*, 1984; Kohmoto *et al.*, 1979). *Alternaria alternata* is also significant as a mycotoxin producing species of *Alternaria* given that it produces a variety of toxic secondary metabolites including alternariol (AOH), alternariol monomethylether (AME), altertoxin I (ATX-I), altenuene (ALT) and tenuazonic acid (TeA) (Scott, 2004). There are numerous other species of *Alternaria* that are capable of producing mycotoxins. AOH and AME have been known to be produced by *A. citri*, *A. cucumerina*, *A. dauci*, *A. tenuissima*, *A. brassicae*, *A. capsici-annui*, *A. kikuchiana*, *A. longipes*, *A. porri*, *A. solani* and *A. tomato* (Bottalico and Logrieco, 1998; Pose *et al.*, 2004). TeA though most commonly produced by strains of *A. alternata*, has also been observed from *A. citri*, *A.*



*longipes*, *A. porri*, *A. japonica*, and *A. tenuissima*. Finally, the ATX toxins are produced by *A. radicina*, *A. tenuissima*, *A. mali* and *A. tomato* (Bottalico and Logrieco, 1998).

Since being discovered there have been a large number species of this genus described with the Index fungorum listing 636 distinct species (CABI, 2004). *Alternaria* fungi are characteristically defined as having dark black or green conidia and mycellium (Pitt and Hocking, 2009). They have multicelled conidia with both transverse and longitudinal septa that occur either in chains or singly and have tapering apical cells or an apical beak (Elliot, 1917; Neergaard, 1945; Simmons, 1992; Wiltshire, 1933). Species of *Alternaria* are typically differentiated by a set of characteristics including size, presence/size of a beak, septation, and pattern of catenation. There have been several attempts at classification of *Alternaria* species, based solely on morphology leading to confusion in the nomenclature (Pastor and Guarro, 2008). It has been suggested that issues have arisen due to significant variation in conidium size, shape, and septation that can be found within a species and even within a single fungal colony (Andersen *et al.*, 2006; Peever *et al.*, 2005). There is also overlap in morphological properties between closely related species that can make it difficult to differentiate. Modern molecular based identification methods are being developed due to their increased speed and accuracy (Andersen *et al.*, 2015). Further research is required in this area, as a complete assessment of the taxonomy has not been completed using these methods (Kordalewska *et al.*, 2015; Patriarca, 2016).

### **2.3 *Alternaria* mycotoxins**

Mycotoxins, including those produced by *Alternaria* species, are secondary metabolites of fungi known to develop on agricultural crops and produce (Chelkowsi and Visconti, 1992;

King and Schade, 1984; Patriarca *et al.*, 2007). *Alternaria* spp. are known to produce more than 120 different secondary metabolites (Panigrahi, 1997). Of these 70 or more are phytotoxins, and a small portion of these have been chemically categorized as mycotoxins (Barkai-Golan and Paster, 2008; Bottalico and Logrieco, 1992; Ostry, 2008a). These metabolites can be classified according to their effect on plants, either host-specific compounds that are toxic to the plant or non-host-specific ones such as mycotoxins that are toxic to humans or animals. While not all the secondary metabolites have been characterized, the following have been listed as possible food contaminants and mycotoxins; altenuene, alternariol, alternariol monomethyl ether, tenuazonic acid, tentoxin, altertoxin I, II, II, stemphytoxin III, and the *Alternaria alternata* f. *sp. lycopersici* toxins (EFSA, 2004).

The *Alternaria* mycotoxins are divided into 5 categories based on their chemical structure (Figure 2.1); dibenzopyrone derivatives (AOH, AME, and ALT), perylene derivatives (ATX-I, -II, -III, and stemphytoxin III), tetramic acid derivatives (TeA), *Alternaria alternata* f. *sp. lycopersici* toxins (AAL toxins), and a fifth class used for toxins with miscellaneous structure such as tentoxin (TEN) (Bottalico and Logrieco, 1998; EFSA, 2004). The most important of the *Alternaria* mycotoxins from a food safety perspective are considered to be AOH, AME, ATX-I, II, III, ALT and TeA (Barkai-Golan and Paster, 2008). The *Alternaria* mycotoxins, AOH, AME, TeA, ALT, and the ATX toxins were isolated and characterized in 1953, 1953, 1957, 1971, and 1973 respectively (Pero *et al.*, 1971, 1973a; Raistrick *et al.*, 1953; Rosset *et al.*, 1957).

Their chemical structures are as follows (CTD, 2008):

AOH: (3,7,9-trihydroxy-1-methyl-6H-dibenzo[b,d]pyran-6-one); MW (molecular weight) 258; molecular formula C<sub>14</sub>H<sub>10</sub>O<sub>5</sub>.

AME: 3,7-dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one; MW 272; molecular formula  $C_{15}H_{12}O_5$ .

ALT: (2 $\alpha$ ,3 $\alpha$ ,4 $\beta$ -tetrahydro-2,3,7-trihydroxy-9-methoxy-4a-methyl-6H-dibenzo[b,d]pyran-6-one); MW 292; molecular formula  $C_{15}H_{16}O_6$ .

ATX-I: 1,2,7,8,12b-pentahydro-1,4,6b,10-tetrahydroperylene-3,9-dione; MW 352; molecular formula  $C_{20}H_{16}O_6$ .

ATX-II: [perlylo(1,2-b)oxirene-7,11-dione,7a,8a,8b,8c,9,10-hexahydro-1,6,8c-trihydroxy-, (7aR,8aR,8bS,8cR)-]; MW 350; molecular formula  $C_{20}H_{14}O_6$ .

ATX-III: [perlylo(1,2-b:7,8-b')bisoxirene-5,10-dione, 1a,1b,5a,6a,6b,10a-hexahydro-4,9-dihydroxy-]; MW 348; molecular formula  $C_{20}H_{12}O_6$ .

TeA: (3-acetyl-5-sec-butyl-4-hydroxy-3-pyrrolin-2-one); MW 197; molecular formula  $C_{10}H_{15}O_3N$ .

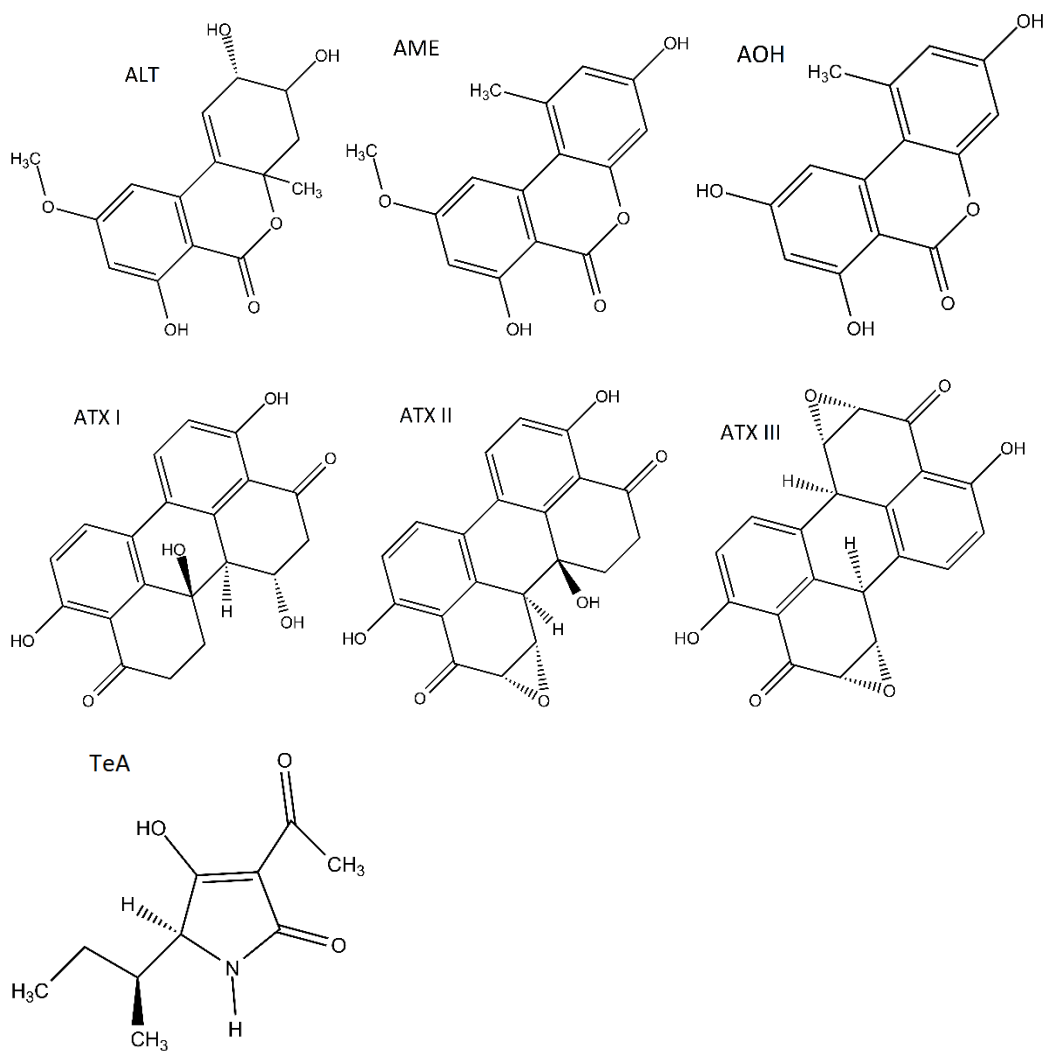


Figure 2.1. Chemical structures *Alternaria* toxins, altenuene (ALT), alternariol (AOH), alternariol monomethyl ether (AME), altertoxin I (ATX-I), altertoxin II (ATX-II), altertoxin III (ATX-III), tenuazonic acid (TeA).

The physical and chemical characteristics have been reported for some of the *Alternaria* toxins. TeA is a colourless, viscous oil, a monobasic acid with pKa 3.5, and it is soluble in chloroform and methanol (EFSA, 2004). AOH and AME will crystallize from ethanol as colourless crystals, with melting points of 350 °C and 267 °C respectively. These mycotoxins are soluble in several organic solvents. ALT and ATX-I have melting points of 190-191 °C and 180

°C, respectively. Under ultraviolet light ATX is known to fluoresce with a yellow-orange colour and AOH, AME, and ALT are characterized by a violet-blue colour (Betina, 1993).

## 2.4 Toxicity

Species in the *Alternaria* genus are toxic to a variety of organisms including bacteria, fungi, and plants (Brian *et al.*, 1949; Gitterman, 1965; Pero and Main, 1970; Raistrick *et al.*, 1953). As previously mentioned some of the *Alternaria* species also produce toxic secondary metabolites. The *Alternaria* mycotoxins have been linked to a number of toxic health effects in both animals and humans.

As compared to other mycotoxins, the *Alternaria* group of toxins have a relatively low acute toxicity. This makes the chances of a case of acute toxicosis of humans by these mycotoxins unlikely (Olsen and Visconti, 1988; Pero *et al.*, 1973b; Pollock *et al.*, 1982). TeA is considered to have the highest toxicity of the *Alternaria* toxins while AOH, AME, and ATX-I, II, and III are more significant for their mutagenic properties (Vinas *et al.*, 1992). Isolates from *Alternaria* culture grown in a laboratory setting have been found to be toxic to a variety of organisms including chicken embryos, chickens and rats and human cell cultures (Griffin and Chu, 1983; Pero *et al.*, 1973b; Sauer *et al.*, 1978). One study found toxic effects on the liver and kidneys of rats fed with *A. alternata* fungus over a period of 28 days (Combina *et al.*, 1999b).

Of the number of toxic secondary metabolites produced, AOH, AME, ALT, ATX-I, II, II, and TeA are considered to be the most important from a food safety perspective. These 5 toxins fall into 3 chemical groups; the perylene derivatives, the tetramic acid derivatives and the dibenzopyrone derivatives (Logrieco *et al.*, 2009a).

#### 2.4.1 Tetramic derivatives (TeA)

This group of mycotoxins can act as both mycotoxin and phytotoxin. TeA is known to exhibit insecticidal, cytotoxic, antibacterial, antitumor, antiviral, phytotoxic, and zootoxic effects (Logrieco et al. 2009). They are produced primarily by *A. alternata*, but has also been found to be produced by other species in the *Alternaria* genus. The tetramic derivatives are considered to have the highest toxicity of the *Alternaria* toxins (Weidenborner, 2011). TeA inhibits protein biosynthesis by suppressing the release of newly formed proteins from the ribosome (Carrasco and Vazquez, 1973)(Shigeura and Gordon, 1963). It has been shown to be acutely toxic for a number of animal species including mice, chickens, and dogs (Griffin and Chu, 1983). It has been found to significantly reduce feed efficiency, causing decreased weight gain as well as internal haemorrhaging in chickens when it is present in their feed. TeA has been reported to be a factor in some haematological disorders in humans such as onyalai, which is a type of thrombocytopenia characterized by a decrease in platelet levels in the blood (Steyn and Rabie, 1976). In dogs, TeA has been observed to cause hemorrhages in organs at doses of 10 mg/kg bw and sub-acute toxicity in chickens when in contaminated feed at 10 mg/kg bw (Scott, 2004). This mycotoxins has also been found to be toxic to a wide variety of other organisms including plants, fungi, bacteria, and viruses (Logrieco *et al.*, 2003). One study included TeA in the diet of mice for 10 months (Yekeler *et al.*, 2001). They reported moderate to severe dysplasia of esophageal cells.

