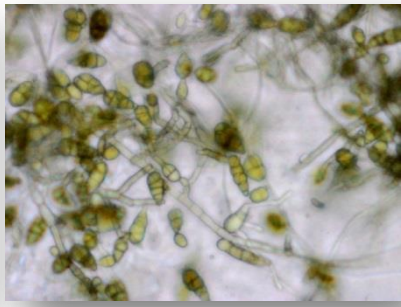

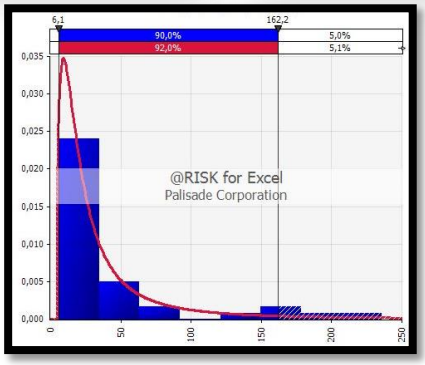

Farm to fork risk assessment of emerging mycotoxins in fresh produce: the case of tomato considering climate change

ir. Evelien Van de Perre

“We should consider every day lost on which we have not danced at least once.”

— Friedrich Nietzsche

Promoters:

Prof. dr. ir. Bruno De Meulenaer
Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University

Prof. dr. ir. Frank Devlieghere
Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University

Prof. dr. ir. Liesbeth Jacxsens
Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University

Dean: Prof. dr. ir. Guido Van Huylenbroeck

Rector: Prof. dr. Anne De Paepe

Ir. Evelien Van de Perre

Farm to fork risk assessment of emerging mycotoxins in fresh produce: the case of tomato considering climate change

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) of Applied Biological Sciences

Dutch translation of the title:

Riek tot vork risicobeoordeling van opkomende mycotoxines in verse groenten en fruit: het geval van tomaten in het licht van klimaatsveranderingen

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List of abbreviations

AF	Aflatoxin
A_f	Accuracy factor
AME	Alternariol monomethyl ether
AOH	Alternariol
B_f	Bias factor
$BMDL_1$	Benchmark dose lower confidence limit 1%
$BMDL_{10}$	Benchmark dose lower confidence limit 10%
BW	Body weight
CPA	Cyclopiazonic acid
DON	Deoxynivalenol
DM	Dry matter
DRBC	Dichloran Rose Bengal Chloramphenicol
FB	Fumonisin
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
IPCC	International Panel on Climate Change
ITS	Internal transcribed spacer
LC-TOF-MS	Liquid chromatography-Time Of Flight- Mass Spectrometry
LB	Lower bound
LOD	Limit of detection
LOQ	Limit of quantification
MEA	Malt Extract Agar
MOE	Margin of exposure
MSE	Mean square error

μ_{max}	Maximum growth rate
NOEL	No observable effect level
OTA	Ochratoxin A
PBS	Phosphate buffered saline
PMTDI	Provisional Maximum Tolerable Daily Intake
RCP	Representative Concentration Pathways
RSME	Root means square error
RV	Rotary evaporator
SEP	Standard error of prediction
TeA	Tenuazonic acid
TDI	Tolerable daily intake
TTC	Threshold of toxicological concern
TTH	Time till harvest
T25	Dose corresponding to a 25% incidence of tumors
UB	Upper bound
UPLC	Ultra High Pressure Liquid Chromatography
WWB	Warm water bath
ZEN	Zearalenone

Summary

The **overall objective** of the presented work was to establish a farm to fork risk assessment of emerging mycotoxins in fresh produce and derived products in view of the pressure of potential climate change scenarios and increasing import across European borders.

Screening and characterization of mycotoxins in fresh produce and their derived products

A first objective of this work was gaining insights in the potential presence and characterization of emerging mycotoxins in fresh produce and their derived products. Therefore, a multiple mycotoxin extraction and detection method was developed. Moulded tomatoes, sweet bell peppers, onions and soft red fruits were screened for alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), ochratoxin A (OTA), fumonisins (FB) and patulin. On moulded tomatoes, onions and soft red fruits, *Alternaria* spp. and their associated mycotoxins were detected. Derived tomato products were also screened and six out of 173 samples and four out of 173 samples were positive for AOH and AME, respectively (Chapter 2). Patulin was present in 11% of the moulded sweet bell peppers and in 8% of the moulded soft red fruits, but not in moulded onions. Patulin was also found in moulded tomatoes, but could not be detected in derived tomato products. It could be concluded that no health risk can be associated with derived tomato products considering patulin (Chapter 3). Because of the rather high prevalence of *Alternaria* mycotoxins in tomatoes, the fact that tomatoes are used frequently in further processing and the high consumption of the derived tomato products, it was decided to study further the presence of *Alternaria* mycotoxins in tomatoes and derived tomato products. From the stability experiment during the production process of

derived tomato products it could be concluded that AOH and AME can be considered very heat stable and are contributing to the fact that it is mainly a pre-harvest problem (Chapter 4). Preventive measures are necessary to avoid the entrances of high percentages of moulded tomatoes in tomato processing industries.

Evaluation of pre-harvest conditions on mould growth and mycotoxin production during tomato cultivation

A second objective of the presented work was the evaluation of pre-harvest conditions on mould growth and mycotoxin production during tomato cultivation. In this study five *Alternaria* spp. strains were isolated from tomatoes and the effects of temperature and Cu concentration on the growth of *Alternaria* spp. and their respective mycotoxin production (AOH and AME) on pre-harvest tomatoes used for further processing in derived tomato products were evaluated. All strains showed a short lag phase, even at lower temperatures (2-107h). There seems to be no effect of Cu-fungicides on the growth rate or mycotoxin production. In view of the activity of copper this result could be expected, as it is a contact fungicide protecting the tomato from infestation. Once the mould is formed, copper will not intervene in the mycelium development nor mycotoxin production. The optimum growth temperature varied from 24-33°C (0.21-0.29 mm/h). Regarding the mycotoxin production it was not possible to develop a quantitative model to describe mycotoxin production as function of temperature and time due to the high variability between the replicates. The optimal production of AOH and AME was reached at 25-30 °C at a mould diameter of 45 mm. At lower temperatures, the production of the mycotoxins was rather limited. At a mould diameter of 25 mm there was almost no production of AOH and AME. This is an important criteria that can be used during

processing of tomatoes. The risk of mycotoxin contamination can be reduced by sorting out the tomatoes and remove tomatoes with a mould diameter of 45 mm before the production of derived tomato products.

The influence of climate change on mould growth was evaluated for two productions regions: Spain and Poland. Four Representative Concentration Pathways (RCPs) were used as input to climate models to generate different climate change scenarios: RPC 2.6 (strong mitigation), 4.5 (mitigation), 6.0 (slowdown in emissions) and 8.5. (no changes). For Spain there were no significant differences for RCP 2.6 and 4.5. For the more extreme RCP scenarios (6.0 and 8.5) the diameter of the mould was significantly lower for the far future compared with the current time frame. In Spain, for the more extreme climate change scenarios, the temperatures are becoming too high for fungal growth. For Poland, the diameter of the mould was for the far future > near future > current time frame. This is due to the predicted higher temperatures in the far future (14-28°C) which becomes closer to the optimal temperature for the growth of *Alternaria* spp. compared with the colder temperatures in the present. According to the results, the situation in Poland in the far future (2081-2100) will became similar as the situation in Spain in the present time frame (1981-2000).

Risk assessment of mycotoxins

The third objective of the presented study was a risk assessment calculation on the emerging mycotoxins and their discussion in view of established mycotoxins in plant products.

For the emerging mycotoxins a farm to fork model was developed and different scenarios were evaluated (Chapter 7). The different climate change scenarios showed that with an increase in temperature the exposure can be lower due to a too high temperature for the growth of *Alternaria* moulds. An increase in moulded tomatoes entering the production line had a significant effect on the exposure of AOH and AME (increasing exposure). This emphasizes the importance of preventive measures at fields to avoid the prevalence of *Alternaria* moulds and the importance of sorting the moulded tomatoes out before entering the production line of derived tomato products and to set process criteria in the tomato processing industry. The hot break procedure resulted in a lower exposure compared with the cold break procedure.

Finally, the risk of established mycotoxins occurring in dried fruits and nuts were compared with the emerging mycotoxins in derived tomato products (Chapter 8). Because different mycotoxins are considered, multiple toxicological thresholds and concepts had to be applied. For OTA it could be concluded that there is no pressure for human health by consuming figs or raisins. For AFB1, the MOE values at the mean exposure level were above 10 000 and can be considered to be of low health concern, based on $BDML_{10}$ for humans, except for figs (MOE = 5782, mean). However, for P95 also the MOE values of almonds, hazelnut and pistachios were below 10 000. The *Alternaria* mycotoxins in derived tomato products are exceeding the set TTC-value both for mean and P95. More information on toxicology is needed to evaluate the exposure to AOH and AME.

Samenvatting

De algemene doelstelling van het gepresenteerde werk was een riek tot vork risico-beoordeling van opkomende mycotoxinen in verse producten en hun afgeleide producten met het oog op de druk van de mogelijke klimaatsverandering scenario's en de toenemende import binnen Europa.

Screening en karakterisering van mycotoxinen in verse plantaardige producten en hun afgeleide producten

Een eerste doelstelling van dit werk was het verkrijgen van inzicht in de mogelijke aanwezigheid en karakterisering van opkomende mycotoxinen in verse plantaardige producten en hun afgeleide producten. Daarom werd een multi-mycotoxine extractie- en detectiemethode ontwikkeld. Beschimmelde tomaten, paprika's, uien en zacht rood fruit werden gescreend op alternariol (AOH), alternariol monomethyl ether (AME), tenuazoonzuur (TeA), ochratoxin A (OTA), fumonisin (FB)en patulineproductie. Op beschimmelde tomaten, uien en zacht rood fruit werden *Alternaria* spp. en bijbehorende mycotoxinen gedetecteerd. Afgeleide tomatenproducten werden ook gescreend en zes van de 173 monsters en vier van de 173 monsters waren positief voor respectievelijk AOH en AME (hoofdstuk 2). Patuline was aanwezig in 11% van de beschimmelde paprika's en in 8% van het beschimmeld zacht rood fruit, maar niet in beschimmelde uien. Patuline werd ook gevonden in beschimmelde tomaten, maar kon niet worden gedetecteerd in de afgeleide tomatenproducten.

Door de vrij hoge aanwezigheid van *Alternaria* mycotoxinen in beschimmelde tomaten, het veelvuldig gebruik voor de verdere verwerking en de hoge consumptie van afgeleide tomatenproducten, werd besloten de aanwezigheid van *Alternaria* mycotoxinen in tomaten en afgeleide tomatenproducten verder te bestuderen. Uit de stabiliteitsproef gedurende het productieproces van afgeleide tomatenproducten

kon worden geconcludeerd dat AOH en AME zeer warmtestabiel zijn, wat bijdraagt tot het gegeven dat deze mycotoxins vooral een pre-oogst probleem vormen (hoofdstuk 4). Daarom zijn preventieve maatregelen nodig om te voorkomen dat een hoog percentage van beschimmelde tomaten in de verdere productie van afgeleide tomatenproducten terecht komt.

Evaluatie van pre-oogst condities op de schimmelgroei en mycotoxineproductie tijdens de tomatenteelt

Een tweede doelstelling van dit werk was de evaluatie van de pre-oogst condities op de schimmelgroei en mycotoxineproductie tijdens de tomatenteelt.

Alle stammen vertoonden een korte lag-fase, zelfs bij lagere temperaturen (2-107h). Er lijkt geen effect van koperfungiciden op de groeisnelheid of op de mycotoxineproductie te zijn. De optimale temperatuur varieerde van 24 tot 33 °C (0.21-0.29 mm/h). Wat de mycotoxineproductie betreft, was het niet mogelijk om een kwantitatief model van de mycotoxineproductie in functie van temperatuur en tijd te ontwikkelen vanwege de hoge variabiliteit tussen de herhalingen. De optimale productie van AOH en AME werd bereikt bij 25-30 °C met een schimmeldiameter van 45 mm. Bij lagere temperaturen was de productie van mycotoxinen vrij beperkt. Bij een schimmeldiameter van 25 mm was er bijna geen productie van AOH en AME. Dit is een belangrijk criterium dat gebruikt kan worden tijdens de verdere productie van afgeleide tomatenproducten. Het risico van mycotoxinecontaminatie kan worden verminderd door het sorteren van de tomaten en door tomaten met een schimmeldiameter van meer dan 45 mm te verwijderen voor de productie van afgeleide tomatenproducten.

De invloed van de klimaatverandering werd geëvalueerd voor twee productie regio's: Spanje en Polen. Vier 'Representatieve Concentration Pathways' (RCP) werden gebruikt als input voor klimaatmodellen om verschillende klimaatscenario's te

modelleren: RPC 2.6 (sterke mitigatie), 4.5 (mitigatie), 6.0 (vertraging van de emissies) en 8.5. (geen veranderingen). Voor Spanje waren er geen significante verschillen voor RCP 2.6 en 4.5. Voor de meer extreme RCP's (6.0 en 8.5) was de diameter van de schimmel significant lager voor de verre toekomst ten opzichte van het heden. Dit kan worden verklaard door de hogere temperaturen (18-38 °C) die te hoog zijn voor schimmelgroei. Voor Polen was er een significant verschil in de verschillende tijdperiodes, de diameter van de schimmel was voor de verre toekomst> nabije toekomst> heden. Dit komt door de voorspelde hogere temperaturen in de verre toekomst (14-28 °C) die dichter bij de optimale temperatuur voor de groei van *Alternaria* spp liggen. Volgens de resultaten, zal de situatie in Polen in de verre toekomst (2081-2100) gelijkaardig worden als de situatie in Spanje in het heden (1981-2000).

Risicobeoordeling van mycotoxinen

De derde doelstelling van de gepresenteerde studie was de risicobeoordeling van opkomende mycotoxinen en de vergelijking met reeds bekende mycotoxinen in noten en gedroogd fruit.

Voor de opkomende mycotoxinen werd een riek tot vork model ontwikkeld en werden verschillende scenario's geëvalueerd (hoofdstuk 7). Uit de verschillende klimaatveranderings- scenario's bleek dat bij een temperatuurstijging de blootstelling lager kan zijn als gevolg van een te hoge temperatuur voor de groei van de *Alternaria* schimmels.

Een toename van beschimmelde tomaten die de productielijn binnenkomen, zal een significant effect hebben op de blootstelling van AOH en AME (toenemende blootstelling). Dit benadrukt het belang van preventieve maatregelen op de velden om de prevalentie van *Alternaria* schimmels en het belang van het uitsorteren van de beschimmelde tomaten voordat ze in de productielijn van afgeleide tomatenproducten terechtkomen.