#### 2.4.2 Dibenzopyrone derivatives (AOH, AME, ALT)

These mycotoxins are produced primarily by *A. alternata* but have also been shown to be produced by other species such as *A. brassicae*, *A. capsici-annui*, *A. citri*, *A. cucumerina*, and *A.*

*tenuissima* (Logrieco et al. 2009). AOH and AME have been reported to be genotoxic, cytotoxic, and mutagenic. AOH is thought to be more genotoxic than AME as shown by assays with human colon carcinoma cells (Brugger *et al.*, 2006; Fehr *et al.*, 2007; Ostry, 2008a). These 2 mycotoxins also have been shown some carcinogenic properties (Yekeler *et al.*, 2001). The acute toxicity of AOH and AME is low (LD<sub>50</sub>: 400 mg/kg bw), however both compounds have shown high cytotoxicity in cell culture (Pero *et al.*, 1973b). It has been reported that AOH and AME may be a factor in increased incidence of oesophageal cancer in China (Liu *et al.*, 1992; Pero *et al.*, 1973b).

#### 2.4.3 Perylene derivatives (altertoxins)

This group is comprised mainly of the altertoxins, ATX-I, II, and III. They are produced primarily by *Alternaria alternata* but have been isolated from other species including *Alternaria tenuissima* and *Alternaria radicina* (Logrieco et al. 2009). The main toxic property of this group is their mutagenicity as demonstrated in the Ames test (Stack and Prival, 1986). In this experiment, it was found that ATX-III was approximately 1.5 times more mutagenic than ATX-II, which in turn is 23 times more mutagenic than ATX-I. ATX-I and ATX-III have a role in cell transformation (Ostry, 2008a). The ATX toxins are thought to be more mutagenic than AOH or AME (Chelkowsi and Visconti, 1992). ATX-I has acute toxic effects, it has shown toxicity in mice and is highly mutagenic in mammalian cell lines.

### 2.5 Regulation

Currently there are no regulations concerning *Alternaria* toxins in food stuffs or feed anywhere in the world (EFSA, 2004). The potential risk of *Alternaria* mycotoxins has been assessed by the Federal Institute of Risk Assessment in Germany (BfR), the Agence Francaise

De Securite Sanitaire Des Aliments (AFSSA), and the European Food Safety Authority (EFSA). The BfR concluded that there is insufficient data to properly carry out an assessment of risk and more research is required concerning the occurrence and toxicology of the *Alternaria* mycotoxins (BfR, 2003). In France, the AFSSA determined that *Alternaria* toxins are not a priority health risk for produce and animal feed. They did state that due to the mutagenic effects seen in in vitro studies, further genotoxicity studies were recommended. Similarly, the EFSA recommends further research into the toxic effects of the *Alternaria* toxins as well as the extent of their presence in food for both animal and human consumption (EFSA, 2004). While they recognized the potential threat of these mycotoxins, given the lack of thorough toxicological and occurrence data, there was no significant justification for the creation of regulatory limits.

## **2.6 Methods of analysis**

### **2.6.1 Extraction methods**

Prior to analysis, the *Alternaria* mycotoxins must be removed and isolated from the food matrix they are in. For this purpose, either a liquid/liquid extraction (LLE) or a solid phase extraction (SPE) method is used. LLE has been used for several different food products including oil seeds (Nawaz *et al.*, 1997), cereals (Siegel *et al.*, 2009; Yu *et al.*, 1999), tomatoes (Harwig *et al.*, 1979), and fruit juices (Lau *et al.*, 2003a). Organic solvents such methanol, ethyl acetate, and chloroform are most commonly used. Extraction with SPE has been used for food products such as fruit and vegetable juices (Asam *et al.*, 2009; Delgado *et al.*, 1996), tomato purees (Zhao *et al.*, 2015a), and whole fruit (Prelle *et al.*, 2013). SPE methods for *Alternaria* mycotoxins typically use aminopropyl, hydrophilic-lipophilic balance, or C18 columns or a combination of the three resins.



## 2.6.2 Chromatographic methods

Most analytical methods for the detection and quantification of *Alternaria* mycotoxins involve some form of chromatography. Thin layer chromatography (TLC) is one of the simpler and cost effective methods available (Man *et al.*, 2017). A few methods of TLC have been developed for some of the *Alternaria* mycotoxins (Hasan, 1996; Matysik and Giryn, 1996; Ostry *et al.*, 2005). TLC has been used for AOH, AME, ALT, ATX-I, and TeA, with limits of detection (LOD) ranging from 100 µg/kg to 2 µg/kg. Compared to other forms of chromatography TLC has the lowest sensitivity, though it can still be an effective tool.

Gas chromatography (GC) has also been used for the analysis of mycotoxins (Pero *et al.*, 1973a; Scott *et al.*, 2006). High sensitivity and selectivity of this method make it suitable for analysis of complex food matrices. The major downside to using GC is that because the *Alternaria* mycotoxins are nonpolar and non-volatile, they must be derivatized before analysis (Pero *et al.*, 1973a). This increases the cost and time of analysis and decreases repeatability. In apple juice a LOD of 1 µg/kg for AOH and AME has been reported (Scott *et al.*, 2006).

The most common means of analyzing *Alternaria* toxins is the use of liquid chromatography (LC) coupled with some form of detector. Historically LC or high performance liquid chromatography (HPLC) coupled with UV detection (Stack and Prival, 1986), fluorescence detection (FLD) (Nawaz *et al.*, 1997) and diode array detection (DAD) (Delgado *et al.*, 1996) have all been studied. The LOD has been reported to be as low as 0.7 µg/kg in apple juice (Delgado *et al.*, 1996). Many types of *Alternaria* mycotoxin have been studied with these methods including AOH, AME, ALT, ATX-I, ATX-II, TEN and TeA. To achieve even high levels of sensitivity more recent innovations include the use of reversed phase HPLC or ultra

high performance liquid chromatography (UHPLC) coupled with single or tandem mass spectrometry (MS) (Lau *et al.*, 2003a; Magnani *et al.*, 2007; Siegel *et al.*, 2009). This technique has allowed for LOD as  $< 0.13 \mu\text{g}/\text{kg}$  (Magnani *et al.*, 2007).

More recent research has looked into the development of immunochemical detection methods. These methods are known for their relatively low cost and high speed, and ease of use, however have only been used for a select few *Alternaria* toxins. Enzyme linked immunosorbent assays (ELISA) have been used for detection of AAL toxins in feed with a reported limit of quantification of  $50 \mu\text{g}/\text{kg}$  (Yu *et al.*, 1999). Another study similarly used ELISA to detect AOH in feed with an LOQ of  $20 \mu\text{g}/\text{kg}$ . A different study developed an enzyme immunoassay using rabbit and mouse antibodies to detect AOH with a LOD of  $1 \mu\text{g}/\text{kg}$ . The only other *Alternaria* mycotoxin to be detected with an immunoassay is TeA in apples and tomatoes with an LOD of  $25 \mu\text{g}/\text{kg}$  (Gross *et al.*, 2011).

## **2.7 Occurrence in food products**

The occurrence of *Alternaria* toxins has been reviewed before and is summarized in table 2.1 (Ostry, 2008a)(Logrieco *et al.* 2009). The maximum level of the toxins in commercial products was reported to range from  $1 - 1000 \mu\text{g}/\text{kg}$ . Higher levels are found in foods with visible signs of infection by *Alternaria* rot, making them undesirable for human consumption whether there is mycotoxin contamination or not.

AOH has been found in a wide variety of products including both raw produce and processed foods and beverages. Samples of pulses, oilseeds, tomatoes, cereal grains, and carrots as well as fruit juice, wine, tomato products and oils have all been found with some level of

contamination (Ackermann *et al.*, 2011; Asam *et al.*, 2009; Delgado *et al.*, 1996; Kravlova *et al.*, 2006; Ostry *et al.*, 2004; Solfrizzo *et al.*, 2004; Visconti *et al.*, 1986; Wittkowski *et al.*, 1983). The highest levels reported were found in tomato puree and lentils at 8756 µg/kg and 290 µg/kg respectively. The lowest quantifiable levels were generally found in fruit and vegetable juices.

Occurrence of AME has been reported in oilseed, tomato products, fruit juice, wine, cereal grains, and vegetables (Asam *et al.*, 2009; Delgado *et al.*, 1996; Kravlova *et al.*, 2006; Olsen and Visconti, 1988; Ostry, 2008a; Ostry *et al.*, 2004; Scott *et al.*, 1997; Solfrizzo *et al.*, 2004; Terminiello *et al.*, 2006b; Wittkowski *et al.*, 1983). The highest concentrations seen have been in grape juice and tomato puree at 39.5 µg/kg and 1734 µg/kg respectively. In contrast to this, a different study looking at 142 tomato products including puree, found no evidence of AME in any of the samples (Motta and Valente Soares, 2001).

The toxin ALT has been observed in grains and processed grain products and to a lesser extent in legumes and oilseed (Kravlova *et al.*, 2006). The amount found is typically in very low concentrations. Other products such as wine, fruits, vegetables, and oils have been examined for ALT content, but none was found to be present (Ostry *et al.*, 2004; Solfrizzo *et al.*, 2004; Visconti *et al.*, 1986; Wittkowski *et al.*, 1983).

TeA was present in a variety of products including cereal grains, beer, processed grain products, and tomato products (Ostry *et al.*, 2004; Siegel *et al.*, 2009; Solfrizzo *et al.*, 2004; Visconti *et al.*, 1986). The highest concentrations of TeA were observed in tomato products, with amounts greater than 1000 µg/kg (Terminiello *et al.*, 2006b). However a different survey with tomato products found no TeA content (Motta and Valente Soares, 2001).

ATX-I has not been found in significant amount in fresh fruits or vegetables, processed foods, or in cereal and oil grains (Ostry *et al.*, 2004; Solfrizzo *et al.*, 2004; Visconti *et al.*, 1986; Wittkowski *et al.*, 1983).

Table 2.1. Occurrence of *Alternaria* toxins in commercial food products.

Food Commodity	Location	TeA Range (µg/kg) (% Positive)	AOH Range (µg/kg) (% Positive)	AME Range (µg/kg) (% Positive)	ALT Range (µg/kg) (% Positive)	ATX-I Range (µg/kg) (% Positive)	Reference
Wheat	Serbia	2.5-2676 (68)	0.75-48.9 (12)	0.49-70.2 (6.5)	-	-	(Janić Hajnal <i>et al.</i> , 2015)
Wheat	Germany	4224 (30)	832 (8)	905 (23)	-	-	(Müller and Korn, 2013)
Wheat	Argentina	1001-8814 (19)	645-1348 (6)	546-7451 (0.6)	-	-	(Azcarate <i>et al.</i> , 2008)
Sunflower meal	United Kingdom	350-6000 (100)	40-1000 (64)	40-1000 (73)	0	0	(Nawaz <i>et al.</i> , 1997)
Oilseed	United Kingdom	500-999 (30)	50-99 (7)	40-99 (7)	0	0	(Nawaz <i>et al.</i> , 1997)
Olive fruit	Italy	109-262 (15)	109-2320 (31)	30-2870 (31)	1400 (8)	0	(Bottalico and Logrieco, 1993)
Beer	Germany	8-175 (86)	-	-	-	-	(Siegel <i>et al.</i> , 2010b)
Soya beans	Argentina	-	25-211 (46)	62-1153 (44)	-	-	(Oviedo <i>et al.</i> 2012)
Dried wine berries	Slovakia	4-18973 (77)	52-1308 (85)	26-776 (77)	48-4120 (54)	7.7-159 (85)	(Mikusova <i>et al.</i> , 2012)
Citrus juice	China	1.21-4.3 (25)	0	0.11-0.2 (11)	-	-	(Zhao <i>et al.</i> , 2015)
Orange juice	Germany	-	0.16-0.24 (100)	0	-	-	(Asam <i>et al.</i> , 2009)
Apple juice	Italy	24.3-45.3 (20)	0	0	45.6 (10)	-	(Prelle <i>et al.</i> , 2013)
Apple juice concentrate	Spain	-	1.35-5.42 (50)	1.71 (50)	-	-	(Delgado and Gómez-Cordovés 1998)
Apple juice	Germany	-	0.16-0.22 (75)	0	-	-	(Asam <i>et al.</i> , 2009)
Ketchup	China	10.2-1787 (100)	2.5-300 (45)	0.32-38 (90)	-	-	(Zhao <i>et al.</i> , 2015)
Tomato puree	Argentina	29-4012 (36)	8.8-187 (7.5)	1.7-84 (32)	-	-	(Terminiello 2006)
Tomato sauces	Italy	0	4.0-6.8 (50)	0	3.8-4.8 (80)	-	(Prelle <i>et al.</i> , 2013)
Tomato sauces	Switzerland	0	4-33 (65)	1-9 (71)	0	0	(Noser <i>et al.</i> , 2011)
Ketchup	Switzerland	0	4-5 (16)	1 (16)	0	0	(Noser <i>et al.</i> , 2011)
Tomato puree	Switzerland	0	4-10 (33)	1-4 (29)	0	0	(Noser <i>et al.</i> , 2011)
Red wine	Germany	-	0.36-7.5 (100)	0.04-0.15 (100)	-	-	(Asam <i>et al.</i> , 2009)
White wine	Germany	-	0.1-7.59 (100)	0	-	-	(Asam <i>et al.</i> , 2009)
Grape juice	Germany	-	0.1-1.05 (100)	0	-	-	(Asam <i>et al.</i> , 2009)
Red wine	Canada	-	0.03-7.41 (80)	0.01-0.23 (80)	-	-	(Scott <i>et al.</i> , 2006)
White wine	Canada	-	0.67-1.48 (9)	0.02-0.06 (9)	-	-	(Scott <i>et al.</i> , 2006)
Grape juice	Canada	-	0.06-0.46 (50)	0.01-39.5 (50)	-	-	(Scott <i>et al.</i> , 2006)

## 2.8 Factors affecting fungal growth/mycotoxin production

The growth of fungi and the production of mycotoxins are influenced by a number of different factors and conditions (Chamley *et al.*, 1994). Fungi and mycotoxins are dependent primarily on 3 factors; the environmental conditions (such as temperature and  $a_w$ ), the fungal species and strain, and the substrate or growth medium (Magan and Baxter, 1994; Young *et al.*, 1980).