Op het einde van het onderzoek werd een rangschikking gemaakt van de mycotoxinen die voorkomen in gedroogde vruchten en noten en de opkomende mycotoxinen in afgeleide tomatenproducten (hoofdstuk 8). Omdat we te maken hebben met verschillende mycotoxinen, moeten meerdere toxicologische drempelwaarden en concepten worden toegepast. Voor OTA kunnen we concluderen dat er geen gevaar is voor de menselijke gezondheid door het consumeren van vijgen of rozijnen. Voor AFB1, waren de MOE waarden gemiddeld boven 10000 en dit kan dus beschouwd worden als een laag gezondheidsprobleem, gebaseerd op $BDML_{10}$ voor mensen, behalve voor vijgen (MOE = 5782, gemiddeld). Echter, voor de P95 waren ook de MOE waarden van amandelen, hazelnoten en pistachenoten lager dan 10000. De blootstelling van *Alternaria* mycotoxinen in afgeleide tomatenproducten was groter dan de TTC-waarde zowel voor de gemiddelde als voor de P95 waarden. Meer informatie betreffende de toxicologie van AOH en AME is nodig.

Chapter 1:

Introduction and objectives

Chapter 1: Introduction and objectives

Fruit and vegetables are important components of a healthy diet. They provide a dietary source of fiber, vitamins, minerals and phytochemicals (Agudo et al., 2002; Soerjomataram et al., 2010). As dietary guidelines recommend the consumption of at least five portions of fruits or vegetables per day and the demand for convenient, healthy and tasty food has grown, the request for fresh and derived food products has increased (WHO/FAO, 2003). Consumption of fresh produce is expected to increase further in coming years because of health promotion by government campaigns. Also, the economic status is an important factor, with a higher income resulting in the consumption of more processed foods (Quested et al., 2010). Next to his, in the Western world, people now spend less time to prepare food so the convenience of processed foods is a very important factor in buying the derived products (Cheng et al., 2007).

Sufficient daily consumption of fruit and vegetables could prevent major diseases. Cardiovascular diseases and certain cancers are associated with the insufficient intake of fresh produce (WHO, 2003). A study on the effect of fruit and vegetable intake on risk for coronary heart disease showed that a one-serving/day increase in fruit or vegetable intake was associated with a 6% lower risk for ischemic stroke (Joshipura et al., 2001). Worldwide, insufficient intake of fruit and vegetables is estimated to cause around 14% of gastrointestinal cancer deaths, about 11% of ischemic heart disease deaths and about 9% of stroke deaths (WHO, 2003).

1.1. Safety of fresh produce

At present, Europe is the largest importer and exporter of food products in the world. EUs food policy is developed to support and manage the safety and the consumer's confidence in it (Van Boxstael et al., 2013). For example, the EU General Food Law states, that "a high level of protection of human life and health should be assured in the pursuit of Community policies" (EU, 2002a).

Moreover, international, European and national concerns have emerged with regard to safety of fresh produce in response to recent outbreaks and reported emerging hazards linked to fresh produce and derived food products. Related to the consumption of these foods, several safety hazards can be attributed, both (micro) biological and chemical. Enteric bacteria such as *Salmonella* spp. and *E. coli* 0157:H7, enteric viruses such as Norovirus and protozoa such as *Cyclospora* have been identified as of concern in fresh produce (EFSA, 2013). Residues of pesticides or application of unauthorized pesticides are an established legal problem and in some cases also human health can be endangered (EFSA, 2011a; Van Boxstael et al., 2013). For Europe, the mycotoxin problem is mainly a border problem from imported dried plant products as nuts, raisins or staple foods as cereals etc. Some of these more established hazards are actually taken into consideration in risk assessment studies and regulations (e.g. microbial hazards in EU Regulation 2073/2005 (EC, 2005a), chemical hazards in EU Regulation 1881/2006 (EC, 2006) and pesticide residues in EU Regulation 396/2005 (EC, 2005b).

1.2. Pressures on the safety of fresh produce

Identified pressures on safety of fresh produce are climate change and globalization (Van Boxstael et al., 2013). Due to climate change and globalization of trade in fresh

produce and derived food products new and emerging (micro-)biological hazards and chemical hazards possibly jeopardize the safety of food products that are eaten raw or minimally processed (Jacxsens et al., 2010b; Kirezieva et al., 2014; Liu et al., 2013). Climate change can affect the development and presence of insects, resulting in a change of pesticides use. Also the increase of rainfall, associated with climate change, can lead to a change in pesticide use (Chen et al., 2001; Dalcero et al., 1995). Additionally, a change in climate will also influence the microbial hazards in food products due to the impact of climate change on contamination sources and pathways of bacteria e.g. flooding of fields with contaminated water or lack of irrigation water at a certain quality (Liu et al., 2013).

International trade expands the variety and lowers the cost of food, benefiting both the exporting and importing countries. The fastest growth in imported food was reported for fruits and vegetable. Global trade with stakeholders in many different countries will make the management of food safety more difficult, especially at the initial stages of the food chain, the agricultural production (Florkowski, 2008). Specifically, large differences exist in fresh produce production processes due to different climate conditions but also due to different regional organization of the food chain as a result of social and cultural aspects. This is already the case within European countries but is even more pronounced on a world-wide scale (Kirezieva et al., 2014).

1.3. Emerging risks in fresh produce: mycotoxins

Mycotoxins are substances produced as secondary metabolites by moulds, contaminating various agricultural commodities both at the pre- and post- harvest stage. Their toxicity is described as acute toxic, carcinogenic, mutagenic, teratogenic,

immunotoxic and oestrogenic in humans and animals, depending upon the mycotoxin involved (Bhat et al., 2010; van Egmond et al., 2007). Mycotoxins can occur, depending on the species of fungi, both in tropical or temperate regions.

Up to date, risk assessment and science-based legislation are available for various mycotoxins, especially for intermediate moisture foods vulnerable to fungi infestation such as aflatoxin (AF), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), fumonisins, T-2 and HT-2 toxin in cereals, maize and dried plant products (such as nuts, spices and dried fruits) (EC, 2010). In addition, patulin in apple and apple products is widely described (Baert, 2007). But there is little information on mycotoxins in fresh produce apart from apples. In the EU a clear regulation is available for the known mycotoxins such as AF, OTA and fumonisins, but there is no legislation for new upcoming mycotoxins such as *Alternaria* mycotoxins. Also, no legislation is available for most fruits and vegetables, with exception for dried spices, dried herbs, dried fruits, juices and apple products such as juices and compotes. Inside the EU there is the obligation of implementing a food safety management system to prevent or control the contamination of mycotoxins (EU Regulation 178/2002; EU Regulation 852/2004). The EU protects itself against contamination of mycotoxins in imported products through the strict border controls (EU Regulation 669/2009) for sensitive products and/or regions. The Rapid Alert System for Food and Feed (RASFF) was put in place to provide food and feed control authorities with an effective tool to exchange information about measures taken, responding to serious risks detected in relation to food or feed. This exchange of information helps Member States to act more rapidly and in a coordinated manner to a health threat caused by food or feed. Table 1.1 gives an overview of the numbers of mycotoxins found in food products during the last years (RASFF, 2003-

2012). AF and OTA seemed to be the most important mycotoxins found on dried fruits and dried vegetables up to now. However, it should be taken into account that the analysis were only performed on food products which have a limit for the presence of mycotoxins in the EU regulation 1881/2006, such as cereals, maize, dried fruits, nuts and nut products. Due to the global trade of food products, the European Commission protects its consumers by increasing border controls on mycotoxins in specific crops imported from certain regions (EC, 2009a). However, no systematic border controls are conducted, so there is a potential risk that food products with higher mycotoxin levels are present on the European market.

Table 1.1: Mycotoxins reported as rapid alert from 2003-2012 (RASFF, 2003-2012)

Year 20**	03	04	05	06	07	08	09	10	11	12
Total mycotoxins detected in food products	**80 5**	**88 1**	**99 7**	**87 7**	**76 0**	**93 3**	**66 9**	**68 8**	**63 5**	**525**
Aflatoxins (sum of AFB1, AFB2, AFG1 and AFG2)	76 5	83 7	94 7	80 0	70 5	90 2	63 8	64 9	58 5	484
Deoxynivalenol	-	-	-	-	10	4	3	2	11	4
Fumonisins	16	18	2	15	9	2	1	3	4	4
Ochratoxin A	24	26	42	54	30	20	27	34	35	32
Patulin	-	-	6	7	-	3	-	-	-	-
Zearalenone	-	-	-	1	6	2	-	-	-	4

1.3.1. Established mycotoxins

An extensive database was construced during this research in collaboration with the partners of the Veg-i-Trade project[1], to evaluate the status of established mycotoxins

[1] VEG-i-TRADE: European Community's Seventh Framework Programme (FP7) under grant agreement no. 244994; June 2010-May 2014

in dried plant food products. A database file was developed in Microsoft Excel (2007) and sent to the different involved Veg-i-Trade partners (UFRGS (Brazil), RIIL (Egypt), UGent (Belgium)) to collect the world-wide available data on mycotoxins in fresh produce and on (dried) derived products (i.e. nuts, dried fruits, spices and herbs, fermented olives). Data were received from different partners from own research projects : UFRGS (Brazil) (7 samples grapes, nuts, apples, tomatoes, patulin), RILL (Egypt) (2508 samples of groundnuts, aflatoxins) and UGent (Belgium) (170 samples of apple juice, patulin). Additionally, 826 data points from scientific literature data from over the world were collected: dried fruits (186), herbs (64), nuts (250), olives (97) and spices (229). Also, data from RASFF reporting (border rejections 2008-2010) were included: nuts (1360 cases), dried fruits (292 cases) and spices (136 cases).

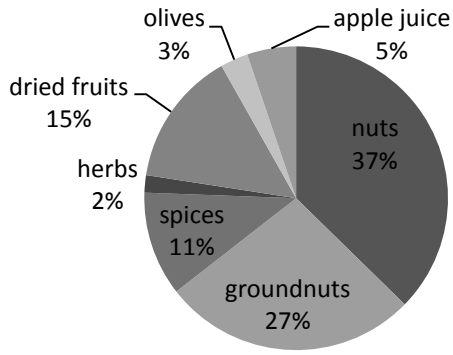

Figure 1.1: Overview of the percentage of different food groups extracted from the database

Table 1.2. Number of data points for the different mycotoxins extracted from the database

Mycotoxin	No. of available data points
Aflatoxin (total, B1, B2, G1, G2)	4754
Ochratoxin	612
Patulin	183
Deoxynivalenol	40
Zearalenone	43
Fumonisin	9
T-2	40
HT-2	0
Citrinin	54
Ergot alkaloids	0
Cyclopiazonic acid	2
Alternariol	0

Information collected were product, matrix, preservation technique, date of the analysis, method used, equipment used, LOD, LOQ and concentration (expressed as µg/kg). Figure 1.1 gives an overview of the percentage of the different product groups in the database available and Table 1.2 shows the number of data points for the different mycotoxins. Nuts and groundnuts are the commodities for which most data were collected. AF were the most analysed mycotoxins. This is logic, since AF are known to occur on nuts and nut products and a lot of research is performed on these mycotoxins (EFSA, 2007b).

Pistachio contains often AF as seen in different studies (Abdulkadar et al., 2000; Cheraghali et al., 2007; Chun et al., 2007; Thuvander et al., 2001; Zaied et al., 2010), but also almonds (Thuvander et al., 2001; Zaied et al., 2010), groundnuts and peanuts (Blesa et al., 2003; Chun et al., 2007; Sahar et al., 2009; Thuvander et al.,

2001; Zaied et al., 2010) are reported to contain levels of AF. The European Commission has set the maximum level for AFB1 at 2µg/kg-12µg/kg (depending on the type of commodity) (EU Regulation 1881/2006). Also Codex Alimentarius Commission has specified limits to 15µg/kg for peanuts, almonds, shelled Brazil nuts, hazelnuts and pistachios intended for further processing (Alimentarius, 1995). From the close to 400 mycotoxins identified to date, AF are the most ubiquitous and toxic ones. There are four important aflatoxins: AFB1, AFB2, AFG1 and AFG2 out of which AFB1 is the most toxic mycotoxin. The adverse health effects of aflatoxins include carcinogenicity, mutagenicity, hepatotoxicity, and impaired growth in animals and consequently, the International Agency for Research on Cancer (IARC) classified AF as class 1 carcinogen (IARC, 1985). Therefore, no TDI can be defined and a different approach is needed to evaluate the risk of this carcinogenic mycotoxin. EFSA recommended using the margin of exposure (MOE) approach, and concluded that if MOE values are higher than 10.000 (based on the BMDL10 from animal studies) it is considered to be of low concern from a public health point (EFSA, 2005). Some studies use a PMTDI (Kuiper-Goodman, 1998; Villa et al., 2009).

OTA was present in Sweden in currents and raisins (Moller et al., 2003), in Brazil in different dried fruits (dried black sultanas, dried white sultanas, dried dates, dried plums, dried apricots, dried figs) (Iamanaka et al., 2005), in Canada in currants, raisins and sultanas (Lombaert et al., 2004) and in Hungary in raisins (Varga et al., 2006). The maximum level set by the European commission is 10 µg/kg (EU Regulation 1881/2006). For OTA in dried fruits and nuts, there is not yet a level set by Codex Alimentarius. OTA was classified by IARC as possible carcinogenic for humans (group 2B) (IARC 1993). OTA is found nephrotoxic, carcinogenic, teratogenic, immunotoxic and hepatotoxic in animals. In humans it is probably involved in the

development of Balkan Endemic Nephropathy (BEN), which is a chronic progressive kidney disease, and urethelial tumors (UT) (Clark et al., 2006; Dai et al., 2004; O'Brien et al., 2005; Pfohl-Leszkowicz et al., 2007). The target organ is the kidney (Clark et al., 2006; JECFA, 2007; Reddy et al., 2010; Richard, 2007). In 1998 The Scientific Committee for Food (SCF) set the tolerable daily intake (TDI) for OTA on 5ng/kg BW.day (SCF, 2002).