It has been reported that the optimal temperature range for *Alternaria* spp. growth is 22-28 °C, the minimal growth temperature is reported as anywhere between -3 °C and 6.5 °C with a maximum of around 36 °C (Hasija, 1970; Pitt and Hocking, 1997; Sommer, 1985). Due to this temperature behaviour, this pathogen is of particular risk to fruits and vegetables exposed to chill injury (Ostry, 2008a). Generally, a high water activity is necessary for the occurrence of the *Alternaria* toxins, maximum production of the mycotoxins AOH, AME, and ALT has been found to occur at 25 °C and 0.98  $a_w$  (Magan *et al.*, 1984). The minimum  $a_w$  for growth has been reported at 0.88 (Hocking *et al.*, 1994) and the pH range for growth is 2.7 – 8.0, with optimal conditions at pH of 4-4.5 (Hasija, 1970). Temperature at which produce is kept is a large factor in the production of mycotoxins. It has been reported that the optimum temperatures for AOH, AME, TeA, ATX-I, and ATX-II production by *Alternaria* fungi are 28 °C, 28 °C 21 °C, 14 °C, and 14 °C respectively (Hasan, 1996). Significant growth of the fungi and production of the toxins occurred at 7 °C but decreased at 35 °C. Others have suggested that the optimal conditions for production of the mycotoxin TeA occurred at 0.98  $a_w$  and between 25 and 30 °C depending on the strain of fungus (Oviedo *et al.*, 2009). The interactions of different water activities, temperatures and incubation times play a large role in the production of TeA. For example, the

mycotoxin was produced at various temperatures ranging from 5 °C to 30 °C and at  $a_w$  from 0.92 to 0.995, however at  $a_w$  below 0.94 little to no TeA was produced in the temperature range of 5-18 °C. The effect of temperature on the production of mycotoxins has also been studied in fruit models (Ozcelik *et al.*, 1990). In this study tomatoes were stored at varying temperatures and tested for presence of ALT, AOH, AME, and TeA after being inoculated with a strain of *Alternaria alternata*. Growth of the fungi and production of the mycotoxins occurred in a range of temperatures from 4 °C to 25 °C. The fastest rate of mycotoxin production occurred at 25 °C. The effect of storing apples in a polyethylene film wrap was also studied here (Ozcelik *et al.*, 1990). When wrapped in film, none of the *Alternaria* mycotoxins were detected in apples stored at either 5 °C or 15 °C. However, at 25 °C *Alternaria* toxins were produced and in similar amounts to that found in the unwrapped apples. This suggests that the polyethylene film created an atmospheric environment that inhibited the growth of the fungi, but did little to prevent the actual production of mycotoxins. Another study looked at the occurrence of *Alternaria* spp. and their related mycotoxins in decayed apples stored at different temperatures (Vinas *et al.*, 1992). A total of 157 samples of apples from a variety of sources were tested. It was found that 110 different strains of *Alternaria* could be isolated from these samples. Of the apples, 47% had AOH, 41% had AME and 38% had both when stored at 25 °C for 7 days. Although found in few samples, both AOH and AME were found in apples stored as low as 2 °C. *Alternaria alternata* is able to grow in low oxygen conditions as well, reported as low as 0.25% (v/v), though with slower growth rate (Follstad, 1966; Wells and Uota, 1970).

The composition of the media on which the *Alternaria* is grown also appears to have an effect on the mycotoxin production. Fourteen different *Alternaria* isolates were investigated for

their ability to produce AME and AOH on different substrates (Maas et al 1981). It was found that AOH and AME could be produced on both a solid rice and a complex liquid media. The amount of the toxins produced varied between the media as well as among the different *Alternaria* strains, with the highest yield of 417  $\mu\text{g/g}$  AOH and 53  $\mu\text{g/g}$  AME being found on the rice medium. A similar study examined the growth and mycotoxin production of an *Alternaria alternata* strain in synthetic, semisynthetic and rice culture mediums (Wei and Swartz, 1985). They found that the toxins were produced in the later stages of fungal growth and that higher levels were produced in the semisynthetic medium.

Most fresh produce samples that are highly contaminated with mycotoxins also show visible signs of damage, such as rot, mold, and bruising. Therefore there is little risk of direct consumption by consumers. However, it is common practice to utilize damaged produce for the production of processed foods including juices or sauces. This suggests that it is the processed goods that are the highest risk group. While less commonly observed, the toxins are not necessarily limited to the damaged portions of the produce (Barkai-Golan and Paster, 2008). In an examination of apples infected with *Alternaria alternata*, AOH and AME were found in undamaged tissue (Robiglio and Lopez, 1995). Furthermore, the toxins were found in mycelium-free tissues, suggesting that the toxins are able to migrate throughout the fruit on their own. This brings up the potential danger of even apparently sound looking fruit.

## **2.9 Mitigation of *Alternaria* toxins in food**

Various means have been studied for their ability to suppress the growth of *Alternaria* fungi and reduce the production of mycotoxins in food. The presence of *Alternaria* spp. and related toxins is both a health concern as well as an economic one due to potential losses from



harvest and post-harvest decay (Andersen et al. 2006; Patriarca et al. 2007; Logrieco et al. 2009). Prevention of fungal contamination is considered by some to be a more effective means of control than removal or destruction of the mycotoxins by processing (Aldred and Magnan, 2004). *Alternaria* spp. is unable to penetrate sound plant tissue, thus for contamination to occur, tissue damage is required (Barkai-Golan and Paster, 2008). This would suggest that the most effective means of prevention of *Alternaria* mycotoxins is in the proper care and handling of the produce. A system of Good Agriculture Practices (GAP), Good Manufacturing Practices and implementation of Hazard Analysis Critical Control Point (HACCP) concepts has been suggested to improve the quality of crops and to reduce the risk of contamination (Codex Alimentarius Commission, 2002).

The effect of agricultural practices as well as environmental conditions on the production of *Alternaria* toxins has studied in maize (Mansfield *et al.*, 2007). Maize was tested for 2 *Alternaria alternata* f. *sp. lycopersici* toxins (AAL-TA, AAL-TB) which were found in 23% and 13% of samples respectively. During maize growth temperature was negatively correlated with mycotoxin occurrence but moisture was positively correlated with mycotoxin production. Use of silo storage techniques and agricultural methods such as a tillage system, different silo types or inoculant use, did not appear to have any effect on the toxin concentration in samples. During the ensiling and storage of sunflower seeds, AOH and TeA levels decreased over the course of 2 – 4 months (Dalcero *et al.*, 1996). After 4 months however, 20% of the samples showed an increase in AOH concentration. AME was found after 2 months and in an increased amount after 4. This suggests that infection or growth of *Alternaria* fungi can occur during storage. The stability of *Alternaria* mycotoxins in fruit juice and wine has also been studied (Scott and Kanhere, 2001).

AOH and AME were found to be stable, suffering no significant losses when kept in apple juice for 20 d at room temperature or at 80 °C for 20 min. Similarly, ATX-I was stable in apple juice over a 27 d period. These findings suggest that steps must be taken to eliminate the mycotoxins before packaging to prevent consumer consumption of the toxins. While AOH and AME are found in fruit beverages, it is quite uncommon to find TeA. This is likely explained by the increased stability to heat processing of AOH and AME as compared to TeA. Based on these studies as well as data on the natural occurrence of *Alternaria* toxins in foods and feeds as previously discussed, it is evident that current agricultural practices are not sufficient to completely control these toxins. Compared to other mycotoxins, only limited research has been conducted to study mitigation, and decomposition of *Alternaria* toxins in food stuffs. Heat treatment is a common food processing technique that is used to increase food safety. In flour made from sunflower seeds heated to 100 °C, AOH and AME were found to be stable for 90 min with no significant losses, whereas TeA was reduced by 50% (Combina *et al.*, 1999a). In order to reduce AME and AOH content effectively, a treatment of 121 °C for 60 min was required. The fate of the mycotoxins AOH, AME, and ALT was examined during the bread baking process of inoculated wheat flour (Siegel *et al.* 2010). Wheat was chosen due to the known occurrence of *Alternaria* contamination in this crop (Li and Yoshizawa, 2000; Ostry *et al.*, 2005; Patriarca *et al.*, 2007). It was found that only a small amount of any of the toxins degraded during wet baking process but a significant amount occurred during dry baking (Siegel *et al.*, 2010a). The stability, in decreasing order, of the mycotoxins was observed to be AME > AOH > ALT. AOH and AME were also found to produce degradation products through the process, thought to be formed by a series of hydrolysis and decarboxylation reactions during heat processing. The toxicity of these

breakdown products was not studied. A second portion of the study examined commercial baked goods for the presence of the same three mycotoxins as well as some of their breakdown products. Of the 24 samples tested, none were found to have AOH, 1 had AME, 2 had ALT, and 4 had one or more of the breakdown products of AOH and AME. These two studies suggest that some of the *Alternaria* mycotoxins are at least partially stable to heat processing. Further study is required using different media and different heat treatments to better understand the fate of these toxins.

There has been some research into the inhibition of fungal growth and mycotoxin production through a chemical means. One study found that significant inhibition of the production of *Alternaria* mycotoxins was attained with the addition of 500 ppm cinnamon oil (Hasan, 1996). The chemical stability of AOH, AME and ALT was studied at different pHs (Siegel *et al.*, 2010a). All 3 toxins were stable in a 0.15 M phosphate buffer at pH 5, but were entirely degraded in a 0.1 M KOH solution. ALT was found to be stable in a 0.18 M phosphate/citrate solution of pH 7, but both AOH and AME were degraded. The mechanism of degradation is thought to be hydrolysis of the lactone group and a decarboxylation reaction. Further study is required to determine the chemical and toxicological nature of these breakdown products.

Sodium benzoate, potassium sorbate, and sodium propionate are all commonly used in food processing as preservative agents. Due to their solubility, taste and low toxicity these chemicals are suitable for use as food additives. They function as antifungal agents because of their ability to decrease intracellular pH and/or disrupt the substrate transport of fungal cells by altering cell membrane permeability (Fresse *et al.*, 1973; Liewen and Marth, 1985). Organic

acids can also inhibit nicotinamide adenine diphosphate oxidation, causing the elimination of reducing agents to the electron transport system in fungi and inhibiting growth. These preservatives have been tested at various concentrations from 1 mg/kg to 200 mg/kg on both TeA and on *Alternaria alternata* grown on an agar culture medium (Combina *et al.*, 1999b). None of the treatments had any effect on detoxifying or destroying the mycotoxin itself, but all of the treatments had an effect on the fungi. Concentrations of sodium benzoate and potassium sorbate at about 10 mg/kg caused total inhibition of fungal growth and TeA production and sodium propionate could cause up to 59% inhibition at 200 mg/kg. Similarly, minimal effects on the growth of *Alternaria alternata* and production of TeA were seen when insecticides and fungicides were applied (Dalcero *et al.*, 1995, 1996). Variable results were observed with some chemical agents having no effect, some causing the inhibition of fungal growth, and some stimulating fungal growth and TeA production.

While the control means discussed so far are of common use in the food industry, other more non-conventional means of control have also been studied. Biological control has been achieved through the competitive growth of other microorganisms. One study found that *Alternaria* and its' associated mycotoxins are not of major concern in strawberries due to the presence of fast growing molds such as *Botrytis* and *Rhizopus* species (Tournas and Stack, 2001). This suggests a potential means of biocontrol of *Alternaria*, however it is likely not feasible as the fruit would be unusable if infected with the other molds. Production of the *Alternaria* mycotoxins on wheat grain by *Alternaria alternata* and the effects of temperature, water activity and gamma radiation was examined over a 3-day incubation period (El Aal, 1997). AME and ATX-I were not detected in the wheat under any set of conditions and ALT was found but only

in insignificant concentrations. It was found that the optimal conditions for the production of TeA and AOH were 25 °C and 0.98  $a_w$ . Usage of gamma radiation at a dosage of 1.0 to 3.0 kGy in combination with storage conditions of 15 °C and 0.90  $a_w$  significantly decreased the concentrations of AOH and TeA in the wheat samples. This reduction method, despite its effectiveness is limited. The use of gamma radiation in food is highly regulated with strict conditions and may not be legal in all countries (World Health Organization, 1999)

## **2.10 Masked mycotoxins and co-occurrence of mycotoxins**

One of the difficulties in properly assessing the impact of mycotoxins in a foodstuff is in accurately detecting and quantifying the toxins present in a sample. This problem is made more difficult by the presence of masked mycotoxins and co-occurrence of mycotoxins.

It has been shown that *Alternaria* mycotoxins can be partially metabolized in plants causing the formation of conjugated metabolites (Berthiller *et al.*, 2005). These conjugated metabolites, or masked/modified mycotoxins, are typically bound to a polar substance in the plant such as glucose, amino acids, or sulfates. They are a concern because they may revert to their original state following enzymatic hydrolysis in the digestive tract after consumption. Current analytical techniques may not account for these modified mycotoxins and so the actual concentration of mycotoxins in food stuff may be underestimated. Most research is focused on the parent mycotoxin and little data exists as to the extent of modified mycotoxin presence in food and feedstuffs. This phenomenon has been seen with the binding of zearalenone and deoxynivalenol to glucose to form zearalenone-4-glucoside and deoxynivalenol-3-glucoside respectively (Berthiller *et al.*, 2005; Gareis *et al.*, 1990). It has been suggested that similar conjugates could occur with *Alternaria* mycotoxins (Ostry, 2008a).

*Alternaria* mycotoxins have been found to be produced in a variety of raw and processed food products including apples (Stinson *et al.*, 1980), tomatoes (Bottalico and Logrieco, 1998), and wheat (Azcarate *et al.*, 2008). Other fungi and other mycotoxins have been known to grow on these crops as well. For example, patulin is commonly found in apples (Barkai-Golan and Paster, 2008), ochratoxin A has been seen in tomatoes (Engelhardt *et al.*, 1999), and the *Fusarium* mycotoxins are often seen in wheat and other cereal grains (Rodríguez-Carrasco *et al.*, 2013). As of yet no studies have been done on the co-occurrence of *Alternaria* mycotoxins and other mycotoxins. Other combinations of mycotoxins have been observed in a variety of crops. For example, aflatoxins, trichothecenes, fumonisins, and zearalenone in maize, ochratoxin and zearalenone in barley, and aflatoxins, fumonisins, ochratoxin, and zearalenone have been observed in cereal grains and peanuts (EFSA, 2004; Ibáñez-Vea *et al.*, 2012; Sangare-Tigori *et al.*, 2006). The co-occurrence of mycotoxins is significant because the combination of different toxins could cause a wide range of antagonistic, synergistic, or additive effects. This phenomenon has been demonstrated with AOH and DON metabolites combined in different concentrations (Juan-Garcia *et al.*, 2014). Several regulations exist for the control of mycotoxins in foodstuffs, however the potential effects of multiple mycotoxins are not widely considered by the policy makers and regulatory agencies.

## **2.11 Conclusion**

*Alternaria* mycotoxins have not received that same attention as other toxins produced by fungal species. These toxins are produced by fungi found in soil and air around the world and are associated with causing disease in crops with significant agricultural value. The *Alternaria* mycotoxins can be found in a range of key food products with a potentially wide exposure range.

Several reports have observed high levels of these toxins in fresh and processed foods or beverages such as olive oil, tomato, products, and apple juice suggesting that current processing methods are not sufficient to remove the toxins. The data on the occurrence of *Alternaria* mycotoxins in foodstuffs is too incomplete to make an accurate assessment, however it is evident that contamination does occur. *Alternaria* mycotoxins typically occur in produce that is visibly damaged or decayed. This type of product is considered unfit for human consumption and sale, limiting the potential exposure. However, this grade of produce is commonly used in the production of processed foods. There is little research as to the efficacy of processing techniques such as pasteurization or filtration on the destruction or removal of *Alternaria* toxins from foods. Research that has been done indicates that some of these toxins may be resistant to heat treatment and other processing methods. Furthermore, there is evidence that potentially toxic breakdown products may be released as a result of processing. These products have not been sufficiently investigated and are not typically taken into consideration when assessing the occurrence of toxins in foods. There are other confounding factors that need to be taken into consideration. Few studies assess for the presence of multiple mycotoxins, not accounting for the potential synergistic effect of multiple mycotoxins. There is also evidence that suggests that mycotoxins, including those produced by *Alternaria*, may be present in a conjugated form. This would effectively mask their appearance from standard analytical methods and could cause an underestimation of mycotoxin content in a sample.

While no regulations have been set yet, there is enough toxicological data available to warrant concern regarding the potential impact of the *Alternaria* mycotoxins on human health. The chances of acute toxicosis appear to be small, however the mutagenic properties of AOH,

AME, and ALT as well as the correlation with oesophageal cancer are of concern and require further investigation. More research is required before a specific set of regulatory guidelines can be created. Specifically, information on the occurrence of these toxins in commercial foods is needed to generate population consumption data and to determine the probability that someone may ingest a dangerous amount. On a related note, more information is also required on the stability of *Alternaria* toxins to process. Past studies indicate that they may be resistant to some common processing methods.



## **Chapter 3: Occurrence of alternariol and alternariol monomethyl ether in apple and tomato products from the Canadian market**

## **Abstract**

Alternariol (AOH) and alternariol monomethyl ether (AME) are mycotoxins produced by *Alternaria* species of fungi. Currently there is insufficient occurrence data on these toxins in food products to properly assess the human health risks they pose. A survey was conducted to examine apple and tomato products from the Canadian market for the presence of AOH and AME. Based on the obtained results, the average exposure of Canadians to AOH and AME from apple and tomato products was calculated. A total of 116 food products including apple juice, apple cider, apple puree, tomato juice, and tomato puree were examined. Fifty percent of total products were found to contain AOH above the limit of quantification and 30% with AME. The maximum concentration found in any sample was 75 µg/kg for AOH and 30 µg/kg for AME. This occurrence survey was combined with apple and tomato consumption data in Canada to assess the potential exposure to these mycotoxins. The estimated intake of AOH and AME was found to be below the threshold of toxicological concern regarding the consumption of each individual product type.

## **3.1 Introduction**

*Alternaria* species are a pathogenic and saprophytic genus of fungi found ubiquitously distributed in soil, water, and air. These fungi have been found to contaminate a variety of crops including cereals, oilseeds, tomatoes, apples, citrus fruit, and olives (EFSA, 2011). *Alternaria* fungi are known to be capable of growth at refrigeration temperatures, and are a spoilage risk during both transport and storage (Ostry, 2008a).

In addition to being spoilage organisms, *Alternaria* fungi are also known to produce over 70 different mycotoxins, some of which are harmful to animal and human health (Solfrizzo *et al.*,

2005). The toxicological properties of these mycotoxins have been studied, and 2 of particular concern are alternariol (AOH), and alternariol monomethyl ether (AME) (Figure 3.1) (EFSA, 2011; Ostry, 2008a). AOH and AME do not have strong acute toxic effects, however they have been linked to chronic effects such as fetotoxicity, and teratogenicity (Brugger *et al.*, 2006; Pero *et al.*, 1973b). They have also been linked oesophageal cancer in China and can cause DNA strand breakage (Liu *et al.*, 1992; Pfeiffer *et al.*, 2007).

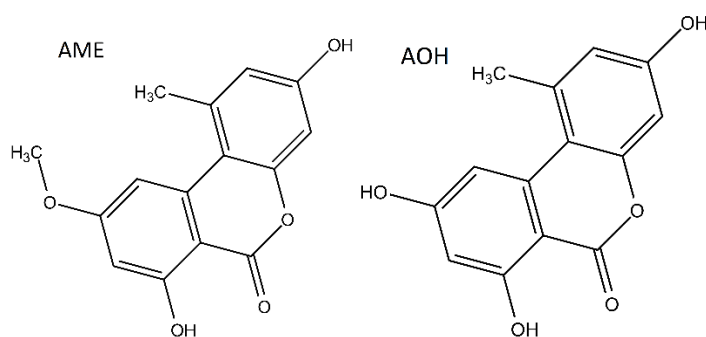


Figure 3.1. Chemical structures of *Alternaria* toxins alternariol (AOH) and alternariol monomethyl ether (AME).

Previous studies have shown that *Alternaria* fungi are able to produce mycotoxins while growing on food stuffs (Logrieco *et al.*, 2009a; Ostry, 2008a; Scott and Kanhere, 2001). Common foods associated with the *Alternaria* toxins include cereals, pulses, oilseeds, tomatoes, carrots, and apples. The concentrations of AOH and AME found vary greatly, ranging from 1 – 1000 µg/kg. Visibly mouldy produce tends to contain the greatest concentrations of these mycotoxins. However, *Alternaria* species are also capable of infecting fruit and growing in the internal tissues with little to no visible signs (Logrieco *et al.*, 2009a).

A variety of methods exist for the detection and quantification of *Alternaria* mycotoxins. An organic solvent is commonly used to extract the mycotoxins from the food matrices, with

solvent partitioning or solid-phase extraction (SPE) used for cleanup and purification. Detection methods involving thin layer chromatography (TLC) (Hasan, 1996), gas chromatography (GC) (Pero *et al.*, 1973b), high performance liquid chromatography (HPLC) (Delgado *et al.*, 1996), and liquid chromatography-mass spectrometry (LC-MS) (Lau *et al.*, 2003b) have all been developed. Due to functionality, sensitivity, and selectivity, most current research uses either HPLC or LC-MS systems for analysis.

There are currently no regulations concerning the presence of these toxins in foodstuffs. In 2011, the European Food Safety Authority (EFSA) reviewed the potential hazards associated with *Alternaria* toxins in the food supply. They found that in order to more accurately assess the potential risk, there is a need for more data regarding the presence of these toxins in commercial food products. This work aims to expand upon the currently available occurrence information on AOH and AME. Commercial tomato and apple products purchased from markets in Canada were analysed for the presence of AOH and AME. Using statistical studies performed by Stats Canada, the potential risk of exposure of the average Canadian to these toxins was assessed.

## **3.2 Materials and methods**

### **3.2.1 Reagents**

Solvents for mycotoxin extraction and HPLC detection, methanol and acetonitrile, were acquired from VWR International (Mississauga, ON, CA). They were both of analytical grades. Formic acid was purchased from Sigma-Aldrich (St.Louis, MO, USA) Pure water was obtained from a Millipore Milli-Q water filtration system (Bedford, MA, USA). Authentic AOH and AME standards in crystallized form (purity >95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 3.2.2 Preparation of standard solutions

Individual standard solution of AOH and AME were prepared by dissolving commercially purchased mycotoxin standards in methanol to obtain concentrations of 250 µg/mL. Solutions were stored in amber bottles at -20°C. No decomposition was observed after a period of 3 months. HPLC calibration solutions were produced by drying AOH and AME standard solutions under nitrogen stream, dissolving in matrix-matched solutions and then performing serial dilutions to concentrations content below the limit of detection (LOD). Validation solutions were prepared by diluting the AOH and AME standard solutions with methanol.

### 3.2.3 Samples

A total of 116 samples were purchased from retail stores in Guelph, Ontario, Canada in the spring of 2016. The samples consisted of processed apple and tomato products including apple juice, cider, apple puree, tomato juice, tomato puree, and tomato sauce. All samples were stored at 4°C prior to analysis and were tested before their best before date.

### 3.2.4 Sample preparation

Prior to mycotoxin extraction, 40 g of each food product were weighed into a centrifuge tube. To aid in extraction and clarify cloudy samples, 300 µL of pectinase was added to each centrifuge tube (Trucksess and Pohland, 2001). Tubes were vigorously shaken for 60 sec and incubated at 23 °C for 8 h before being centrifuged at 4500 g for 5 min. The supernatant was collected and used for mycotoxin extraction.

### 3.2.5 Mycotoxin extraction

The extraction of AOH and AME from samples was based on a modified version of the SPE method described by Solfrizzo et al (2004). In brief, a Strata C18-U SPE cartridge (Phenomenex, Torrance, CA, USA) was preconditioned with 5 mL of acetonitrile and 5 mL of water. The supernatant was drawn off from centrifuged sample tubes and 5 ml was passed through the cartridge, followed by a wash of 2 mL water and 2 mL of acetonitrile – water (30:70, v/v). AOH and AME were eluted together with 3 mL of acetonitrile. The eluent was evaporated to dryness under a stream of nitrogen gas and the dried extract was finally dissolved in 0.25 mL of methanol. To avoid clogging of the HPLC column, the sample was filtered with a 0.2 µm polyvinylidene difluoride (PVDF) syringe filter before analysis.