Patulin is a well-known mycotoxin in several fruits, especially in apple and apple derived products such as apple juice, cider or apple puree (Baert et al., 2006; Beretta et al., 2000; de Sylos et al., 1999; Lai et al., 2000; Tangni et al., 2003). It is also found in other fruits or fruit derived products such as blueberry, cranberry, raspberry syrup and grape juice (Rychlik et al., 1999; Weidenbörner, 2001). When contaminated fruits are used as raw material for the manufacture of derived products, the final product is likely to be contaminated as well, because patulin is not completely degraded during the processing of fruits (Boonzaaijer et al., 2005). Different treatments during the production process can have an influence on the final patulin concentration in the product. In apple juices it is shown that an average loss of patulin of 39% is obtained for conventional clarification (Acar et al., 1998). A study on the effect of heat treatments showed that as the heating and evaporation time increased, the concentration of patulin in apple juice samples decreased (Kadakal et al., 2003). Patulin is produced by more than sixty fungal species, of which *Penicillium expansum* is the most important (Lai et al., 2000). Growth of the fungi on fruits occurs mainly if the surface tissue of the fruit is damaged and is thus mostly a post-harvest problem (Baert et al., 2012; Battilani et al., 2008). In contrast to its original description as an antibiotic, patulin can cause symptoms of acute and chronic toxicity and has teratogenic properties (Speijers, 2004). After absorption in the

gastrointestinal tract patulin degrades quickly before reaching other tissues (Rychlik, 2003). The International Agency for Research on Cancer (IARC) concluded that there is inadequate evidence that patulin is carcinogenic in animals (IARC, 1998). The "no observable effect level" (NOEL) was set at 43 µg/kg BW/d and a Provisional Maximum Tolerable Daily Intake (PMTDI) of 0.4 µg/kg BW was established (JECFA, 1995). The maximum permitted level of patulin in apples and derived products in Europe is 50 µg/kg, and 10 µg/kg for baby food (EC, 2006). Thus the presence of patulin in fruits and derived products is a potential and relevant food related health risk, especially for vulnerable consumers (Baert, De Meulenaer, Verdonck, et al., 2007; Tangni et al., 2003). As stated before, little studies have been performed and little information is available on mycotoxins in fresh produce.

1.3.2. Emerging mycotoxins in fresh produce

EFSA defines 'emerging risk' as follows: "Having regard to Articles 23f and 34 of Regulation (EC) 178/2002, an emerging risk to human, animal and/or plant health is understood as a risk resulting from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard." (EFSA, 2007a).

In order to estimate the prevalence of emerging mycotoxins on fresh produce and derived products a number of mycotoxins and matrices were selected in this research. This selection was made based on (1) literature research, (2) expert opinion and (3) their frequent use in derived food products and thus the theoretical risk of exposure for consumers to the possible presence of mycotoxins in derived food products (a consumer likely does not eat a moulded product (assumption author)). A first product that meets these criteria are tomatoes. Tomatoes are not only eaten

fresh, they are also processed into a variety of products, such as sauces, ketchup, pulp, paste, juices and dried tomatoes. There is a large consumption of these products by the population, which means that the possible presence of mycotoxins could have a significant impact on public health (Mariutti et al., 2009; Muhammad et al., 2004). In addition, tomatoes are highly susceptible to fungal infestation due to their soft epidermis (Moss, 1984). The most common fungi that infect the tomato plant and the tomatoes are *Alternaria* species. The occurrence of *Alternaria* mycotoxins in tomato products has been reported in Argentina (Somma et al., 2011; Terminiello et al., 2006), Switzerland (Noser et al., 2011), Brazil (da Motta et al., 2001) and Germany (Ackermann et al., 2011; Stefan Asam et al., 2011). Tenuazonic acid (TeA) is reported to occur in higher concentration compared to other *Alternaria* mycotoxins such as alternariol (AOH) and alternariol monomethyl ether (AME) (EFSA, 2011b). AOH and AME, on the other hand, are possibly genotoxic, whereas TeA is not (EFSA, 2011b). Figure 1.2 shows the chemical structure of these compounds.

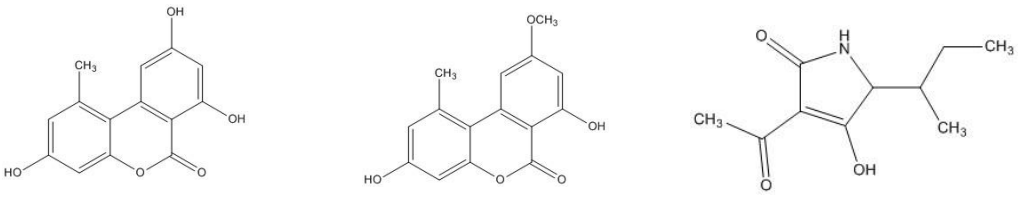

Figure 1.2: Chemical structure of alternariol (AOH) (left) alternariol monomethyl ether (AME) (middle) and tenuazonic acid (TeA) (right)

Alternaria species can produce several mycotoxins which can have adverse effects on humans such as salivation, vomiting, erythema, convulsions and gastrointestinal hemorrhage (Kralova et al., 2006). *Alternaria alternata* plays an important role in human esophageal cancer. AOH and AME are not very acutely toxic, where

tenuazonic acid (TeA) is toxic for several animal species (Ostry, 2008). AOH and AME are very weak acute toxins (LD50>>400 mg/kg BW), but they have chronic and sub-acute effects (Siegel et al., 2010b) and are mutagenic (Scott, 2001). TeA possibly inhibits the protein biosynthesis in cells (Zhou et al., 2008) and has been shown to be acutely toxic in several animal species and is more toxic then the other *Alternaria* mycotoxins (Ostry, 2008; Siegel et al., 2010b).

In 2011, an EFSA report on *Alternaria* mycotoxins was published. Data from Member States and published data were considered in these risk study. The panel used the threshold of toxicological concern (TTC) to give an opinion on the risks for public health. For the genotoxic toxins such as AOH and AME, the estimated dietary exposure exceeded the TTC (=2.5 ng/kg BW/day), for TeA, a non-genotoxic mycotoxin the exposure was below the set TTC (= 1500 ng/kg BW/day) (EFSA, 2011b). Next to tomatoes, also sweet bell peppers can be associated with mycotoxins. Recently, internal fruit rot of sweet bell peppers has become a problem in several European countries (i.e. Belgium, the Netherlands, and UK) and Canada. Several *Fusarium* species are suspected of causing this phenomenon (Mondale et al., 2009; Monbaliu et al., 2010). Also onions can be of concern. Since the phytopathogenic *Fusarium proliferatum* (which can produce fumonisin B1 (FB1)) was associated with bulb rot on onion plants (Dissanayake et al., 2009; Dugan et al., 2003; Stankovic et al., 2007) and a study in Serbia showed that there is also a potential mycotoxin risk in onion plants which are contaminated with *Fusarium proliferatum* (Stankovic et al., 2007), also onions can be of concern. At this moment, there are no data available of contaminated onions with mycotoxins.

Last groups of fresh produce potentially at risk are soft red fruits (i.e. small berries such as strawberries, blueberries, blackberries, red currants and raspberries). They

have a soft skin and are thus susceptible for small lesions which allow the growth of spoilage fungi both in the pre- and post-harvest stage. So far, little information is known on the presence of mycotoxins in soft red fruits or derived products. A study in Austria analyzed seven berry juices for patulin but none of the samples were contaminated. In Sweden 42 samples of blueberry soup and purees were tested for patulin but none of them were contaminated (EU, 2002b). A study on strawberries showed the presence of ochratoxin A (Engelhardt et al., 1999).

1.3.3. *Alternaria* mycotoxins

Alternaria is a genus of fungi, of which several species contaminates a wide variety of crops in fruits, vegetables and grains. *Alternaria* species produce several mycotoxins which can have adverse effects on humans such as salivation, vomiting, erythema, convulsions, gastrointestinal hemorrhage (Kralova et al., 2006). *Alternaria alternata* is the most common *Alternaria* species in fruits and vegetables and the most mycotoxin producing (Bhat et al., 2010; EFSA, 2011b) and plays an important role in human esophageal cancer (Liu et al. 1992). It occurs on cereals, sunflower seeds, oilseed rape, olives and various fruits (EFSA, 2011b).

The most frequent *Alternaria* toxin is AOH, which is sometimes found together with AME (EFSA, 2011b; Kralova et al., 2006). Other toxins are TeA, altertoxins (ATX), tentoxin (TEN), altenuene (ALT) *Alternaria alternata* f. sp. *lycopersici* toxins (AAL-toxin). The lather is known to be produced by Alternaria arborescens (previously named as *Alternaria alternata* f. sp. *Lycopersici)*.*Alternaria* spp. is able to contaminate tomatoes due to physical alterations such as sunburn, nutritional deficiency, cracks, etc. (Logrieco et al., 2009). Economic losses by the disease are

especially important in production processes for derived tomato products (such as concentrates, canned tomatoes, ketchups and sauces) (Logrieco et al., 2009).

1.4. Impact of climate change on the safety of fresh produce

1.4.1. Climate change scenarios

Climate change is a significant and lasting modification in the statistical distribution of weather patterns. Causes of climate change range from human to non-human activities and its impacts are experienced globally. The Intergovernmental Panel on Climate Change states that the warming up of the planet is very likely (IPCC, 2007). Climate change includes changes in overall weather patterns, temperature and rain patters whereas the consequences include increased surface temperatures, raised sea levels and increased intensity of extreme weather events, such as heat waves, hurricanes, heavy rainfall. As a result, the quantity and quality of food products can be affected, leading to food insecurity. The effects on the food safety and quality are depending on the regions but climate change will have an effect on the soil quality, crop yields and variations in seasons affecting the growth of crops (Paterson et al., 2010). This calls for scientific attention, especially from a risk analysis perspective (Magan et al., 2011). Climate change is important in food safety research and management (Liu et al., 2013). To identify and quantify climate change impacts on food safety, different climate scenarios are needed (Jacxsens et al., 2010).

Such climate change models are assessed by the Intergovernmental Panel on Climate Change (IPCC) for specific future scenarios. Four Representative Concentration Pathways (RCPs) are used to model climate changes: RPC 2.6, 4.5, 6.0 and 8.5. The different RCP levels are expected to stimulate research on the mitigation action in terms of technology change and the policy conditions (van Vuuren et al., 2011). RCP

8.5 represents the worst case scenario, suggesting that we carry on ('business as usual') with strong economic development for the rest of this century, using fossil fuels. RCP 6 (slowdown in emissions) is the scenario without a global coordinated climate policy, but with many localized clean energy initiatives which will manage to stabilize emissions by the latter half of the century. RCP 4.5 (mitigation) is the scenario when strong limits on fossil fuel emissions are set, so that greenhouse gas emissions peak by mid-century and then start to fall. RCP 2.6 (strong mitigation) is a world in which emissions peak in the next few years, and then fall dramatically, so that the world becomes carbon neutral by about mid-century (IPCC, 2013). Figure 1.3 gives a graphical view of the four different RCPs scenarios and the expected global surface temperature change.

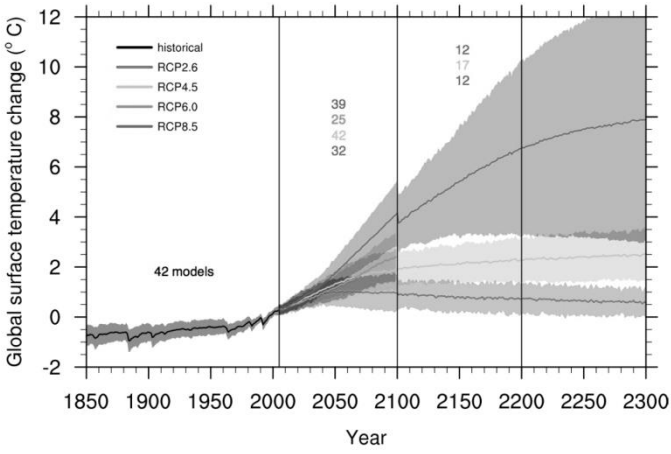

Figure 1.3: Time series of global annual mean surface air temperature anomalies (relative to 1986-2005) from CMIP5 (Coupled Model Intercomparison Project Phase 5) concentration-driven experiments. Projections are shown for each RCP for the multi-model mean (solid lines) and the 5 to 95% range (±1.64 standard deviation) across the distribution of individual models (shading). The numbers in the figure

indicate the number of different models applied for the projection to the different time periods (Collins, 2013).

1.4.2. Climate change impact on the growth of moulds and the presence of mycotoxins

Several published reviews suggest that climate change will affect the amount and presence of mycotoxin in crops (Magan et al., 2011; Paterson et al., 2010; Paterson et al., 2011). These reviews address the problem of climate change, but these are only hypothesis and not based on effective scientific data. Figure 1.4 gives an overview of the potential factors that may affect mycotoxin occurrence in the tomato production chain. Climate change can have an effect on several factors which are influencing the pre-harvest mould development and mycotoxin production. Biological factors, such as the change in tomato fruit and plant physiology due to adaptation to higher temperatures or water stress can affect the development of *Alternaria* moulds and mycotoxin production on tomatoes (Magan et al., 2011). Environmental factors, such as the presence of altered insects, pests and plant diseases may impact the susceptibility towards mould infestation (Rosenzweig, 2001). Also a shift in microbiological ecology on the plants can be expected and pathogenic moulds are gained/lost in this competition (IPCC, 2007). Change in temperature and water stress due to climate changes can influence the mould development and mycotoxin production. Once tomatoes are harvested and without intermediate storage entering (industrial) production processed, climate will not be an issue anymore but stability of the mycotoxins in production process will become relevant.

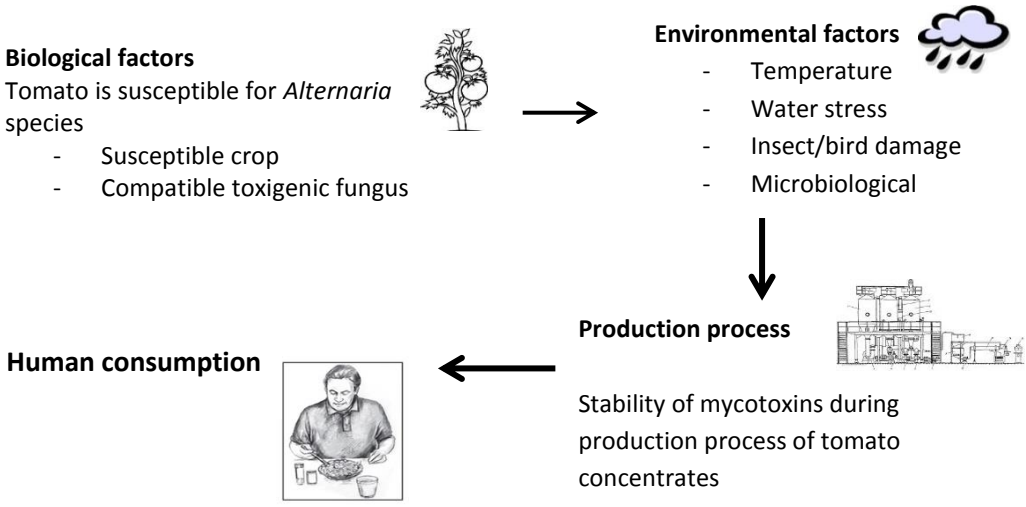

Figure 1.4: Factors affecting mycotoxin occurrence in the food chain (adapted for the tomato case based on CAST (2003))

To have mould growth and mycotoxin production, optimal temperatures and water activities are crucial (Magan et al., 1984), so the effect of climate change on the temperature will be of particular interest with respect to the pre-harvest situation of tomato production. Table 1.3 gives some examples of the consequences of climate change regarding to the production of mycotoxins as an effect of change in temperature. On the one hand, in areas where the temperature becomes too high for relevant fungi to grow and to produce mycotoxins, a decrease in mycotoxin production can occur. On the other hand, in areas where it is now too cold for the fungi, the increase of the temperature due to climate change can lead to the presence of fungi on crops which did not have those problems before (EFSA, 2012b; Miraglia et al., 2009). Moreover, extreme weather events can lead to more damaged crops, and thus the crops are potentially more vulnerable for the growth of fungi

(Marvin et al., 2013). Such events however, are more difficult to predict via modelling.