### 3.2.6 HPLC conditions

Chromatographic detection and quantification of AOH and AME was performed using an HPLC system (Agilent 1260, Waldbronn, Germany). This consisted of a quaternary pump, auto sampler, and diode array detector. The column used for separation was a Phenomenex C18 column (100 x 4.6mm) with 2.6 µm particle size. Mobile phases consisted of 0.02% formic acid:water (solvent A) and methanol (solvent B). Samples were injected at a volume of 10 µL and eluted with a binary gradient starting at 40% mobile phase A and reaching 100% B in 12 min with a flow rate of 0.6 mL/min. Presence and quantity of *Alternaria* mycotoxins was determined at a wavelength of 256 nm.

### 3.2.7 Method validation

The performance of the extraction and detection methods described here was evaluated based on linearity, limit of quantification (LOQ), LOD, and recovery. Linearity of results was assessed by injecting serially diluted standard solutions into the HPLC. Mycotoxin concentration

of solutions ranged from 1 to 100 µg/kg. Results were assessed based on the correlation coefficient of the resulting curve. Extraction efficiency experiments were done by spiking matrix-matched samples that were found to contain AOH and AME below the LOD. Concentration of spiked solution ranged from 1 to 100 µg/kg. The spiked solutions were processed according to the mycotoxin extraction method prior to injection in the HPLC. Matrix effects and mycotoxin recovery were determined by comparing chromatograms of spiked matrix-matched solutions with HPLC validation solutions. The LOQ and LOD were determined with signal to noise ratios of 3/1 and 10/1, respectively.

### 3.2.8 Exposure assessment

In order to characterise the exposure of the Canadian population to AOH and AME, consumption data for Canadians was taken from a survey by Statistics Canada (Statistics Canada, 2002) and combined with the occurrence data from the current study. The Statistics Canada study provided estimation of the average Canadian's yearly consumption of apple and tomato products in kg/yr. This was converted to kg/day and added to the average contamination data from this study to estimate the daily intake of AOH and AME from apple and tomato products. Average contamination was defined as the average amount of AOH/AME found in the products surveyed. Calculations were done in accordance to the methods used in the EFSA report on *Alternaria* toxins (EFSA, 2011) Amounts <LOQ were taken as LOQ/2 and those <LOD were counted as LOD/2. To evaluate whether the daily intake was a potential danger, it was compared to the threshold of toxicological concern (TTC) as defined by EFSA. The TTC for both AOH and AME is 2.5ng/kg/b.w. per day, and an average body weight of 60 kg was used. Risk was expressed as the percentage of the TTC that the daily intake equates to (TTC %).

### **3.3 Results and discussion**

#### **3.3.1 Method development**

For separation and purification of AOH and AME an SPE method was used with a C18 cartridge. For the cleanup step from the SPE cartridge acetonitrile/water solutions in different concentrations (20, 30, 40, and 100% acetonitrile) were tested. Optimal cleanup efficiency was found with a solution of 30% (v/v basis) acetonitrile, where the lowest LOD and high recovery % could be obtained. Figure 3.2 shows typical chromatograms for extracts of apple and tomato juice, both with and without AOH and AME spiking. The apple chromatograms are less impacted by interferences from matrix as compared to the tomato ones. These interfering compounds might be amino acids, polyphenols or other chromophores present in tomato juice but not in apple juice that have similar absorbance to AOH, and AME. With this method, the retention time for AOH was found to be 4.9 min and for AME 7.8 min.



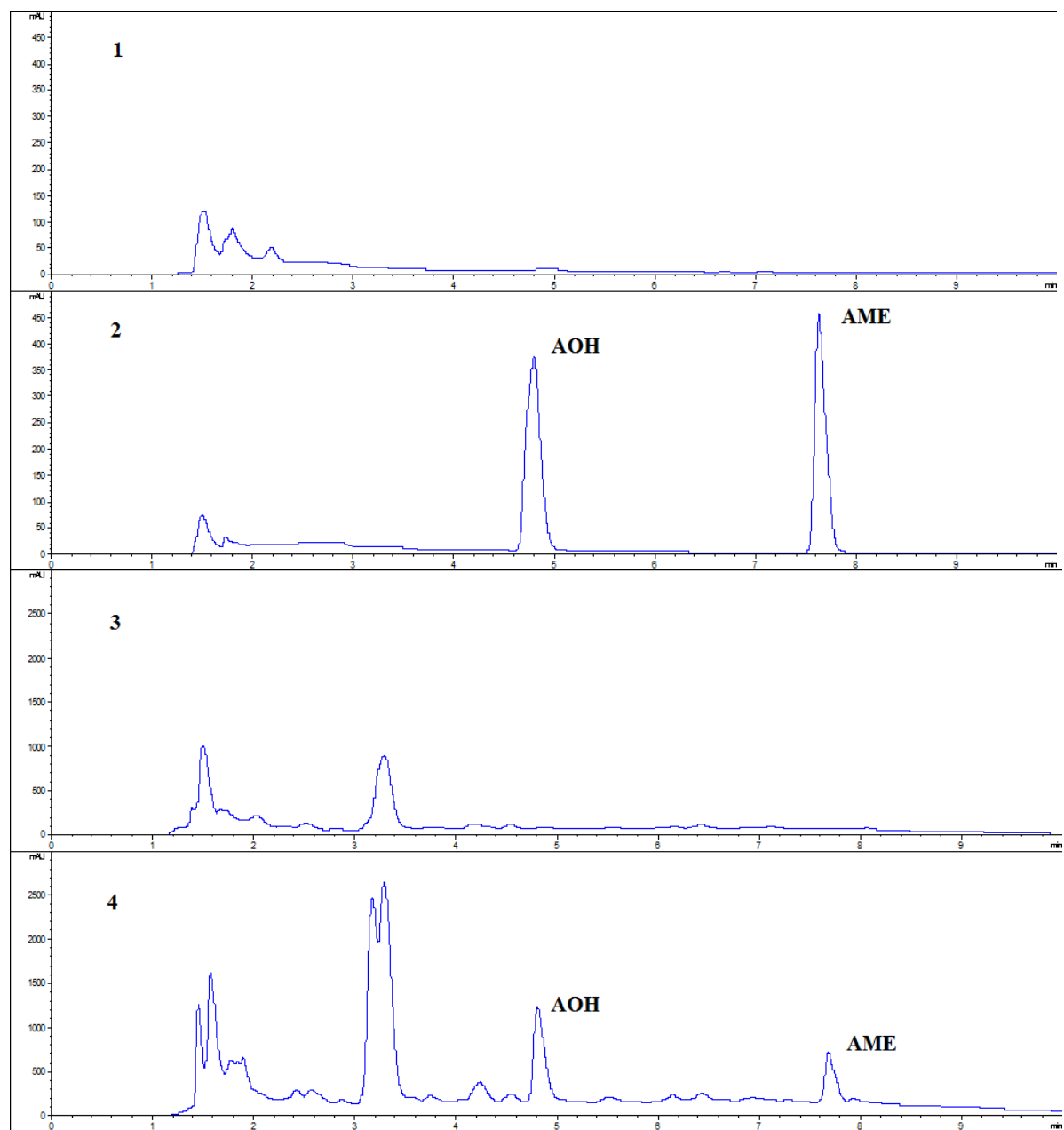


Figure 3.2. HPLC-DAD chromatograms of 1: apple juice with no AOH or AME, 2: apple juice spiked with 15  $\mu\text{g}/\text{kg}$  AOH and 15  $\mu\text{g}/\text{kg}$  AME, 3: tomato juice with no AOH or AME, and 4: tomato juice spiked with 15  $\mu\text{g}/\text{kg}$  AOH and 15  $\mu\text{g}/\text{kg}$  AME.

### 3.3.2 Method validation

To assess the performance of the method used, the characteristics of linearity, LOD, LOQ, and recovery were measured and are summarized in Table 3.1. No regulations have been

set for the presence of AOH or AME in foods in Canada or anywhere else in the world (EFSA, 2011). Accordingly no performance guidelines have been established either. Acceptable performance characteristics limits were set as those defined for other mycotoxins by the European Commission (European Commission, 2006). This document allows for a recovery % ranging from 50 – 120, and RSD % of  $\leq 40$ . The linearity of the calibration curve was defined as acceptable at a coefficient ( $r^2$ ) value of  $< 0.99$ . The results of the performance tests in the current study are in line with these limits. LOD, LOQ, and recovery of the mycotoxins were better in apple juice than tomato juice. This is related to the fact that more interfering compounds were found in the tomato juice than the apple. The performance characteristics for analysis of AME were also better than those for AOH. AME eluted later than AOH, and so the greater results can be attributed to less influence from matrix effects which were typically more hydrophilic, i.e. eluted earlier. The linearity of all calibration tests exceeded an  $r^2$  value of 0.99.

*Table 3.1. Performance characteristics for the method used for extraction and detection of AOH and AME.*

<b>Sample</b>	<b>Compound</b>	<b>LOD (<math>\mu\text{g}/\text{kg}</math>)</b>	<b>LOQ (<math>\mu\text{g}/\text{kg}</math>)</b>	<b>Mean recovery <math>\pm</math>RSD (%)</b>
<b>Apple Juice</b>	AOH	1.1	3.3	80.2 $\pm$ 7.5
	AME	0.5	1.5	87.6 $\pm$ 8.4
<b>Tomato Juice</b>	AOH	2.1	6.3	76.8 $\pm$ 7.8
	AME	1.9	5.7	85.9 $\pm$ 5.6

### 3.3.3 Occurrence

The occurrence of AOH and AME in Canadian apple and tomato products is shown in Table 3.2. Regarding apple products, AOH was detected in 33% of juices, 8% of ciders, and 62% of purees. The highest concentration seen was in apple puree at 54  $\mu\text{g}/\text{kg}$ . AME was detected in fewer samples than AOH, with only 22% of apple juice, 8% of ciders, and no purees found to contain AME above the LOQ. These results differ from most other studies, which have failed to

find quantifiable amounts of AOH or AME in apple juice samples (López *et al.*, 2016; Prella *et al.*, 2013; Zhao *et al.*, 2015a). One other Canadian study also detected both AOH and AME in apple juice, but at significantly lower concentrations than those seen in the current study (Lau *et al.*, 2003b). The conflicting results in occurrence can be attributed to a combination of factors including the location of the survey, origin of the fruit, and sample size.

The levels of contamination in tomato products were similar to those found in the apple products. AOH was found in 50% of tomato juice samples and 92% of tomato sauce, while AME was found in 17% of tomato juice and 15% of tomato sauce. The highest concentration of AOH was found in a tomato sauce with 75 µg/kg, and the highest AME in a tomato juice with 30 µg/kg. In a recent survey examining *Alternaria* toxins in German food products, AOH was found in 71% of tomato products tested and AME in 79% (Hickert *et al.*, 2016). Another study found AOH and AME present in 50% of tomato sauces sampled (López *et al.*, 2016). The highest levels of mycotoxins found in both these studies were AOH at a concentration of 25 µg/kg. Other research has shown significantly higher concentration of AOH and AME in tomato products. A study in China found tomato sauce with 1787 µg/kg AOH, and a different study based in Argentina detected AOH in a tomato sauce at a level of 8756 µg/kg (Terminiello *et al.*, 2006a; Zhao *et al.*, 2015a).

Table 3.2. Occurrence of AOH and AME in apple and tomato products.

Sample (n=)	AOH Incidence (%)	AME Incidence (%)	AOH & AME Incidence (%)	AOH Concentration (µg/kg)		AME Concentration (µg/kg)	
				Maximum	Mean of pos. samples	Maximum	Mean of pos. samples
Apple Juice (n=36)	33	22	11	18	7.2	15	11.6
Apple Cider (n=26)	8	8	0	26	24.5	14	12.1
Apple Puree (n=14)	62	0	0	54	34.6	0	0.0
Tomato Juice (n=24)	50	17	13	38	23.4	30	16.4
Tomato Sauce (n=26)	92	15	15	75	24.9	8	4.1

Only samples >LOQ were taken into account when calculating the percent incidence and the concentrations of AOH and AME.

### 3.3.4 Exposure assessment

The exposure of Canadians to AOH and AME through apple and tomato products was calculated with results shown in Table 3.3. Combining the results of a food consumption study by Statistics Canada (Statistics Canada, 2002) with the contamination data from this study, the average daily intake of AOH, and AME was estimated. To determine if the intake of *Alternaria* toxins was hazardous, these values were compared to the TTC for AOH and AME as set out by EFSA. TTC are a means of assessing the potential health hazards associated with the ingestion of toxic substances. They are commonly used for chemicals in which little toxicological information is available. The TTC for both AOH and AME as defined by EFSA is 2.5 ng/kg/b.w. per day (EFSA, 2011). Based on these criteria, neither toxin was present in any product in sufficient quantities on average to surpass the TTC. The highest level of exposure was in tomato puree, with average intake of 0.11 µg/d AOH, which accounted for 73.7% of the TTC. It should be noted that these calculations represent only a rough estimate of the potential exposure of Canadians to these toxins. The consumptions data is based on the Canadian average yearly

consumption as a whole; it does not take into account different consumer demographics. The mycotoxin intake of high volume consumer could possibly surpass the TTC, however this consumption data was unavailable. Similarly, those individual more at risk for disease, the young, old, or infirm, may require an entirely different TTC for accurate assessment. The current study only examined the potential of consuming a single product; the additive effect of consumption of multiple of these products or others could potentially increase the intake of these toxins to levels higher than the TTC. There is also potentially increased risk with consumption of products contaminated with multiple mycotoxins. In this study a portion of products were found to contain both AOH and AME. While the possible synergistic, antagonistic, and/or additive effects of AOH and AME, have not yet been studied, some interactions have been seen with AOH and deoxynivalenol (DON) (Juan-Garcia *et al.*, 2014). In this case depending on the ratio of AOH to DON, the additive cytotoxicity changed.

As compared to other analyses, the results in this study show lower levels of estimated toxin ingestion. The calculations done by EFSA in 2011 estimate an intake of 0.22 – 1.56 µg/day AOH and 0.01 – 0.2 µg/day AME across 15 European countries. A study on the exposure of the German population to *Alternaria* toxins found that from a combination of bakery products, juices, tomato products, vegetable oil, and sunflower seeds the average person would ingest a 2.1 µg/day of AOH and 0.4 µg/day of AME per day (Hickert *et al.*, 2016). Regarding a 60 kg individual this is equivalent to 1400% of the TTC% for AOH and 280% for AME.

Table 3.3. Average daily intake of AOH and AME from apple and tomato products, based on the results of this study. TTC values were defined by EFSA (EFSA, 2011), and food consumption data is taken from Statistics Canada (Statistics Canada, 2002).

Toxin	Sample	Food Consumption (kg/yr)	Toxin consumption (µg/d)	TTC %	TTC (ng/kg/b.w.)
AOH	Apple juice	6.08	0.06	38.87	2.5
	Apple cider	6.08	0.02	15.55	2.5
	Apple puree	0.46	0.02	14.96	2.5
AOH	Tomato juice	1.54	0.05	34.59	2.5
	Tomato puree	2.23	0.11	73.72	2.5
AME	Apple juice	6.08	0.02	12.22	2.5
	Apple cider	6.08	0.01	5.55	2.5
	Apple puree	0.46	0.00	0	2.5
AME	Tomato juice	1.54	0.01	9.28	2.5
	Tomato puree	2.23	0.01	4.89	2.5

### 3.4 Conclusion

This study examined apple and tomato products available in the Canadian market for the presence of the *Alternaria* mycotoxins AOH and AME. Both of these toxins were found in low concentrations in commercially available foods. Further study is required to determine the source of the contamination within the food processing chain as well as the effectiveness of food processing and food handling methods on reducing *Alternaria* toxin content.

The results of the exposure assessment suggest that AOH and AME intake does not represent a health concern to Canadian consumers of these food products. However, consumption of multiple types of contaminated products in a day or consumption of these products in a quantity above the average could result in ingestion of *Alternaria* toxins above the TTC. The TTC as defined by EFSA is based off estimated toxicity and may not be an accurate assessment when considering those with weakened immune systems, and does not consider the effects of multi-mycotoxin ingestion. In order to under a more thorough analysis of the total

intake of *Alternaria* toxins, other food products in the Canadian market should be assessed for contamination.

**Chapter 4: Comparison of high temperature and high pressure processing treatments for the reduction of alternariol and alternariol monomethyl ether in tomato juice**



## **Abstract**

The purpose of this study was to investigate the possibility of reducing *Alternaria* toxins in tomato juice using high temperature pasteurisation and high hydrostatic pressure (HHP) processing techniques. Fresh tomato juice was spiked with different concentrations of alternariol (AOH) and alternariol monomethyl ether (AME) (80, 200, and 500 µg/kg). The heat-treated samples were then processed at 90 or 121 °C for 10 or 20 min. Samples processed by HHP were subjected to either 300 or 600 MPa, for 3 or 5 min. The highest reduction of toxins was seen in samples spiked with 500 µg/kg and was 25.3% for AOH and 15.3% for AME. HHP was more effective than high temperature processing. No significant patterns could be seen when comparing the effect of individual processing variables. All treatments caused a significant reduction in total phenolic content of the tomato juice, but all treatments caused similar reductions.

## **4.1 Introduction**

The *Alternaria* genus of fungi is known to produce upwards of 70 different mycotoxins, 7 of which are commonly associated with foods (Solfrizzo *et al.*, 2005). Alternariol (AOH) and alternariol monomethyl ether (AME) are 2 *Alternaria* mycotoxins of significance due to their association with human and animal illness.

As compared to other mycotoxins AOH and AME have relatively weak acute toxic effects (Olsen and Visconti, 1988; Pollock *et al.*, 1982). The LD<sub>50</sub> for these toxins has been reported as 400 mg/kg of b.w. for mice, making the risk for acute toxicosis low. These compounds have also been shown to have cytotoxicity in cell cultures and there is concern regarding synergistic effects (Pero *et al.*, 1973b). AOH and AME are primarily significant for

their genotoxic, mutagenic, and cytotoxic effects. They have been associated with an increased rate of oesophageal cancer in China and may cause mutagenicity in human fetal cells (Ostry, 2008a)(Liu *et al.*, 1992).

The occurrence of AOH and AME in foodstuffs has been reviewed (Logrieco *et al.*, 2009a; Ostry, 2008a; Scott and Kanhere, 2001). They have been found in a wide range of cereals, fruits, and vegetables including: pulses, oilseeds, tomatoes, cereal grains, carrots, and their derivative products. Despite this there are currently no regulatory guidelines or limits concerning any of the *Alternaria* mycotoxins in food.

Limited research is available as to the effect of food processing on the degradation of AOH and AME. Under baking conditions the mycotoxins were found to be stable for 60 min at an oven temperatures of 200 °C and 45 min at 230 °C in a wheat flour dough (Siegel *et al.*, 2010a). Up to 70% degradation of AOH and 50% degradation of AME were achieved in dry flour using a baking process of 230 °C for 60 min. In a second study, after a heat treatment of 100 °C for 90 min, AOH and AME were not found to have degraded in sunflower flour (Combina *et al.*, 1999a). Reduction of the *Alternaria* mycotoxins in sunflower flour was achieved using a process of 121 °C for 60 min, causing degradation of 100% AME and 75% AOH. Using a cereal extrusion process, a reduction of 87.9% AOH and 94.5% AME content was achieved in whole wheat flour (Janić Hajnal *et al.*, 2016). The extrusion process exposed the wheat to conditions of 114°C and 0.17 MPa for 0.5 min. Only one study has examined the stability of AOH and AME in a liquid medium, where no degradation was found after 20 min at 80 °C in apple juice (Scott and Kanhere, 2001).

There is concern regarding mycotoxin contamination of high water content products such as juices, sauces and purees (Terminiello *et al.*, 2006a). This is due to the stability of *Alternaria* mycotoxins in these products and the potential for cross contamination during processing (Scott and Kanhere, 2001). Juices are typically subjected to a form of preservation process to reduce the microbial load in fruit juices. Utilization of heat is the most common and traditional means of pasteurization. This method has seen a decline in use due to the potential for degradation of desirable nutritional components (Rawson *et al.*, 2011). High hydrostatic pressure (HHP) processing is a novel method that has been shown to be effective in destroying pathogens (Jayathunge *et al.*, 2015). HHP works by subjecting a food to high pressure which acts to suppress cell component functionality and integrity (Rendueles *et al.*, 2011). HHP processing has been approved by Health Canada for use as an alternative to pasteurisation in fruit and vegetable juices (Health Canada, 2015). Both high temperature and HHP techniques have also been investigated for their ability to reduce the concentration of some mycotoxins such as patulin and citrin (Avsaroglu *et al.*, 2015; Hao *et al.*, 2016; Tokuşoğlu *et al.*, 2010).

The objective of the following study was to investigate high temperature and pressure processing treatments for their ability to degrade AOH and AME in tomato juice.

## **4.2 Materials and methods**

### **4.2.1 Reagents**

AOH and AME standards as well as formic acid, gallic acid, Folin-Ciocalteu's reagent, and Na<sub>2</sub>CO<sub>3</sub> were purchased from Sigma-Aldrich (St.Louis, MO, USA). Methanol and acetonitrile were acquired from VWR International (Mississauga, ON, CA). AOH and AME

standards were dissolved in methanol to obtain stock solutions of 1 mg/mL (AOH) and 0.2mg/mL (AME), respectively.

#### 4.2.2 Sample Preparation

Fresh tomato juice was prepared by blending and sieving washed tomatoes (12 kg) obtained from a local supermarket. Tomatoes were of the beefsteak variety and purchased in January of 2017. The juice was standardized to pH 4.0 with the addition of 1 M NaOH and then divided into 4 equal sized batches. Each batch was then spiked with to an initial concentration of 0, 80, 200, or 500 µg/kg of both AOH and AME. Samples were packaged in 50 mL aliquots into polyethylene pouches. The pouches were heat sealed and stored at 4 °C until processing.

#### 4.2.3 Tomato juice characteristics

The total phenolic content of the juice samples was measured before and after heat/pressure processing using Folin-Ciocalteu's method. Briefly, 25 µL aliquots of juice were treated with 125 µL of 1/10 diluted Folin-Ciocalteu's phenol reagent followed by 125 µL of 7.5% Na<sub>2</sub>CO<sub>3</sub>. Absorbance was read at 765 nm using a microplate reader (PowerWave XS2, Biotek Canada, Winooski, VT, USA). The measured absorbance was compared to gallic acid standards to determine the gallic acid equivalent concentration (GAE) (mg/mL) of the tomato juice samples.

#### 4.2.4 High temperature treatment

High temperature processing treatments were done using a water bath (Heidolph, Elk Grove Village, IL, USA) and a steam jacketed autoclave. Four different treatments were applied, with temperatures of 90 and 121 °C, and for 10 min and 20 min. Samples were kept refrigerated prior to testing. Each processing treatment was performed twice, on separate days, in order to assess day-to-day variability.

#### 4.2.5 HHP treatment

HHP applications were performed using a high pressure food processor (QFP, Flow Autoclave Systems Inc, Columbus, OH, USA). Four different treatments were applied using different combination of pressure and time. Pressures of 300 and 600 MPa were tested, each for both 3 and 5 min. The processing took place at ambient room temperatures; however because of pressure on temperature, the internal temperature of the HHP was increased. HHP applications at 300 MPa occurred at 20 °C and 600 MPa at 29 °C. Samples were kept refrigerated prior to testing. Two trials of the experiment were performed, with all treatments repeated on a second day.

#### 4.2.6 Extraction of toxins

The extraction of AOH and AME in tomato juice samples followed the same method used in Chapter 2 of this thesis. In short 5 mL of sample was passed through a Strata C18-U SPE cartridge (Phenomenex, Torrence, CA, USA) that was preconditioned with 5 mL of acetonitrile and 5 mL of water. The cartridge was washed with 2 mL of water and 2 mL of acetonitrile – water (30:70, v/v). The *Alternaria* toxins were eluted with 3 mL of acetonitrile and then evaporated to dryness under nitrogen gas. The dried extract was dissolved in 0.25 mL of methanol.

#### 4.2.7 HPLC-DAD analysis

AOH and AME were quantified with an HPLC system (Agilent 1260, Waldbronn, Germany) consisting of a quaternary pump, auto sampler, and diode array detector. Data was acquired in the form of chromatograms taken at a wavelength of 256 nm. A Phenomenex C18 column (100 x 4.6mm) with 2.6 µm particle size was used for separation. Mobile phase A consisted of 0.02% formic acid:water, and mobile phase B was methanol. A binary gradient was

used starting at 40% mobile phase A and reaching 100% B in 12 minutes with a flow rate of 0.6 mL/min. Quantities of AOH and AME were determined using calibration curves using solutions spiked with AOH and AME in the range of 1 – 500 µg/kg ( $R^2 = 0.9998$  and  $R^2 = 0.9997$ ).

Standards of AOH and AME were prepared by serial dilution of stock solution with methanol.

#### 4.2.8 Data analysis

The results were reported as mean reduction (%)  $\pm$  standard deviation. SPSS 17 for Windows (IBM Corporation, Armonk, New York, USA) was used for statistical analysis. One-way analysis of variance and Tukeys HSD ( $p < 0.05$ ) were used to test for significance in differences among pressure and heat treatments, and mycotoxin concentrations.

### 4.3 Results

#### 4.3.1 Effect of temperature on mycotoxin reduction

Spiked tomato juice samples were subjected to 4 different high temperature processing treatments; 90 °C and 121 °C for either 10 or 20 min. The reduction in mycotoxins concentration as a result of these treatments can be seen in Figure 4.1 for AOH and Figure 4.2 for AME. AME was significantly more stable than AOH under high temperature conditions in all scenarios except one ( $p < 0.05$ ). Generally, the reduction in mycotoxin content increased as both the temperature and processing time increased. Depending on the combination of variables the reductions of AOH ranged from 0.9% to 14.5% and AME from 1.0% to 15.3%. Temperature had a greater effect on mycotoxin reduction than time. However, individually increasing either factor did not always cause a significant increase in mycotoxin reduction at  $p < 0.05$ , changing both variables was necessary. Processing at 121 °C for 20 min caused the highest reduction of AME, and 121 °C for 10 for AOH.