Many of the factors, discussed in Figure 1.4, will interact which each other, so the effect of climate change on mycotoxin production is very complex and difficult to predict completely. By using already the impact of temperature changes, however, a good indication of what could happen in the future can be made (EFSA, 2012b; Paterson et al., 210; Van Der Fels-Klercx et al., 2012).

Table 1.3: Summary of the consequences of climate change with focus on temperature changes regarding to the production of mycotoxins

Mycotoxin	Increase in mycotoxin production	Decrease in mycotoxin production	Reference
Aflatoxin	Temperature >25 °C	Temperatures <20 °C	Cotty et al.(2007)
Deoxynivalenol	25-30 °C	>32 °C <10 °C	Miraglia et al (2009); Ramirez et al. (2006)
Fumonisins	15-25 °C	>30 °C <15 °C	Samapundo et al. (2005); Schaafsma et al. (2007)
Ochratoxin	*Aspergillus ochraceus* (tropical and subtropical climates) *Penicillium verrucosum* (temperate climate)		Walker (2002)

Alternaria mycotoxins	Optimal temperature: 21-25 °C	No production <6°C	Paterson et al. (2011); Pose et al. (2010)

1.5. Derived tomato production process

A flow chart of derived tomato product production process is shown in Figure 3.3 (Chapter 3). Tomato processing factories are implanted within tomato fields to reduced transportation time. Tomatoes are harvested from the field and within 48h are entering the production line. Depending on the factory different ways of handling the incoming tomatoes are handled. Some screen the whole bath and accepted the whole batch when the number of stones, green and moulded tomatoes is under a certain level. Others do a manual sorting and/or using a colorimeter to remove stones, green and black tomatoes. After washing the tomatoes are pre-heated. Depending on the further application the pre-heating is performed at 60-65 °C (cold break procedure) or 90-95 °C (hot break procedure). The cold break procedure uses lower temperatures, resulting in a less viscous product, where the color and flavor is preserved. Cold break products are used for juices or higher value products. The hot break procedure results in a thicker and less quality product, used for ketchups and sauces. After the pre-heating the products is going through a sieve where seeds and peels are removed. The juice enters then an evaporator, where the product is thickened at 77 °C. Afterwards the product is sterilized and cooled down to fill in bags and cans. The product can be directly brought on the market for consumption or further used to produce sauces, ketchups and other products.

1.6 Conclusion

The overall objective of the presented work is to establish a farm to fork risk assessment study of emerging mycotoxins in fresh produce and derived products in view of the pressure of potential climate change scenarios and import across European borders.

The outline of this research is illustrated in Figure 1.5. A **first objective** of this work was gaining insights in the potential presence and characterization of emerging mycotoxins in fresh produce and their derived products. Therefore, a multiple mycotoxin extraction and detection method was developed. Moulded tomatoes, sweet bell peppers, onions and soft red fruits were screened for emerging mycotoxins (alternariol (AOH), alternariol monomethylether (AME) and tenuazonic acid (TeA)) and more established mycotoxins (OTA, patulin and fumonisins (FB1, FB2 and FB3)) (Chapter 2 and 3). Focus was further laid on *Alternaria* mycotoxins AOH and AME in derived tomato products. The stability of AOH and AME was evaluated throughout the (industrial) production of tomato concentrates (Chapter 3 and 4).

A **second objective** of the presented work was the evaluation of pre-harvest conditions on mould growth and mycotoxin production during tomato cultivation. In Chapter 5 the effect of temperature and fungicide concentration on the growth and mycotoxin production of *Alternaria* spp. was evaluated and used as an input for Chapter 6, were the impact of climate change and its scenarios on the growth of *Alternaria* spp. and its mycotoxin production in the tomato chain was investigated.

The **third objective** of the presented study was a risk assessment calculation on the emerging mycotoxins and their discussion in view of established mycotoxins in plant products. In Chapter 7 a farm to fork risk exposure of alternariol (AOH) and

alternariol monomethyl ether (AME) in derived tomato products is presented, based on the data gained in previous chapters. In Chapter 8 the exposure assessment calculations from the screening of tomato derived products (from Chapter 2 and 4) on emerging mycotoxins AOH, AME and TeA are combined with exposure output from established mycotoxins on dried plant products in order to perform a risk ranking and evaluate the relevant mycotoxins related to different plant products.

The work presented in this PhD thesis was part of a research funded by European Community's Seventh Framework Programme (FP7) under grant agreement no. 244994 (project VEG-i-TRADE). This project assessed the impact of climate change and globalization on the safety issues concerning fresh produce and derived food products.

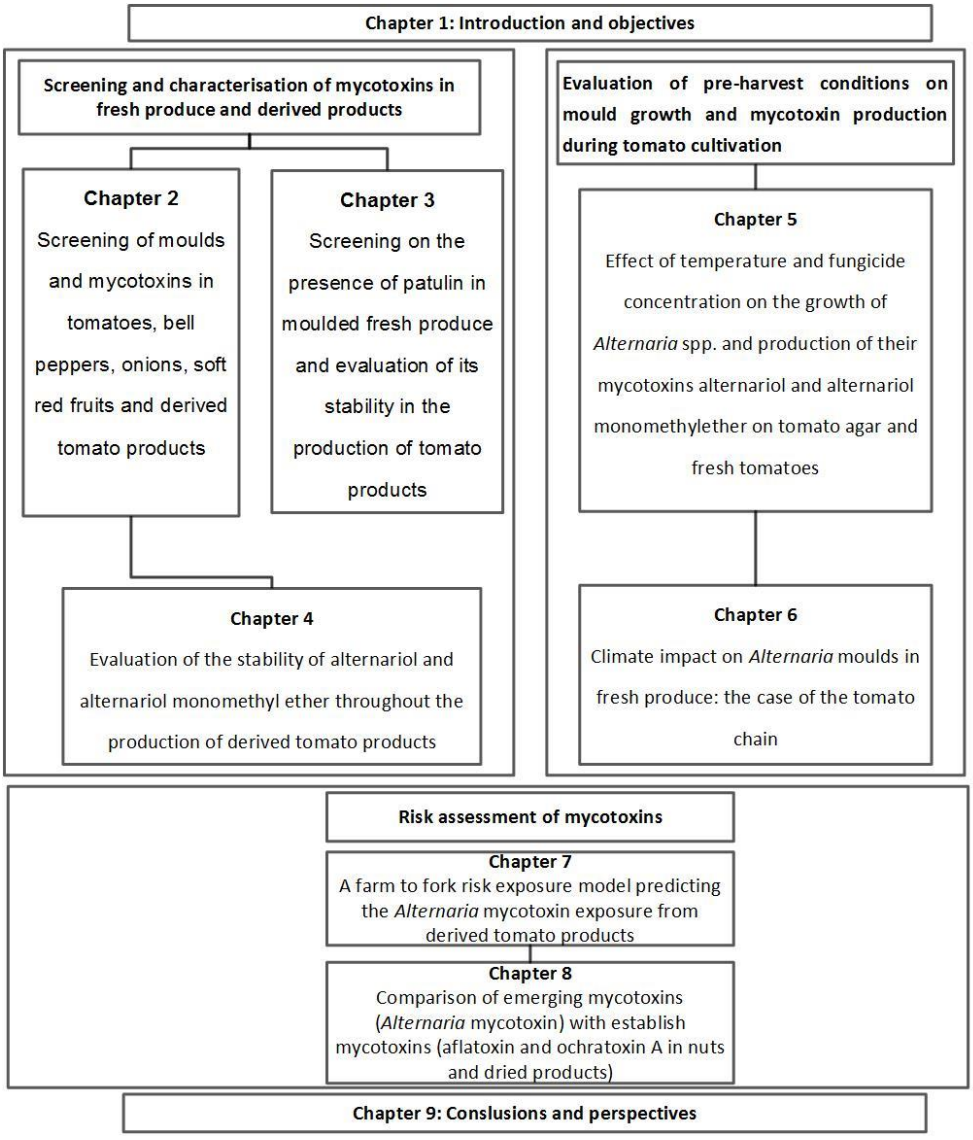

Figure 1.5: Outline of this research

Chapter 2:

Screening of moulds and mycotoxins in tomatoes, bell peppers, onions, soft red fruits and derived tomato products

Redrafted from: Van de Perre, E., Deschuyffeleer, N., Jacxsens, L., Vekeman, F., Van Der Hauwaert, W., Asam, S., Rychlik, M., Devlieghere, F., & De Meulenaer, B. (2014). Screening of moulds and mycotoxins in tomatoes, bell peppers, onions, soft red fruits and derived tomato products. *Food Control, 37*(0), 165-17

Chapter 2: Screening of moulds and mycotoxins in tomatoes, bell peppers, onions, soft red fruits and derived tomato products

2.1. Abstract

International standards and European legislation are available for established mycotoxins such as aflatoxins (AF), ochratoxin A (OTA) and trichothecenes in a variety of dried plant products. However, information of mycotoxins in fresh fruit and vegetable produce and derived products is limited. A semi-quantitative screening method was developed to screen for six mycotoxins (alternariol (AOH), alternariol monomethyl ether (AME), OTA and fumonisin B1, B2 and B3 (FB)) relevant in different matrices (tomatoes, bell peppers, onions and soft red fruits). On tomatoes, onions and soft red fruits, *Alternaria* spp. and their associated mycotoxins were detected. Derived tomato products were also screened and six out of 173 samples and four out of 173 samples were positive for AOH and AME, respectively. Moreover, 11/11 derived tomato products, containing AOH or AME, were positive for tenuazonic acid (TeA) as well. This study demonstrates the necessity for further mycotoxin research in the fresh produce chain in order to guarantee the safety and health of the consumers.

Introduction

Scientific literature, risk assessment studies and legislation are available on established mycotoxins such as aflatoxins (AF), ochratoxin A (OTA) and trichothecenes in different dried plant products (e.g. cereals, maize) (EC, 2010; van Egmond et al., 2007). However, information for mycotoxins in fresh produce or derived processed products (e.g. pasteurized or canned products) is limited. In order to estimate the prevalence of emerging mycotoxins on fresh produce and derived products a number of mycotoxins and matrices were selected in this research. This selection was made based on (1) a literature research, (2) expert opinion and knowledge and (3) their frequent use in derived food products and thus the theoretical risk of exposure for consumers to the possible presence of mycotoxins in derived food products (see Chapter 1).

In this study, tomatoes, sweet bell peppers, onions and soft red fruits were screened for emerging mycotoxins (AOH, AME, TeA) and more established mycotoxins (OTA and fumonisins (FB1, FB2 and FB3)) in order to have a first insight in the potential risks for public health related to the presence of mycotoxins in those fresh produce and their derived products. Next to the screening for mycotoxins, also the moulds present on those fresh produce were isolated and identified. The presence of patulin in these products was evaluated separately and will be discussed in Chapter 3.

2.2. Material and methods

2.2.1. Chemicals and reagents

LC-MS grade water; acetic acid and formic acid were supplied by Fluka (Sigma-Aldrich, Bornem, Belgium). LC-MS grade methanol, ethyl acetate and acetonitrile

were supplied by VWR (Leuven, Belgium). Standards of AOH, AME, OTA, FB1, FB2 and FB3 were supplied by Sigma-Aldrich (Bornem, Belgium). The standards were dissolved in methanol, except for FB1 and FB3, FB1 was dissolved in water/acetonitrile 1:1 (v:v) and FB3 was bought as a solution (water/acetonitrile, 1(v):1(v)). The solutions were dried under nitrogen and kept at -28°C.

2.2.2. Sample preparation

2.2.2.1. Sample collection

Moulded samples of fresh tomatoes (n=161), bell peppers (n=47), onions (n=61) and soft red fruits (n=50) were collected on several markets in different countries (i.e. Belgium, Spain, Egypt, Brazil, India and South-Africa). Moulded samples were kept at room temperature for some more days. After they were completely moulded, the samples were stored at -28°C. Derived products of tomatoes such as ketchups, concentrates, pulp, dried tomatoes and juices were collected on local markets in different countries (i.e. Belgium, Spain, Egypt, Brazil and South-Africa). A total of 144 samples of derived tomato products were screened.

2.2.2.2. Spiking of blank samples

The matrices (fresh tomatoes, bell peppers, onions and soft red fruits with no visible moulds contamination) were homogenized by use of a blender. Before extraction the blank samples were spiked with a working solution at different levels. The samples were mixed during 1h using a rotary shaker (Labinco, Breda, The Netherlands) to allow equilibration with the matrix.

2.2.2.3. Extraction

Two ml of extraction solvent (acetonitrile/ethyl acetate/formic acid 60:39:1) was added to 1 gram of the homogenized sample. After vortexing, it was mixed using a rotary shaker (Labinco, Breda, The Netherlands). The mixture was centrifuged during 10 min at 9000 rpm (Sigma 4k15, Buckinghamshire, England). A volume of 750 µl of extract was transferred into Eppendorf tubes and dried under a gentle stream of nitrogen and reconstituted in 750 µl of methanol. After vortexing and sonication, the sample was filtered through a 0.45µm syringe filter and analyzed by LC-TOF-MS.

2.2.3. Instrumental parameters

High resolution liquid chromatographic separation was achieved on an Ultimate 3000 RSLC system (Dionex, Amsterdam, The Netherlands), consisting of a vacuum degasser, binary pump, cooled auto sampler, column oven (30°C), and equipped with a Zorbax SB-C8 column (Agilent Technologies, Diegem, Belgium). Mobile phase A consisted of water/methanol/acetic acid 95:4.9:0.1 and mobile phase B of methanol/water /acetic acid 97:2.9:0.1. The gradient was a linear increase from 30 to 100% of mobile phase B in 23min and back to 30% mobile phase A at a flow rate of 0.2 ml/min. One run took 29 min. Injection volume was 20 µl.