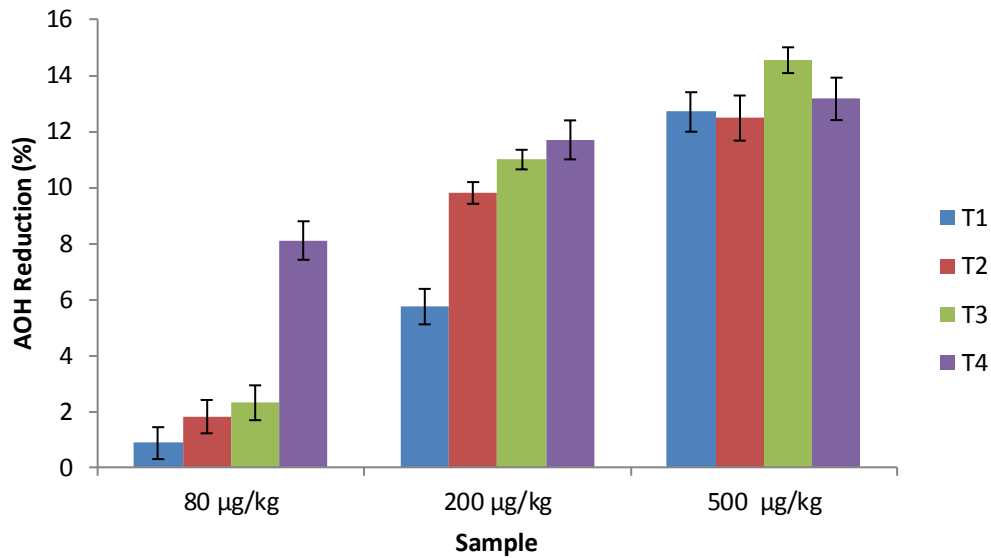


Figure 4.1. Reduction as a percent of initial concentration of AOH in tomato juice after high temperature processing. Abbreviations: T1 - 90 °C for 10 min, T2 - 90 °C for 20 min, T3 - 121 °C for 10 min, T4 - 121 °C for 20 min.

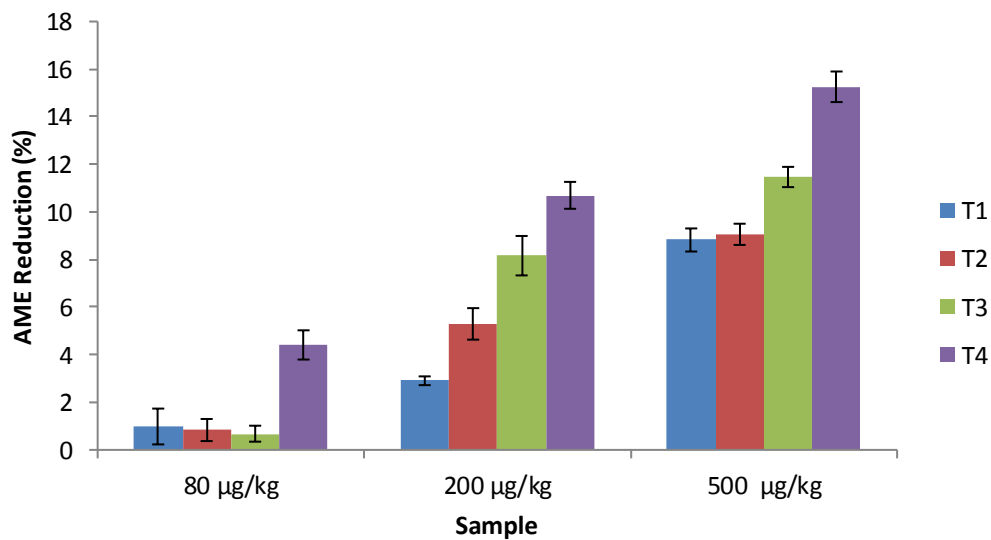


Figure 4.2. Reduction as a percent of initial concentration of AME in tomato juice after high temperature processing. Abbreviations: T1 - 90 °C for 10 min, T2 - 90 °C for 20 min, T3 - 121 °C for 10 min, T4 - 121 °C for 20 min.

#### 4.3.2 Effect of pressure on mycotoxin reduction

The effect of different pressure treatments on AOH and AME content in tomato juice was studied. Spiked tomato juice was subjected to 300 or 600 MPa for either 3 min or 5 min. Reduction of both mycotoxins was seen in all samples (Figures 4.3 and 4.4). Both AOH and AME have shown some resistance to pressure processing; no treatment caused a reduction in toxin content of more than 25.3%. As with the high temperature processing, AME was more stable to pressure than AOH as shown by the significantly lower reductions ( $p < 0.05$ ). Increasing pressure generally resulted in an increased reduction in both AOH and AME. The reduction in AOH ranged from 5.8% to 25.3% and AME reduction from 1.7% to 12.9%. The extent of mycotoxin reduction was dependent on both processing pressure and time. Processing time did not have a large solo impact on mycotoxin reduction, increasing the time alone did not cause any significant differences at  $p < 0.05$  in mycotoxin reduction. The most effective treatment was at 600 MPa, but there was no significant difference at  $p < 0.05$  between processing for 3 min or 5 min.



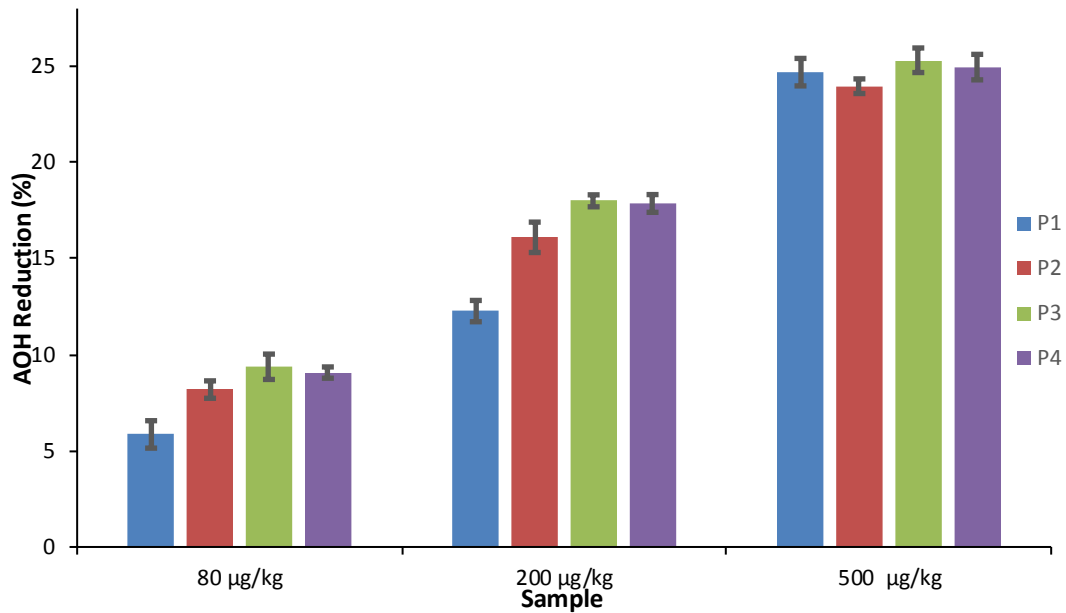


Figure 4.3. Reduction as a percent of initial concentration of AOH in tomato juice after high pressure processing. Abbreviations: P1 – 300 MPa for 3 min, P2 – 300 MPa for 5 min, P3 – 600 MPa for 3 min, P4 – 600 MPa for 5 min.

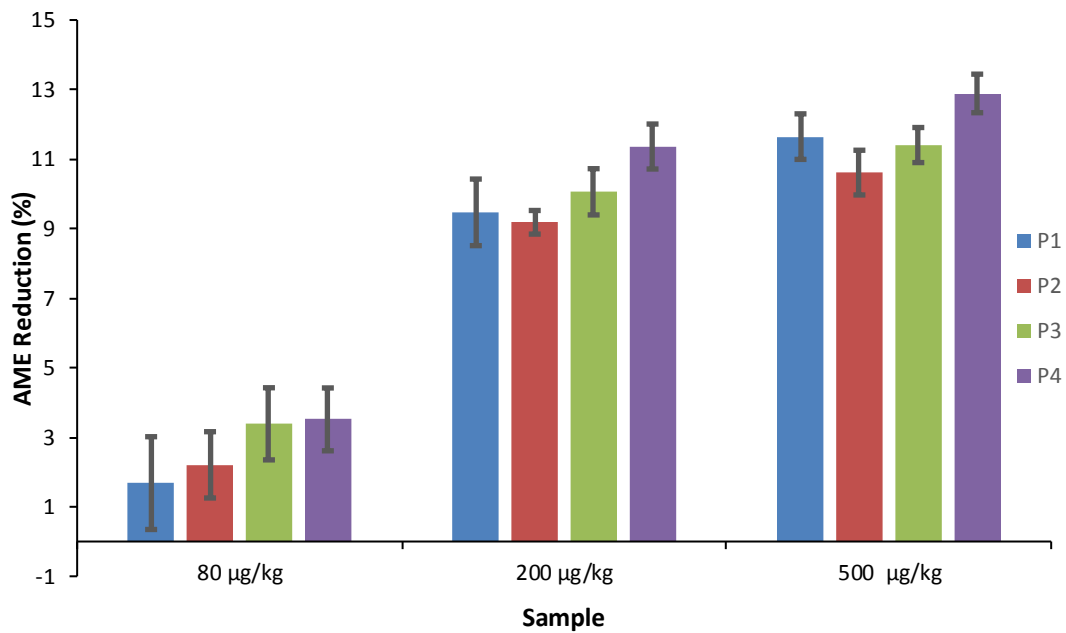


Figure 4.4. Reduction as a percent of initial concentration of AOH in tomato juice after high pressure processing. Abbreviations: P1 – 300 MPa for 3 min, P2 – 300 MPa for 5 min, P3 – 600 MPa for 3 min, P4 – 600 MPa for 5 min.

#### 4.3.3 Effect of initial concentration

Tomato juice was spiked with 80, 200, or 500  $\mu\text{g}/\text{kg}$  prior to heat or pressure treatment. The effects of the initial concentration of mycotoxins can be seen in Figures 4.1 – 4.4. When comparing each individual processing treatment, the percent reduction of both AOH and AME increased with higher levels of initial concentration. For any given treatment, the percent reduction of both AOH and AME was significantly higher ( $p < 0.05$ ) in juice spiked with 500  $\mu\text{g}/\text{kg}$  as compared to that with 80  $\mu\text{g}/\text{kg}$ .

#### 4.3.4 Effect of trial

The 8 processing treatments were each done twice, on separate days. Tables 4.1 and 4.2 compares the results from each trial. There were no significant differences in the reduction of AOH or AME from a trial to trial basis ( $p > 0.05$ ).

Table 4.1. Percent reduction of AOH and AME in tomato juice following processing by high pressure treatments.

<b>Initial Concentration (µg/kg)</b>	<b>Time (min)</b>	<b>Temperature (C°)</b>	<b>Pressure (MPa)</b>	<b>Trial</b>	<b>AOH (%)</b>	<b>AME (%)</b>
<b>80</b>	3	20	300	1	6.22 ± 0.80	2.74 ± 0.90
<b>80</b>	3	20	300	2	5.49 ± 0.48	0.62 ± 0.54
<b>80</b>	5	20	300	1	7.84 ± 0.24	1.88 ± 0.86
<b>80</b>	5	20	300	2	8.53 ± 0.31	2.53 ± 1.10
<b>80</b>	3	29	600	1	9.32 ± 0.60	3.93 ± 0.68
<b>80</b>	3	29	600	2	9.43 ± 0.85	2.84 ± 1.16
<b>80</b>	5	29	600	1	9.29 ± 0.16	4.02 ± 0.57
<b>80</b>	5	29	600	2	8.85 ± 0.22	3.00 ± 0.97
<b>200</b>	3	20	300	1	12.26 ± 0.67	10.22 ± 0.70
<b>200</b>	3	20	300	2	12.27 ± 0.56	8.72 ± 0.34
<b>200</b>	5	20	300	1	16.74 ± 0.30	9.09 ± 0.24
<b>200</b>	5	20	300	2	15.47 ± 0.50	9.28 ± 0.45
<b>200</b>	3	29	600	1	18.01 ± 0.41	9.88 ± 0.62
<b>200</b>	3	29	600	2	18.00 ± 0.27	10.24 ± 0.79
<b>200</b>	5	29	600	1	17.82 ± 0.71	11.93 ± 0.16
<b>200</b>	5	29	600	2	17.91 ± 0.17	10.79 ± 0.19
<b>500</b>	3	20	300	1	24.16 ± 0.57	12.02 ± 0.70
<b>500</b>	3	20	300	2	25.23 ± 0.30	11.27 ± 0.40
<b>500</b>	5	20	300	1	24.21 ± 0.23	10.05 ± 0.21
<b>500</b>	5	20	300	2	23.72 ± 0.37	11.17 ± 0.23
<b>500</b>	3	29	600	1	25.00 ± 0.12	11.19 ± 0.28
<b>500</b>	3	29	600	2	25.63 ± 0.84	11.61 ± 0.65
<b>500</b>	5	29	600	1	24.47 ± 0.15	12.51 ± 0.45
<b>500</b>	5	29	600	2	25.44 ± 0.61	13.26 ± 0.38

Table 4.2. Percent reduction of AOH and AME in tomato juice following processing by high temperature treatments.

Initial Concentration (µg/kg)	Time (min)	Temperature (C°)	Trial	AOH (%)	AME (%)
80	10	90	1	0.44 ± 0.33	1.66 ± 0.08
80	10	90	2	1.29 ± 0.41	0.31 ± 0.19
80	20	90	1	1.87 ± 0.86	0.50 ± 0.11
80	20	90	2	1.75 ± 0.37	1.17 ± 0.43
80	10	121	1	2.81 ± 0.34	0.87 ± 0.35
80	10	121	2	1.80 ± 0.28	0.49 ± 0.23
80	20	121	1	7.56 ± 0.31	4.88 ± 0.43
80	20	121	2	8.63 ± 0.47	3.94 ± 0.32
200	10	90	1	5.26 ± 0.20	2.83 ± 0.19
200	10	90	2	6.21 ± 0.53	2.98 ± 0.18
200	20	90	1	10.08 ± 0.31	5.77 ± 0.51
200	20	90	2	9.51 ± 0.21	4.82 ± 0.39
200	10	121	1	11.08 ± 0.50	8.55 ± 0.94
200	10	121	2	10.90 ± 0.18	7.77 ± 0.62
200	20	121	1	12.13 ± 0.65	11.03 ± 0.64
200	20	121	2	11.25 ± 0.46	10.36 ± 0.21
500	10	90	1	12.12 ± 0.40	9.02 ± 0.50
500	10	90	2	13.26 ± 0.33	8.62 ± 0.47
500	20	90	1	12.00 ± 0.80	8.86 ± 0.55
500	20	90	2	12.94 ± 0.55	9.26 ± 0.28
500	10	121	1	14.83 ± 0.47	11.59 ± 0.20
500	10	121	2	14.24 ± 0.22	11.34 ± 0.61
500	20	121	1	12.56 ± 0.58	15.50 ± 0.49
500	20	121	2	13.76 ± 0.14	15.02 ± 0.79

#### 4.3.5 Effect of processing on pH and TPC

Total phenolic contents (TPC) and pH of tomato juice samples were tested before and after each temperature and pressure treatment as a means of assessing effect of processing on quality. No significant changes occurred to pH during any of the treatments (Table 4.3). The TPC is a collective measure of the all compounds with phenolic feature in a solution. Comparing the effect of time on TPC, when all other conditions were the same, no significant difference in TPC could be seen. Increasing pressure from 300 to 600 MPa significantly decreased the TPC. An increase in temperature from 90 °C to 121 °C however did not have any significant impact.

Every treatment caused a significant reduction ( $p < 0.05$ ) in phenolic content as compared to the non-treated control juice. The TPC of the heat-treated juices was lower than that in the pressure treated ones but not by a significant degree.

*Table 4.3. Effect of high temperature and high pressure processing on tomato juice quality characteristics.*

<b>Time (min)</b>	<b>Temperature (C°)</b>	<b>Pressure (MPa)</b>	<b>TPC (GAE mg/mL)</b>	<b>Average pH</b>
0	0	0	$0.083 \pm 0.002^e$	4
3	20	300	$0.064 \pm 0.004^c$	4.1
5	20	300	$0.061 \pm 0.002^d$	3.9
3	29	600	$0.059 \pm 0.002^{a, b}$	4
5	29	600	$0.059 \pm 0.003^{a, b}$	4
10	90	-	$0.058 \pm 0.002^{a, b}$	4.1
20	90	-	$0.055 \pm 0.002^a$	3.9
10	121	-	$0.057 \pm 0.003^{a, b}$	4
20	121	-	$0.056 \pm 0.003^{b, c}$	4.1

Values in the same column with the same letter are not significantly different at  $p < 0.05$

#### **4.4 Discussion**

Pasteurization and other preservation processing treatments in juices are primarily designed for the inactivation of microorganisms. The operating conditions in this study were chosen based on those used for this purpose i.e. to maximise juice quality and reduction of pathogens (Hayes *et al.*, 1998; Jayathunge *et al.*, 2015). Under these conditions the max reduction was 25.3% for AOH and 15.3% for AME. This suggests that the processing conditions required to kill foodborne pathogens are not sufficient for the destruction of mycotoxins. When

developing a food safety plan, pathogenic bacteria and mycotoxin should both be considered. Additional processing steps or critical control points may be required to ensure food safety.

Previous research has determined that AOH and AME are resistant to heat processing of juice (Scott and Kanhere, 2001). In apple juice, no reduction in AOH and AME content was seen after processing at 80 °C for 20 min (Scott and Kanhere, 2001). The results from the current study showed a similar trend, with processing at 90 °C for 20 min, causing a reduction of 12.5% AOH and 9% AME. All of the processing variables studied in the current experiment, time, temperature, and pressure, had an effect on the reduction of AOH and AME. Increasing both processing temperature and time significantly improved the reduction rate for both AOH and AME ( $p < 0.05$ ), however, changing just one of these variables at a time did not always show significant difference. Both AOH and AME reacted similarly to high temperature processing, with AOH having only slightly higher reductions.

From Tables 4.1 and 4.2, we can see that pressure treatments had a significantly higher effect on mycotoxin reduction than heat treatments. There was no consistent, linear trend concerning the effect of pressure and time on mycotoxin reduction. Increasing either variable did not always lead to a significant increase in reduction of AOH or AME. This suggests that either a more extreme set of processing conditions are required to increase reduction of these mycotoxins or that this is the limit of what HHP can achieve. Due to the lack of a cooling system in the HHP equipment used, the processing temperature increased with increased pressure. This means that the HHP treatments at 600 MPa worked under an operating temperature 9 °C higher than those done at 300MPa. Based on the fact that processing at 600 MPa did not always cause higher degradation of AOH and AME, this difference is likely insignificant.

Others have noted that the optimum conditions for reduction of *Alternaria* mycotoxins is through the use of a combination of temperature and pressure treatments (Combina *et al.*, 1999a; Janić Hajnal *et al.*, 2016). Using 121 °C, 0.1 MPa for 60 min allowed one study to reduce AOH by 100% and AME by 75% (Combina *et al.*, 1999a). In contrast, the current study found that using each of 600 MPa for 5 min and 121 °C for 20 min individually caused a max reduction of 25.3% for AOH and 15.3% respectively. While it is possible there is a synergistic effect of combining these processing treatments, the more likely cause of this discrepancy is in the processing time.

The initial spiking concentration of AOH and AME influenced the reduction of AOH and AME with both HHP and high temperature treatments. In all treatments, the reduction of both AOH and AME was significantly higher in the juice spiked with 500 µg/kg as compared to that spiked with 80 µg/kg. This phenomenon has been reported before with regards to a different mycotoxin, patulin. It was found that reduction of the mycotoxin was dependent on initial concentration when processing with HHP (Avsaroglu *et al.*, 2015), and high temperatures pasteurisation (Janotová *et al.*, 2011). In both studies, there was a significant difference in the percent reduction when comparing the lowest spiking level to the highest. It has been suggested that other components in the food matrix such as sulphites, thiols, or other compounds, may be involved in rate limiting reaction kinetics and may be the cause (Janotová *et al.*, 2011).

One of the concerns with any form of processing is in the potential loss of quality of the food product. No pH change was seen in juice after any of the processing treatments, but both HHP and heat treatments caused a significant reduction in TPC as compared to the unprocessed control sample. TPC is a measure of the antioxidant activities of fruits and vegetables, and

reduction in TPC may be a concern. HHP is thought to be a less harsh form of processing as compared to heat treatments and better at preserving juice quality (Rawson *et al.*, 2011). This was not seen in this study, with no significant differences ( $p < 0.05$ ) in TPC or pH among the processing treatments. Other have reported that there are only minimal effects on TPC as a result of tomato processing by high temperatures (Dewanto *et al.*, 2002) or by HHP (Garcia-Fernandez *et al.*, 2001). The Folin-Ciocalteu total phenolic content assay used in this study has been criticized as being too general (Sánchez-Rangel *et al.*, 2013). It does not specify the type of phenolic compound and may suffer false positives due to interactions with reducing sugars. Despite this the Folin-Ciocalteu assay has been used in the past as an estimate of tomato juice quality following processing (Gahler *et al.*, 2003; Vallverdu-Queralt *et al.*, 2011). It has been suggested that the sugar content of tomatoes is low enough that the interfering effects are negligible (Spigno *et al.*, 2014).

#### **4.5 Conclusion**

In this experiment, the effect of high temperature pasteurization and HHP treatments on the stability of *Alternaria* mycotoxins in tomato juice was tested. The results suggest that AOH and AME have some level of resistance to heat and pressure processing. HHP processing was more effective, however none of the operating parameters tested could reduce the mycotoxin content by more than 25.3%. Optimal conditions were not determined and more extreme processing, or the use of both heat and pressure may be adopted for increased effective. The occurrence of potentially toxic breakdown products was not determined and requires further study. Based on the findings here it is recommended that the use of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) be heavily implemented as the primary



deterrent of contamination, because processing conditions did not appear to be completely effective in mycotoxin reduction in foods.

## **Chapter 5: Conclusions**

## 5.1 Conclusions

*Alternaria* mycotoxins like AOH and AME are not currently regulated in Canada or any other country in the world (EFSA, 2011). The main reason for this is the lack of data regarding their presence in food products. The purpose of this work was to investigate the occurrence of these mycotoxins in commercial food products and their stability through food processing.

Commercial apple and tomato products were examined for AOH and AME contamination. AOH was found in 50% of the 116 products studied and AME was found in 30%. The highest concentrations of mycotoxins were found in a tomato sauce with 75 µg/kg of AOH and a tomato juice with 30 µg/kg of AME. There is no set maximum allowable level for either of these toxins. To assess whether the concentrations found were significant, the occurrence data from this study was used in conjunction with consumption information from a Statistics Canada study to determine the average daily intake for the mycotoxins. The calculated intake of both AOH and AME for each individual food product was below the TTC as defined by EFSA. This suggests that these mycotoxins present a minimal risk to human health. That conclusion may be inaccurate however considering the limitations of the experiment. The daily exposure to these toxins is likely significantly greater than that calculated if you examined different consumer groups. Particularly at risk groups would be those consuming larger than average amounts of a food product, individuals who are consumers of multiple contaminated product types, and those with weaker immune systems such as infants, the elderly, and the infirm.

The second part of this study examined the ability of high temperature and high pressure processing treatments to reduce the AOH and AME content from tomato juice. Both mycotoxins showed some degree of resistance to both heat and pressure. With processing conditions as high

as 121°C for 20 min, and 60 MPa for 5 min, the AOH and AME content of tomato juice could not be completely eliminated. The highest reduction was 25.6% for AOH, and 15.5% for AME. None of the processing methods tested were able to reduce AOH content to below 72 µg/kg, and AME to below 76.5 µg/kg.

## 5.2 Recommendations

Overall this study has provided some understanding as to the presence of AOH and AME in food products. Based on the results here there are several areas that warrant further investigation.

1. The occurrence data determined in this study has shown that AOH and AME can be found in apple and tomato products in Canada. Other food products have been found to contain these mycotoxins (Ostry, 2008a). A more widespread study of the presence of *Alternaria* mycotoxins in the Canadian food supply would help to provide a more detailed exposure analysis.
2. The accuracy of any exposure assessment is dependent on the knowledge of the toxicological properties of the substance. This is necessary to define what constitutes a hazardous quantity. It would be beneficial to have a better understanding of the acute and long term effects of AOH and AME on human health. Similarly, it was not determined in this study what chemical(s) AME and AOH were transformed into because of processing. Further study should examine the possible toxic properties of any breakdown products.
3. While the concentrations of AOH and AME found here were low, some of the samples contained both. Other studies have similarly found food products containing multiple *Alternaria* mycotoxins (Hickert *et al.*, 2016; Zhao *et al.*, 2015b). Mycotoxins

not produced by *Alternaria spp.*, such as patulin, have also been found to occur in apple and tomato products (Barkai-Golan and Paster, 2008). The possible synergistic, additive, and/or antagonistic effects of the co-occurrence of mycotoxins should be considered.

4. AOH and AME were found to be resistant to heat and pressure. Future studies should assess the efficacy of different processing methods such as filtration, radiation, and inactivation by microorganisms.

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