The RSLC system was interfaced split less to a time-of-flight mass spectrometer (microTOF II, Bruker Daltonics, Bremen, Germany) equipped with an orthogonal electrospray ionization (ESI) source operating in both positive and negative mode. At the beginning of each run, the MS was calibrated with a sodium-acetate solution (0.1% acetic acid, 1% 1M NaOH in a water/isopropanol mixture (50/50)).

2.2.4. Validation

The recovery of the extraction method was validated by spiking blank samples of mixed tomatoes, bell peppers, onions, soft red fruits (blueberry, redcurrant, strawberry and blackberry) with a known amount of mixed standards (0-200 µg/kg). This procedure was done in duplicate and used 6 different concentrations (0-20-50-100-150 and 200 µg/kg). The total recovery (TR), signal suppression-enhancement (SSE) and extraction efficiency (EE) were calculated as described by Sulyok et al. (2006): TR (%)=100 x slope c/ slope a, where a is the slope of spiked pure solvents and c is the slope of the calibration curve from blank extracts spiked before extraction ; SSE (%) = 100* slope b/ slope a where b is the slope of the blank extracts spiked before injection and EE (%) = 100 * slope c/slope b.

The limit of detection (LOD) and limit of quantification (LOQ) were determined using the equation LOD = 3 sbl/S (sbl is the standard error of the intercept and S the slope of the respective calibration curve).

2.2.5. Screening for moulds

MEA (Malt Extract Agar) and DRBC (Dichloran Rose Bengal Chloramphenicol) were supplied by Oxoid (Hampshire, England). Each produce was visually examined for the presence of different fungi and those fungi were transferred on the two media. The plates were then incubated for one week at 25°C. With the aid of a stereomicroscope (SZX16, Olympus, Tokyo, Japan) the colonies that developed were studied morphologically. If necessary, further isolation and purification was performed by transferring the different moulds on new plates.

The identification up to genus level was done by observing the conidiophores, the sporongophores and the fruiting bodies of the different mould type using the stereomicroscope. Also an inverse light microscope (view hyphae and spores) (IX 81, Olympus, Tokyo, Japan) was used to visualize the hyphae and the mould spores. By using the identification key by Samson et al. (2004) the different isolates could be identified by comparing the morphological observations with the text book pictures and illustrations.

2.2.6. Screening for TeA

Thirty one moulded tomato samples and eleven derived tomato products, which tested positive for AOH and AME, were screened for TeA with the method explained in Asam et al. (2011) by prof. Michael Rychlik and his team. The samples were freeze dried before sending to Germany.

2.2.7. Data evaluation

Target AnalysisTM software (Bruker Daltonics, Bremen, Germany) was used for target mycotoxin screening, verification was done by Data Analysis TM software (Bruker Daltonics, Bremen, Germany). Each component was evaluated for mass accuracy, retention time and isotopic pattern. This gave the opportunity to distinguish between true and false positive results.

Prevalence data for each type of fresh produce and each country were expressed and the standard deviation of a sample proportion was calculated with:

$$Sp = \sqrt{\frac{p\,(1-p)}{n}} \qquad \text{Equation 2.1}$$

with p the proportion of positive samples and n the number of samples tested (Uyttendaele et al., 2009).

2.3. Results and discussion

2.3.1. Validation

Mixtures of mycotoxin standards were analyzed at different levels from 10 ng/ml to 100 ng/ml (10-25-50-75 and 100 ng/ml). The linearity was assessed by plotting the peak area versus the mycotoxin concentrations, resulting in good linearity for all mycotoxins with R2>0.99. Evaluating the recovery was performed by spiking the different products in duplicate at the following concentrations: 0, 20, 50, 100, 150 en 200 µg/kg. The results are summarized in Table 2.1.

Signal suppression by matrix effects (SSE) was obtained from spiking after extraction and results showed that there was significant signal suppression by matrix effects. For most of the matrices a SSE<70% was achieved. Only a few mycotoxins had higher values, such as AME in sweet bell peppers (75,4%), redcurrants (100,7%), blueberries (90,1%) and FB2 in tomatoes (84,9%). Table 2.1 shows also clearly that the extraction efficiency is much lower for FB compared to AOH, AME and OTA. Also, in the multi screening method with LC tandem MS of Monbaliu at al. (2009) the recovery of fumonisins was lower than the other mycotoxins. Fumonisins are highly polar compounds because of their long hydroxylated hydrocarbon chain. Since fruit and vegetables are very aqueous matrices, it is possible that the FB cannot sufficiently be extracted by the extraction solvent and therefore remains in the matrix. As the method also targeted more hydrophobic compounds, a compromise needed to be found.

Noser et al. (2011) developed an UPLC-MS/MS method for the detection of *Alternaria* mycotoxins in tomatoes. Recoveries for AOH and AME were respectively 29±2 and 87±6 % for fresh tomatoes. These are lower than the recoveries we obtained for the same matrix and mycotoxins (73.7±6.0 and 89.9±4.4% respectively).

The LOD of the mycotoxins obtained are shown in Table 2.2 For AOH and AME there is at this moment no maximum level in EU regulation 1881/2006, neither in other risk assessment nor in management document (e.g. on Codex Alimentarius level, EFSA report). For OTA and fumonisins the EU regulation 1881/2006 has set several limits, but not for tomatoes, onions, sweet bell peppers or soft red fruits. In this study the aim was to identify mycotoxins producing moulds in fresh produce and thus the obtained method performance was considered fit for its purpose. Since patulin is widely known to be a contaminant of apples and derived products (Baert et al., 2006), it was also tried to include patulin in the semi-quantitative method. However, it was analytically not possible to include it in the same method. So, we developed an UPLC-DAD method for the determination of patulin in different products. These results will be discussed in Chapter 3.

Table 2.1: Recovery (signal suppression enhancement (SSE), total recovery (TR) and extraction efficiency (EE)) ±stdev (%) of the different mycotoxins in tomatoes, sweet bell peppers, onions and soft red fruits (i.e. strawberry, blackberry, red currant and blueberry).

Matrix		AOH	AME	OTA	FB1	FB3	FB2
Tomato	**SSE**	77± 4	62±10	66±2	75±2	60±1	85±2
	TR	74±6	90±4	61±5	27±2	37±4	57±1
	EE	83±6	96±4	94±1	37±2	61±5	68±0
Sweet bell pepper	**SSE**	43±2	59±7	51±0	47±2	60 ±5	73±6
	TR	41±7	56±3	42±6	23±3	34±4	60±1
	EE	76±1	94±6	83±12	50±6	57±6	83±1
Onion	**SSE**	45±4	70±16	53±3	56±1	61±8	54±5
	TR	19±2	29±5	39±6	26±1	32±1	34±7
	EE	43±0	42±3	73±14	44±2	53±8	62±7
Strawberry	**SSE**	44±0	73±5	55±2	52±2	53±4	59±7
	TR	39±4	60±10	43±1	26±2	38±4	46±2
	EE	90±9	69±8	74±5	49±4	63±12	75±16
Blackberry	**SSE**	53±0	69±3	52±2	53±3	41±1	64±3
	TR	41±1	41±6	44±0	26±3	53±1	37±3
	EE	78±2	59±7	84±2	49±3	80±1	58±2
Red currant	**SSE**	48±2	101±5	46±1	42±2	42±0	45±2
	TR	39±1	59±1	35±1	22±1	25±1	34±1
	EE	82±5	59±1	76±2	52±3	61±3	76±1
Blueberry	**SSE**	68±5	90±5	48±1	48±2	43±2	40±0
	TR	44±3	54±6	36±1	23±2	26±2	26±2
	EE	65±1	60±1	75±0	48±2	60±2	66±4

Table 2.2: LOD (µg/kg) of the different mycotoxins on different commodities

LOD (µg/kg)	Tomatoes	Sweet bell peppers	Onions	Blueberry	Red currant	Strawberry	Blackberry
AOH	12.2	17.1	17.4	14.4	7.8	14.6	7.4
AME	13.5	30.0	8.2	4.7	19.6	90.0	9.3
OTA	7.2	34.7	7.8	13.9	19.5	43.7	4.3
FB1	28.4	35.0	8.9	9.9	17.6	16.2	7.8
FB3	9.3	10.7	13.1	11.1	20.0	23.8	8.9
FB2	9.2	20.8	16.3	5.6	10.0	1.3	9.9

2.3.2. Identification of moulds

Two media for mould isolation were used. MEA can be considered as a general growth medium for non xeropphilic moulds (aw~0.99 and pH 5.4 ± 0.2), but on the downside there can be a possible overgrowth by mucoraceous fungi. That is why a second growth media was used. DRBC is a selective medium, due to the presence of dichloran Rose Bengal and Chloramphenicol and restricts the growth of fast spreading mucoraceous fungi such as *Rhizopus* spp. and *Mucor* spp. but allows the grow of other genera (Pitt et al., 1997). DRBC is suitable to use for fresh and high aw foods and thus ideal to simulate the intrinsic parameters of fresh produce (Hocking et al., 1992).

Penicillium spp. appears to be the most frequently isolated, over the different commodities and countries (113 out of 188 samples). *Aspergillus* spp., *Fusarium* spp.

and *Alternaria* spp. were also detected on the Belgian tomatoes (10, 3 and 3 out of 188 samples, respectively). Other microorganisms and genera that were found were bacteria, yeasts, *Rhizopus* spp., *Geotrichum* spp., *Cladosporium* spp., *Botrytis* spp. and moulds belonging to the phylum *Zygomycota*. The isolation and identification of the fungi could only be done for the products from Belgium and Spain. The samples sent by the partners from Brazil, South-Africa, India and Egypt contained no more viable moulds or mould spores.

Many studies investigated the presence of moulds on vegetables and fruits. Next to *Botrytis* spp., *Phoma* spp., *Cladosporium* spp. and *Geotrichum* spp., also *Alternaria* spp. and *Penicillium* spp. were reported by several studies (Tournas, 2005; Tournas et al., 2005; Tournas et al., 2001). In our study *Penicillium* spp. was identified on all selected commodities (i.e. tomatoes, onions, sweet bell peppers and soft red fruits). *Penicillium* spp. is a widespread fungus and the different species are one of the most important pathogens of fruits and vegetables. *Penicillium expansum* can produce patulin and *Penicillium verrucosum* is known to produce OTA (Tournas et al., 2005). Besides the presence of *Penicillium* spp., tomatoes were also infected by *Aspergillus* spp., *Fusarium* spp. and *Alternaria* spp.. Although a low number of *Alternaria* spp. was isolated out of the sample, the majority of the fruits and vegetables contained *Alternaria* mycotoxins. This could be related to the application of a growth medium not specific for *Alternaria* species. Moreover, other fungi such as *Mucor* spp. and *Rhizopus* spp. grow very rapidly and can overgrow the agar faster than *Alternaria* species, which makes isolation and subsequent identification impossible (Pitt et al., 1997). Tomato plants and tomatoes are highly associated with the presence of *Alternaria* spp. which explains the presence of the mould on tomatoes (Ostry, 2008; Pose et al., 2010). Moreover, *Alternaria* spp. is able to grow at lower temperatures

and thus can grow during storage and transportation, and also produce mycotoxins. Although moulded onions can be contaminated with *Aspergillus niger* and *Fusarium* spp. (Pitt et al., 1985; Stankovic et al., 2007; Toit et al., 2003; Velez-Rodriguez et al., 2007) and bell peppers are associated with different *Fusarium* species (Monbaliu, 2009), only *Penicillium* species were identified during the screening on onions and sweet bell peppers. These results can be influenced by the low number of samples screened (n=26) and the fact that many *Penicillium* moulds, that were heavily sporulated, were found. These grew faster on the selected media and thus overgrew other species, such as *Fusarium* spp. and *Aspergillus* spp. *Penicillium* spp. was also identified in seven of the 18 soft red fruits samples. Previous studies showed the most common moulds isolated from berries were *Botrytis cinerea*, *Rhizopus* spp. (on strawberries), *Alternaria* spp., *Penicillium* spp., *Cladosporium* spp. and *Fusarium* spp. (Pitt et al., 1997).

2.3.3. Presence of mycotoxins on moulded products

In total 320 moulded samples were screened for multiple mycotoxins with a semi-quantitative LC-TOF-MS method. Prevalence is calculated applying equation 2.1. In 97 samples (30.3%. standard deviation of sample proportion (Sp) 2.6%) AOH was detected and 79 were positive for AME (24.7%. Sp 2.4%). Belgian onions had the highest prevalence of AOH (74.4%. Sp 6.7%) and AME (72.1%. Sp 6.8%). Soft red fruits from Belgium had a prevalence of 66% (Sp 6.7%) for AOH and 62% (Sp 6.9%) for AME. Tomatoes originating from Belgium, Spain and Egypt were positive for the presence of AOH (14.8; 29.6 and 30%; Sp 3.4; 8.8 and 14.5%. respectively). Tomatoes from Belgium and Spain also contained levels of AME (10.2 and 18.5%; Sp 2.9 and 7.5%. respectively). Compared to the other products, bell peppers had a lower AOH

prevalence level (6.7%. Sp 4.6%). The moulded products were negative for OTA, FB1, FB2 and FB3, except for the bell peppers from India which had a 50% prevalence of FB2 (Sp 13.4%). Since the analysis of TeA was done by the group of Technische Universität München, it was decided to only analyze samples which tested positive for AOH. 20 moulded tomatoes from Belgium and 11 moulded tomatoes from Spain were analyzed for TeA. All moulded tomato samples both from Belgium and Spain tested positive for TeA.

It can be concluded from this screening that the *Alternaria* mycotoxins seems to be the major problem compared to OTA and fumonisins in the selected fresh produce. In tomatoes, *Alternaria* mycotoxins only occur in the more moderate climates (Belgium, Spain, South-Africa), and not in more tropical regions (exemplified by Brazil, India), although more samples should be screened to be able to confirm this effect. It could be hypothesized however that these observations are due to the fact that *Alternaria* species grow best at moderate temperatures but also at low temperatures, with an optimal temperature of 23°C. The optimal temperature for the production of AOH and AME is 25°C (Pose et al., 2010). *Alternaria* species are host-specific (Logrieco et al., 2009) and have been reported to occur on several fresh produce such as apples, olives, tomatoes and citrus fruits (EFSA, 2011b). As from the present screening, fumonisins and ochratoxin A do not seem to play a major role as a contaminant in the considered fresh produce.

Because of the rather high prevalence of *Alternaria* mycotoxins in tomatoes, the fact that tomatoes are used frequently in further processing and the high consumption of the derived tomato products, it was decided to screen a number of derived tomato products for AOH, AME and TeA.

2.3.4. Mycotoxins and derived tomato products

The results of the screening for mycotoxins in derived tomato products bought in Belgium are presented in Table 2.3. Five percent of the products bought in Belgium contained concentrations of AOH > LOD (n=6/119), 3.5% samples had concentrations of AME>LOD (n=4/119). In the derived products from other countries only one derived product was positive for AOH (originating from Egypt), but a rather small number of analyses was performed for each country (13 samples from Spain, 11 from Brazil, 7 from Cyprus, 5 from Finland, 15 from Egypt). The derived tomato products screened for TeA (remark: these products were all positive for AOH and/or AME) were concentrates (6), purees (2) and canned tomatoes (3). They were all found to be positive for TeA as well, with concentrations from 0,7-4,8 mg/kg.

Table 2.3: Prevalence (%) of AOH and AME in derived tomato products samples on the Belgian market (Sp= standard deviation of the proportion)

Type of tomato product (n=119)	Total samples (n)	Prevalence (%)			
		AOH	Sp	AME	Sp

Concentrates	18	22.2	9.7	11.1	7.4
Canned tomatoes	28	0		0	
Ketchup	22	0		0	
Tomato puree	15	13	8.6	13	8.6
(sun)dried tomatoes	9	0		0	
Tomato soup	7	0		0	
Tomato powder	7	0		0	
Tomato juice	3	0		0	
Tomato sauce	3	0		0	
Tapenades, pesto	7	0		0	

A remarkable result was that both AOH and AME were found in the tomato concentrates and tomato purees (the product positive for AOH from Egypt was also tomato concentrate), but in the further processed and diluted products such as ketchups and soups no mycotoxins were found anymore. Other studies however found *Alternaria* mycotoxins in tomato products other than concentrates and purees (such as ketchups). A study in Brazil analyzed processed tomato products for cyclopiazonic acid (CPA), TeA, AOH and AME. Both TeA (LOD=8 µg/kg) and CPA (LOD=11 µg/kg) were detected in the samples but neither AME (LOD=2 µg/kg) or AOH (LOD=5 µg/kg) were found in the samples (da Motta et al., 2001). A similar study in Argentina investigated eighty samples of tomato puree processed and sold in Argentina. TeA (LOD=11 µg/kg) was found in twenty-three samples, AOH (LOD=5

µg/kg) in five samples and AME (LOD=2 µg/kg) in twenty-one samples (Terminiello et al., 2006). Different tomato products bought on the Swiss market were screened for different *Alternaria* mycotoxins, TeA (LOD=2 µg/kg) was found in 81/85 samples, AOH (LOD=4 µg/kg) and AME (LOD=1 µg/kg) in 26/85 samples, of which 17/17, 11/17 and 12/17 concentrates/purees tested positive for TeA, AOH and AME, respectively (Noser et al., 2011). Derived tomato products from Germany were screened for AOH (LOD=2 µg/kg), 18/18 ketchups, 10/10 tomato pastes and 9/16 tomato juices were contaminated (Ackermann et al., 2011).

2.4. Conclusions

Screening for moulds showed that *Penicillium* spp. was widely present on all the commodities (i.e. tomatoes, sweet bell peppers, onions and soft red fruits). Additionally, *Aspergillus* spp., *Fusarium* spp. and *Alternaria* spp. were detected on the Belgian tomatoes. A method for the screening of six mycotoxins (i.e. AOH, AME, OTA, FB1, FB2 and FB3) was developed and validated to be able to identify mycotoxins in fresh produce. In total 320 moulded samples were screened for multiple mycotoxins with the semi-quantitative LC-TOF-MS method. The *Alternaria* mycotoxins seemed to be the major problem compared to OTA and fumonisins in the selected fresh produce. Furthermore, derived tomato products such as concentrates and purees were contaminated with AOH, AME and TeA. These derived tomato products did not contain levels of OTA or fumonisin. EFSA reports a potential public health risk due to the presence of AOH and AME in food products. Because the estimated chronic dietary exposure to TeA was below the set TTC value, these levels were unlikely to be of concern for human health (EFSA, 2011b). In order to estimate the risks for public health related to the presence of these *Alternaria* mycotoxins in

derived tomato products a dietary risk exposure is needed (see Chapter 7 and 8). Also, the presence of patulin in these commodities should be evaluated, this will be further discussed in Chapter 3.

Chapter 3:

Screening on the presence of patulin in moulded fresh produce and evaluation of its stability in the production of tomato products

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Chapter 3: Screening on the presence of patulin in moulded fresh produce and evaluation of its stability in the production of tomato products

3.1. Abstract

A screening for the presence of patulin in moulded fresh produce was conducted. Patulin was present in 11% of the sweet bell peppers and in 8% of the soft red fruits, but not in moulded onions. Patulin was also found in fresh moulded tomatoes, but could not be detected in derived tomato products. When simulating the production process of tomato concentrates, none of the evaluated hypotheses could however explain a complete degradation of patulin. It was thus concluded that the tomato varieties used for further processing, are probably less susceptible to infestation by patulin producing moulds compared to tomatoes used for the fresh market in the pre-harvest stage. This was confirmed by screening for patulin throughout the production process of tomato concentrate, gazpacho and salmorejo in three different countries where all 191 samples were negative for patulin. Therefore, it can be concluded that no health risk can be associated with derived tomato products considering this particular mycotoxin.

3.2. Introduction

Patulin is a well-known mycotoxin in several fruits, especially in apple and apple derived products such as apple juice, cider or apple puree (Baert et al., 2006; Beretta et al., 2000; de Sylos et al., 1999; Lai et al., 2000; Tangni et al., 2003). It is also found in other fruits or fruit derived products such as blueberry, cranberry, raspberry syrup and grape juice (Rychlik et al., 1999; Weidenbörner, 2001). When contaminated fruits are used as raw material for the manufacture of derived products, the final product is likely to be contaminated as well, because patulin is not completely degraded during the processing of fruits (Boonzaaijer et al., 2005). Different treatments during the production process can have an influence on the final patulin concentration in the product. In apple juices it is shown that an average loss of patulin of 39% is obtained for conventional clarification (Acar et al., 1998). A study on the effect of heat treatments showed that as the heating and evaporation time increased, the concentration of patulin in apple juice samples decreased (Kadakal et al., 2003).

The first objective of this study was to evaluate the presence of patulin in moulded vegetables and fruits matrices others than previously reported (i.e. tomatoes, onions, sweet bell peppers, soft red fruits (small berries such as strawberries, blueberries, blackberries, red currants and raspberries) and derived tomato products). After the observation that patulin was found in fresh moulded tomatoes, but was absent in derived tomato products hypotheses linked to the potential degradation of patulin during tomato processing were evaluated, both in laboratory and industrial conditions.

3.3. Material and methods

3.3.1. Reagents and chemicals for patulin analysis

Ethyl acetate (pa), ultra-pure water and acetic acid (99-100%) (pa) were purchased from ChemLab, Zedelgem, Belgium. Perchloric acid 70% and acetonitrile were bought from Fisher Scientific, Aalst, Belgium and patulin from Sigma, Diegem, Belgium. A stock of standard solution of patulin was prepared by dissolving 5 mg of patulin in 25 mL of ethyl acetate. The working standards were further prepared by making dilutions in water at pH 4. Standards were kept at -28 °C. Ascorbic acid, malic acid and citric acid were bought from ChemLab, Zedelgem, Belgium. L-glutamate was bought from Fluka, Diegem, Belgium, aspartate was purchased from Merck, Overijse, Belgium.

3.3.2. Analyses for patulin and screening of moulded products and derived tomato products

3.3.2.1. Moulded tomatoes, bell peppers and onions

To evaluate the method for screening of patulin, the protocol described by Sanzani et al. (2009) was adapted. Potato dextrose agar (PDA) petri plates (Oxoid, Drongen, Belgium) were inoculated with *Penicillium expansum* MUM 00.01 and incubated for 10 d at 25 °C. Three different types of samples were taken in order to evaluate which contained the highest patulin concentration: only fungi/mycelium (1 cm^2), only agar (1 cm^2) and combination of fungi and agar (1 cm^3). This procedure was conducted in triplicate. Results showed that when fungi were taken together with some agar (up to 1 cm^3) the highest peak of patulin was obtained. Therefore, it was decided to take a piece of fungi combined with a piece of the moulded matrix for the screening.

Because the objective was to evaluate the possible presence of patulin producing moulds, this qualitative method proved to be sufficient.

One cm^2 fungi together with one cm depth of product was put into a Falcon tube together with three mL of acidified water (pH adjusted to 4 with pure acetic acid). This was centrifuged for 10 min at 9000 g, filtered through a 0.45 µm syringe filter and analyzed by HPLC-UV (Thermo, Aalst, Belgium). The results were expressed as present/absent and are qualitative. This method allowed an easy screening to determine the presence of patulin producing moulds on the moulded products (i.e. tomatoes, sweet bell peppers and onions).

3.3.2.2. Moulded soft red fruits and derived tomato products

Patulin was extracted from frozen soft red fruits and derived tomato products by adding 2 mL ethyl acetate to 1 g of homogenized sample. After vortexing, it was mixed using a rotary shaker for 1h. The mixture was centrifuged during 10 min at 9000 g. A volume of 750 µL of extract was transferred into Eppendorf tubes, dried under a gentle stream of nitrogen and reconstituted in 750 µL of water pH 4. After vortexing and sonication, the reconstituted extract was filtered through a 0.45 µm syringe filter and analyzed by UPLC (Dionex, Aalst, Belgium). This method is quantitative and the results were expressed as µg patulin/kg product.

3.3.3. Instrumental parameters

The HPLC was a Varian Vista 5500 (Thermo, Aalst, Belgium). It was equipped with a Rheodyne Model 7125 six-way injector with a 20 µl loop. The column used was a 250 mm x 4.6 mm i.d., 5 µm, Inertsil 5 ODS-2 Stainless Steel (Varian, Diegem, Belgium) column. As a mobile phase acetonitrile/0.0175% perchloric acid in water (7/93, v/v)

was used at 1 mL/min. After 13 min a change was made to 90% acetonitrile to elute interfering compounds from the extract. After 15 min there was again a shift to acetonitrile/0.0175% perchloric acid in water (7/93, v/v). The column and guard column were maintained at 36 °C during the HPLC analysis. Detection was carried at 276 nm and runtime was 38 min.

UPLC separation was achieved on an Ultimate 3000 RS system (Dionex, Aalst, Belgium), consisting of a vacuum degasser, quaternary pump, cooled auto sampler, column oven (30 °C), and equipped with a 150 mm x 2.1 mm i.d., 2.2 µm Acclaim RSLC 120 C18 column (Dionex, Aalst, Belgium). As a mobile phase acetonitrile/perchloric acid in water (0.0175%) (7/93, v/v) was used at 0.5 mL/min. After 5 min a change was made to 90% acetonitrile to elute polluting compounds from the extract. After 6.6 min there was again a shift to acetonitrile/0.0175% perchloric acid in water (7/93, v/v). The column was maintained at 30 °C during the UPLC analysis. Detection was carried at 276 nm by a diode array detector and runtime was 10 min.

3.3.4. Validation of the quantitative method for determining patulin in moulded soft red fruits and derived tomato products

The analytical method was validated by spiking fresh soft red fruit (strawberry, red currant, blackberry, and blueberry) which was homogenized to a juice and tomato concentrates with patulin (0.1; 0.5; 1 and 1.5 µg/mL). This procedure was done in duplicate. After spiking with patulin, the samples were mixed for 1h using a rotary shaker to allow equilibration with the matrix.

3.3.5. Evaluation of the stability of patulin in the production process of tomato derived products

3.3.5.1. Binding of patulin with compounds present in the tomato matrix

Fresh tomatoes were visually checked for the absence of moulds and then mixed to prepare a tomato juice using a blender. The tomato juice was spiked with three patulin concentrations (0.5; 1.0 and 1.5 µg/mL) and left between 0 and 5 days at 4 °C before starting the extraction procedure. This was done in duplicate.

3.3.5.2. Influence of ascorbic acid (vitamin C) and free amino acids on the stability of patulin

Different tomato simulants were made in order to investigate the influence of vitamin C on patulin**.** Tomato juice was first analyzed for the presence of free amino acids (Mestdagh et al., 2011) and vitamin C (Van Bree et al., 2012). Based on these results, the formulation of the tomato simulants was made in order to evaluate the effect of vitamin C and amino acids on the stability of patulin during the heating process of the production of tomato concentrate. The simulant was made by adding citric acid and malic acid (respectively 9% and 4% on dry matter (DM) (5% DM in tomato)) and vitamin C (0.14g/5g DM) to water. The same was done without adding vitamin C, but adding amino acids, glutamate (0.07g/5g DM) and aspartate (0.02 g/5g DM). These amino acids proved to be the two dominant amino acids representing respectively 63 and 21% of the total free amino acids present in tomatoes. In order to simulate the production process of tomato concentrate, the simulants were thickened from 5% to 12% dry matter using a rotary evaporator (100 °C) (Heidolph, Schwabach, Germany). Each experiment was done in duplicate and analyzed for patulin using the previously presented extraction procedure.

3.3.5.3. Influence of the heating process on the stability of patulin

Tomatoes, visually checked for the absence of moulds, were inoculated with *Penicillium expansum* MUM 00.01 and incubated at 25 °C for 2 weeks. After homogenization, the sample was preheated in a warm water bath at 95 °C for 30 min, and then put on a rotary evaporator at 77 °C for 20 min, thus simulating the hot break procedure. This was done in quintuplet. Samples were taken before preheating, after preheating, after 10 min evaporating and at the end (20 min evaporating). Samples were analyzed for patulin using the previously presented extraction procedure.

3.3.5.4. Sampling in industrial production process of derived tomato products

Samples were taken from the field stage through till the end product in different companies producing tomato concentrate (in Spain, Portugal and Poland), salmorejo (Spain) and gazpacho (Spain). The type and number of samples that were analyzed for patulin were moulded tomatoes on field (pre-harvest) (n=49), moulded tomatoes entering factory (post-harvest) (n=36), skin and seeds (removed from process) (n=17), intermediate products (n=61) and end products (i.e. concentrates, gazpacho and salmorejo) (n=28).

3.3.5.5. Data evaluation

Prevalence data of patulin for each type of fresh produce and each country were expressed and the standard deviation of a sample proportion was calculated with equation 2.1 (Chapter 2).

3.4. Results and discussion

3.4.1. Development and validation of the quantitative method for analyzing patulin in moulded soft red fruits and derived tomato products

Because the moulded soft red fruits were liquid after defrosting, the described qualitative method could not be used to screen for presence of patulin and thus for patulin producing moulds in these samples. Therefore, a more complex method was used to detect patulin. All derived tomato products were also screened with this method.

Patulin standards were analyzed at different levels (0.1; 0.5; 1 and 1.5 µg/mL). The linearity was assessed by plotting the peak area versus the patulin concentration, resulting in a good linearity with $R^2 > 0.99$. The RSD obtained at different levels were 3.9% for 1.5µg/ml and 4.7% for 0.1 µg/ml (n=10: 2 repetitions over 5 days). The recovery of patulin in the different matrices was 96.5 ±11.0 % for tomato concentrate, 77.5 ± 7.4 % for blue berry, 76.1 ±12 % for red currant, and 76.4 ±8.6 % for strawberry and 71.1 ± 6.8 % for blackberry. A good recovery above 70% was achieved for all matrices. The recovery for tomato concentrate was higher than the recoveries for soft red fruit. A LOD of 13 µg/kg and LOQ of 39 µg/kg were obtained. This is below the maximum permitted level of 50 µg patulin/kg in apples and derived products in Europe (EU, 2006).

3.4.2. Screening for patulin in moulded products

In total 44 moulded sweet bell peppers, 61 moulded onions, 50 moulded soft red fruits and 158 moulded tomatoes were screened for the presence of patulin. The results of the screening are shown in Figure 3.1. In moulded onions no patulin was

found. Patulin was present in 16.7% of the moulded sweet bell peppers from Belgium (n=30, Sp = standard deviation of a sample proportion =6.8%) and in none of the moulded sweet bell peppers from India (n=14). Currently, in the literature there is no information on patulin occurrence in sweet bell peppers available. Only by Frank (1977) it is reported that after inoculation with *Penicillium expansum*, patulin was found in sweet bell peppers, but not in onions. Up to 8% of the moulded soft red fruits were contaminated with patulin (n=50, Sp=3.8%). Frank (1977) also mentioned the occurrence of patulin on strawberries after inoculation with patulin producing moulds. Additionally, patulin was present in 11% of moulded tomatoes (n=158, Sp=2.5%). 14% of the tomatoes from the Belgian market were positive for patulin (n=107, Sp=3.4%) and patulin was present in 7.4% of the moulded tomatoes from the Spanish market (n=27, Sp=5%). So far, no information on the presence of patulin in moulded tomatoes is available in literature. Because of the high number of tomatoes contaminated with patulin, it was decided to screen further for the presence of patulin in derived tomato products, since they form a higher risk for exposure to patulin for consumers then the moulded tomatoes, which are not likely to be consumed as such.

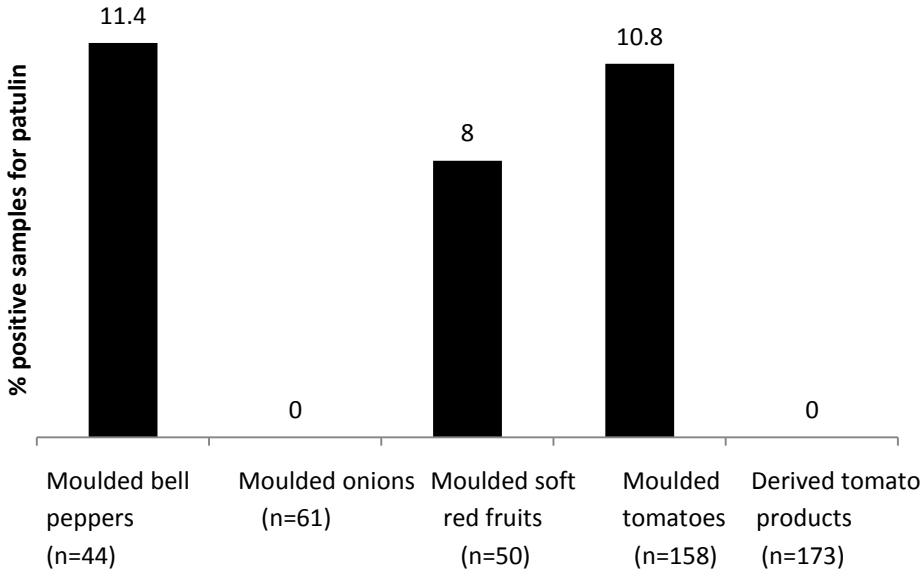

Figure 3.1: Percentage of positive samples for patulin for moulded bell peppers (n=44), moulded onions (n=61), moulded soft red fruits (n=50), moulded tomatoes (n=158) and derived tomato products (n=173).

3.4.3. Screening for patulin in derived tomato products

In total 173 derived tomato samples (purees, concentrates, juices, dried tomatoes, soups) were screened for patulin (from Belgium (n=114), Spain (n=13), South-Africa (n=13), Brazil (n=7), Egypt (n=16), Cyprus (n=7) and Finland (n=5)) applying the qualitative screening method. Although 11% of the moulded fresh tomato samples were positive for patulin, none of the derived tomato samples tested positive (173 samples<LOD). At the moment there is no reported knowledge on the presence of patulin in processed tomato products. A study in Brazil analyzed 48 samples of tomato pulp after processing and no patulin was found in any of the samples (Kawashima et al., 2002). The Ministry of Agriculture, Fisheries and Food (MAFF) of

the UK analyzed 26 samples of tomato products and none of the samples were contaminated with patulin (MAFF, 1995). In view of the fact that patulin was found in 11% of the moulded fresh tomatoes, and none in the derived tomato products different hypotheses on the behavior and stability of patulin throughout the production process of tomato products were evaluated.

3.4.4. Evaluation of the stability of patulin in tomato product production processes

3.4.4.1. Binding of patulin with compounds present in the tomato matrix

In a first experiment the possibility of patulin binding with compounds from the tomato matrix was evaluated. Previously, it was shown that the clarification process reduced significantly the concentration of patulin in apple juice (Bissessur et al., 2001). Moreover, a study on patulin in apple juices showed that patulin was able to bind with the solid particles of cloudy apple juice, probably due to binding with proteins (Baert et al., 2007). It has been reported before that patulin is able to bind with thiol groups from small molecules such as amino acids (Fliege et al., 2000). Therefore, a spiking experiment with patulin was performed on laboratory-made tomato juice. The tomato juice was spiked with three patulin concentrations and left during 0-5 d at 4 °C before starting the extraction procedure. As can be seen in Table 3.1 a reduction of patulin of 18-26% was observed after 1 day. After 5 days no further significant reduction was observed. These results reveal that patulin is binding only partially to compounds in the tomato matrix, probably with proteins, and remains available for detection.

Table 3.1: Results of binding of patulin on tomato matrix in function of days expressed as recovery of spiked tomato juice (n=2).

Spiked concentrations on day 0 (µg/mL of patulin)	Recovery ± S.D. (%)		
	Day 0	**Day 1**	**Day 5**
0.5	108.4±11.4 [a]	82.2±8.5 [b]	80.9±5.8 [b]
1	95.1±0.2 [a]	73.7±0.4 [b]	79.4±1.1 [b]
1.5	85.9±1.5 [a]	67.6±3.4 [b]	7.9±1.8 [b]

[a] and [b] are significantly different within same concentration

3.4.4.2. Influence of respectively ascorbic acid and free amino acids on the stability of patulin during the heating process of the production of tomato concentrate

In a second experiment the influence of specific tomato matrix compounds on the stability of patulin during the production process of tomato concentrate was investigated. Therefore, different tomato simulants were made and spiked with three concentrations of patulin. The simulants were subsequently heated using a rotary evaporator (20 min, 95 °C), mimicking the tomato concentration process. The results showed that the heating process did not induce the binding of patulin with the amino acids evaluated (recovery of 88.3±15%). On the other hand, the average recovery of patulin in the simulant to which vitamin C was added (59.9±9.2%) was significantly lower than the average recovery of patulin in the simulant with only amino acids ($p=0.027<0.05$). This confirms previous studies that ascorbic acid reduces the concentration of patulin (Aytac et al., 1994; Brackett et al., 1979). In one

study the addition of ascorbic acid reduced the presence of patulin by 30% after 34 days at acidic conditions in an aqueous juice-like model system (Drusch et al., 2007).

3.4.4.3. Overall influence of the production process on the stability of patulin in tomatoes

In a third experiment, the impact of the whole production process of tomato concentrates on the stability of patulin in a tomato matrix was evaluated on lab scale. Figure 3.2 shows the evolution of the patulin concentration as function of the different steps in the production of tomato concentrate. The full line shows that the patulin concentration decreased 27% after preheating (30 min at 95 °C), while after 20 minutes in the rotary evaporator the concentration of patulin was increased by 47% compared to the original concentration. However, this increase was due to the concentration process of the tomato juice. In order to investigate the effect of the heating treatments on patulin alone, the evaporation of water was thus taken into account. By doing so, a decrease of the patulin content by 44% is observed in the final product, mainly due to a decrease after the preheating (30 min at 95 °C). Overall however, the observed absence of patulin in the derived tomato products cannot be explained by the impact of the production process on patulin. In the literature it is shown that patulin is relatively heat stable, confirming our results (Acar et al., 1998; Bissessur et al., 2001). Therefore, a screening for patulin in products taken during the industrial production process of tomato concentrates, gazpacho and salmorejo was performed in various production countries.

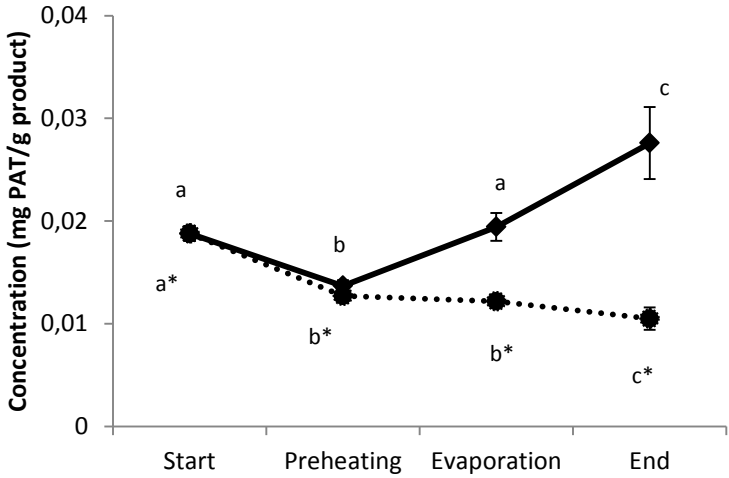

Figure 3.2: Evolution of patulin concentration in tomatoes throughout the production process of concentrated tomatoes, hot break procedure. Solid line: concentration on total product (mg patulin/g tomato product) (mean ± SD, n = 5; a, b, and c are significantly different); Dotted line: concentration taking evaporation into account; (a*, b*, and c* are significantly different. Start, juice molded tomatoes; preheating = 95 °C, 30 min, evaporation = 77 °C, 10 min, and end = final product tomato concentrate (77 °C, 10 min).

3.4.4.4. Screening of patulin during the production process of tomato concentrate, gazpacho and salmorejo

Samples were taken from the field till the end product in the production process of tomato concentrates in different companies in producing regions in Spain, Portugal and Poland, gazpacho (Spain) and salmorejo (Spain). Figure 3.3 gives a flowchart of the production process and shows the steps in the production process where samples were collected. None of the samples tested positive for patulin. The

products tested were moulded tomatoes on field (pre-harvest) (n=49), moulded tomatoes entering factory (post-harvest) (n=36), skin and seeds (removed from process) (n=17), intermediate products (n=61) and end products (i.e. concentrates, gazpacho and salmorejo) (n=28). Agronomists at the different fields in Spain and Portugal, where the tomatoes for further production are cultivated, confirmed the observation that the tomatoes in the field are not infected by *Penicillium expansum*, hence supporting the findings about the absence of patulin in the derived tomato products. However, to confirm this observation, extra studies have to be performed, for example with a genetic approach. It is currently not clear why tomatoes for the fresh market are clearly more susceptible for infestation with patulin producing moulds, while tomatoes which are further processed are not. It is striking though that the dry matter content of the different tomatoes is significantly different: 0.5-0.6% dry matter for the tomatoes of the fresh market vs. 3-4% dry matter for the tomatoes which are used for further processing. Interestingly, Konstantinou et al. (Konstantinou et al., 2011) observed that the susceptibility to *P. expansum* was negatively correlated to the fruit firmness. In addition, *P. expansum* is a post-harvest problem, while the tomatoes used for further processing are stored no longer then 48h after harvesting before further processing. However, it cannot be excluded that other factors are involved in the observed differences in the vulnerability to mould infestation.

3.5. Conclusions

Fresh tomatoes, soft red fruits and sweet bell peppers in the fresh market are likely to be contaminated with patulin upon mould infestation. Remarkably, no patulin was found in processed tomato products, although it was shown that patulin is not likely to be completely degraded during processing. It could be concluded that the tomato varieties used for further processing are not vulnerable for infestation by patulin producing moulds due to the limited time between harvest and processing (no post-harvest storage of these tomatoes) and thus that patulin is not a relevant safety risk in processed tomato products while it is in moulded fresh tomatoes.

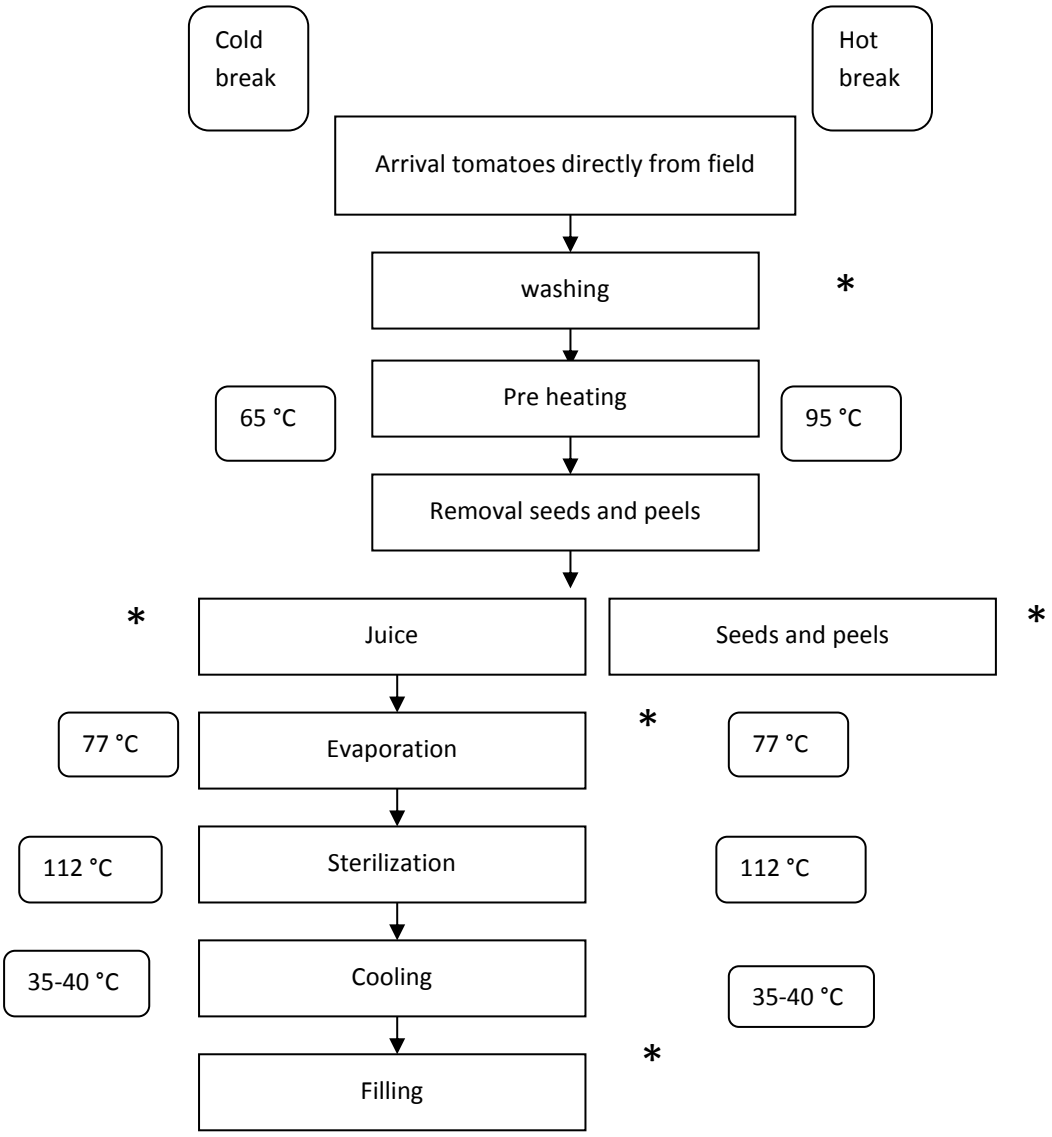

Figure 3.3: Flow chart of derived tomato product production process and indication of sampling in various steps (sampling is indicated by *)

Chapter 4:

Evaluation of the stability of alternariol and alternariol monomethyl ether throughout the production of derived tomato products

Redrafted from: Van de Perre, E., Jacxsens, L., Haesaert, I., Gianaria, A., De Meulenaer, B. (2014). Evaluation of the stability of alternariol and alternariol monomethyl ether throughout the production of derived tomato products. *Submitted*.

Chapter 4: Evaluation of the stability of alternariol and alternariol monomethyl ether throughout the production of derived tomato products

4.1. Abstract

This study evaluated the stability of alternariol (AOH) and alternariol monomethyl ether (AME) throughout the processing of fresh tomatoes into derived tomato products (i.e. concentrates, salmorejo and gazpacho) on industrial samples. Next to sampling of industrial production processes (summer 2012) also lab scale experiments were performed in order to gain additional insight in the stability of both AOH and AME during the production process of tomato concentrates. AME and AOH were both detected in industrially prepared derived tomato products and raw materials applied for further processing. According to the lab experiments results, it could be concluded that they were not fully destroyed when applying cold/hot break treatments. Therefore, AME and AOH have to be considered as potential hazards in derived tomato products.

4.2. Introduction

As discussed in Chapter 2, the presence of *Alternaria* toxins in tomato products is relevant. An EFSA report on the risk of *Alternaria* mycotoxins considering data from Member States and published data was elaborated in 2011. The panel used the threshold of toxicological concern (TTC) to give an opinion on the risks for public health. For the genotoxic toxins, such as AOH and AME, the estimated dietary exposure exceeded the TTC (=2.5 ng/kg BW.day). For tenuazonic acid (TeA), a non-genotoxic mycotoxin the exposure was below the set TTC (= 1500 ng/kg BW.day) (EFSA, 2011b). In order to evaluate the risk of AOH and AME in food, not only the occurrence and toxicity of these mycotoxins needs to be known, also the behavior during food processing needs to be understood. So far, little information is available on the behavior of AOH and AME during food processing. In general, mycotoxins are known to be very heat stable. Several studies investigated the stability during heat treatment and showed that AOH and AME are quite heat resistible in sunflower flour, apple juices and bread (Combina et al., 1999; Scott et al., 2001; Siegel et al., 2010b). No evaluation was yet made on the stability of AOH and AME during the production process of tomato products. And since it was shown that derived tomato products are frequently contaminated with these mycotoxins a study on their behavior during production process of tomato products was performed. Next to sampling of industrial production processes (summer 2012) also lab scale experiments were done in order to gain additional insight in the stability of both AOH and AME during the production process of tomato concentrates.

4.3. Material and methods

4.3.1. Reagents and chemicals

Ethyl acetate (pa), ultra-pure water and acetic acid (99-100%) (pa) were purchased from ChemLab (Brussel, Belgium). Standards of AOH and AME were supplied by Sigma-Aldrich (Bornem, Belgium). The standards were dissolved in methanol. The solutions were dried under nitrogen and kept at -28°C. LC-MS grade water, acetic acid and formic acid were supplied by Fluka (Sigma-Aldrich, Bornem, Belgium). LC-MS grade methanol, ethyl acetate and acetonitrile were supplied by VWR (Leuven, Belgium).

4.3.2. Analyzing LC-TOF-MS method for *Alternaria* mycotoxins AOH and AME

The extraction method and analytical equipment are described in Chapter 2. The gradient was a linear increase from 30 to 100% of mobile phase B in 5 min, stayed at 100% mobile phase B for 3 min and back to 30% mobile phase A in 1 min at a flow rate of 0.2 mL/min for another 6 min. One run took 15min. The injection volume was 20 µL. The LOD for AOH was 12.2 µg/kg and for AME 13.5 µg/kg, and the total recoveries were 82% and 76 % respectively. LOQ was defined as 2 * LOD

4.3.3. Evaluation of the stability AOH/AME in the production process of tomato derived products

4.3.3.1. Screening of AOH and AME during the production process of tomato concentrate, gazpacho and salmorejo

Samples were taken from the field (fresh tomatoes) till the end product in different companies producing tomato concentrate (in Spain (Badajoz), Portugal (Benavente)

and Poland (Krobia)), salmorejo (Spain (Almeria)) and gazpacho (Spain (Murcia and Almeria)). The type and number of samples that were analyzed were moulded tomatoes on field (pre-harvest) (n=49), moulded tomatoes entering the factory (post-harvest) (n=36), skin and seeds (removed from the process) (n=17), intermediate products (n=61) and end products (i.e. concentrates, gazpacho and salmorejo) (n=28).

4.3.3.2. Overall influence of the heating process on the stability of AOH and AME

Different mould strains were isolated from fresh tomatoes. Out of these, four strains were selected based on their ability to produce mycotoxins. All strains were identified by MUCL (Louvain La Neuve, Belgium) as *Alternaria arborescens*. The identification was done by comparison the DNA sequence with reference sequences from their database, cross-checked with the morphology and both ITS (Internal transcribed spacer) and genes coding for elongation factors were compared. The strain which produced the highest amounts of AOH and AME at 25 °C was used to inoculate tomatoes. A spore solution was made as followed. The strain was inoculated on malt extract agar (Oxoid, UK) and after that, incubated at 25 °C to sporulate. In order to harvest the spores, Tween 80 was added to three MEA plates to dissolve the spores and then this solution was filtered over sterilized cotton in a falcon tube. After that, the solution was centrifuged (18K-3 Sigma, Sigma-Aldrich, Germany) and the supernatant was removed. Subsequently, the solution was dissolved in the same volume of PBS-tween and centrifuged. Afterwards, the supernatant was removed and the solution was dissolved in PBS and then centrifuged again. This procedure was repeated again. The final spore solution was standardized using a counting chamber under an inverted light microscope (model

IX2-ILL100, Olympus, Japan). In order to obtain this standardized solution 1 mL of spore solution was transferred to an Eppendorf and then a centrifugation took place. Finally, the tomatoes were inoculated by adding 10 µL of solution (10^5 spores/mL) into an injury spot made at the tomato (visually checked for the absence of moulds). Tomatoes were put in an incubator at 25 °C. After 14 days, tomatoes were collected to produce derived products on lab scale. For each experiment 170 g of homogenized tomatoes was used. After homogenization, the tomato juice was preheated in a warm water bath either at 95 °C for 30 min (hot break procedure) or 65 °C for 30 min (cold break procedure), then put on a rotary evaporator at 77 °C for 20 min. This was done in quintuplet. Samples were taken before preheating, after preheating, after 10 min evaporating and at the end (total 20 min evaporating).

4.4. Results and discussion

4.4.1. Screening during the industrial production process of tomato concentrates, salmorejo and gazpacho

The sixty samples taken in the production line of salmorejo and gazpacho were all below detection limit of AOH (LOD = 12.2 µg/kg) and of AME (LOD = 13.5 µg/kg). This was expected, since the tomatoes entering the factory were of very high quality and there were no moulded tomatoes entering the production line (personal observation). On the other hand, in the factories for the production of tomato concentrates, moulded tomatoes did enter the production line. Depending on the factory, there was a manual and/or mechanically elimination of green or rotten tomatoes but no complete removal of moulded raw materials was observed (personal observation). Samples were collected halfway the harvesting period in August and at the end of harvesting period in September 2012. Samples from the

production process in August were all below the detection limit, with the exception of one sample (end product) which tested positive for AOH (below LOQ of 24.4 µg/kg). At the end of the harvesting season more moulded tomatoes were expected – due to colder and wetter weather which induces cracks in the tomatoes - and thus more samples contaminated with mycotoxins were expected (personal observations and comments agronomists). Table 4.1 shows the number of positive samples for AOH found in tomato concentrates sampled in the different factories in function of the production period. Only AOH was found, all samples were negative (< LOD) for AME. Table 4.1 shows only the positive samples, meaning that most intermediate products were <LOD.

Table 4.1: Overview of positive samples for AOH in tomato concentrates sampled during different time periods (LOQ = 24.4 µg/kg)

Period of sampling	Country	Positive sample	N positive AOH/ total samples	µg AOH/ kg product
August	Spain	end product tomato concentrate	1/20	<LOQ
September	Spain	before evaporation and end product tomato concentrate	2/9	<LOQ
September	Portugal	end product tomato concentrate	9/13	12.2-26.2
September	Poland	end product tomato concentrate	4/12	<LOQ

Some previous studies show the presence of AOH and AME in derived tomato products.. A study from Argentina showed that in respectively 5 and 21 out of 80 analyzed Argentina tomato purees AOH (LOD= 5µg/kg) and AME (LOD= 2µg/kg) were present (Terminiello et al., 2006). Also, Ackermann et al. (2011) found that AOH was present in ketchups, tomato concentrates and tomato juices (LOD=2 µg/kg). In a study of da Motta et al. (2001) eighty samples of tomato products (juice, pulp, paste) from Brazil were tested and were all negative for AOH and AME (LOD AOH=5 µg/kg, LOD AME=2 µg/kg).

Table 4.2 gives an overview of the prevalence of AOH and AME both in the moulded tomatoes, picked from the fields and final tomato products, in order to make a potential link between end product and initial raw material contamination. In contrast with the end products, were no AME was found, AME was found in the moulded tomatoes from the field and entering the production line of tomato concentrate. Also other studies show the presence of AOH and AME in fresh moulded tomatoes (Bottalico and Logrieco 1998; Stinson et al. 1981). A possible explanation for the absence of AME in derived tomato products is dilution or elimination during the production process. Therefore, the production process of tomato concentrate was simulated on lab scale in order to have an insight in the stability of AOH and AME during the different steps of the production process of tomato concentrate.

Table 4.2: Overview of the prevalence of AOH and AME (%) in the moulded tomatoes as raw materials picked from the fields and the final tomato products (LOD AOH=12.2 µg/kg; LOD AME 13.5 µg/kg)

		Positive samples + Sp (%) (range (µg/kg))	
Samples	**N total**	**AOH**	AME
Moulded fresh tomatoes	66	15.2 ± 4.2 (18.3-486)	10.6 ± 3.8 (26.1-73.1)
Tomato concentrate	19	15.8 ± 8.4 (12.2-26.2)	<LOD
Gazpacho	6	<LOD	<LOD
Salmorejo	3	<LOD	<LOD

4.4.2. Influence of the production process on the stability of AOH and AME in tomatoes

The impact of the whole production process of tomato concentrates on the stability of AOH and AME in a tomato matrix was evaluated on lab scale using tomatoes inoculated with *Alternaria arborescens.* Figure 4.1 shows the evolution of the AOH/AME concentration as function of the different steps in the production of tomato concentrate, starting from artificially inoculated tomatoes with toxigenic moulds, both in cold break (65 °C preheating) and hot break (95°C preheating) procedure. The full line shows that both AOH (black) and AME (grey) concentration decreased with respectively 17% and 10.2% after preheating for cold break (30 min at 65 °C) and 19% and 16.3% for hot break (30 min at 95 °C) procedure. After 20 minutes in the rotary evaporator the concentration of AOH was increased with 25.9%

and decreased with 9.3%, respectively for cold and hot break compared to the original concentration. The concentration of AME in the final product in the cold break procedure was not significant different from the starting contamination. For the hot break procedure a total decrease of 16.5% AME was observed. In order to investigate the effect of the heating treatments on the mycotoxins alone, the evaporation of water was taken into account. Doing so, a decrease of AOH and AME content of 78.1 - 87.7% was observed in the final product (dotted lines) both for cold and hot break treatments. Comparing the results for AOH and AME it was observed that both showed a similar pattern in stability during the production process of tomato concentrate. A study on the effect of heat treatment on the stability of AOH and AME in sunflower flour showed that the concentrations stayed constant during 90 min heating of 100 °C (boiling). On the other hand, heating at 121 °C for 60 min (autoclaving) resulted in a decrease of respectively 25 and 100% (Combina et al., 1999). Another study on the stability of AOH and AME showed that AOH and AME were stable in apple juice and white wine after a heat treatment of 80 °C for 20 min (Scott et al., 2001). A study on the stability of *Alternaria* toxins during bread baking indicated that the mycotoxins hardly decreases during wet baking, while there was a significant degradation during dry baking. AME was less stable then AOH (Siegel et al., 2010a). These results show that the stability of AOH and AME is highly depending on parameters such as the matrix, time and temperature of the heating treatment.

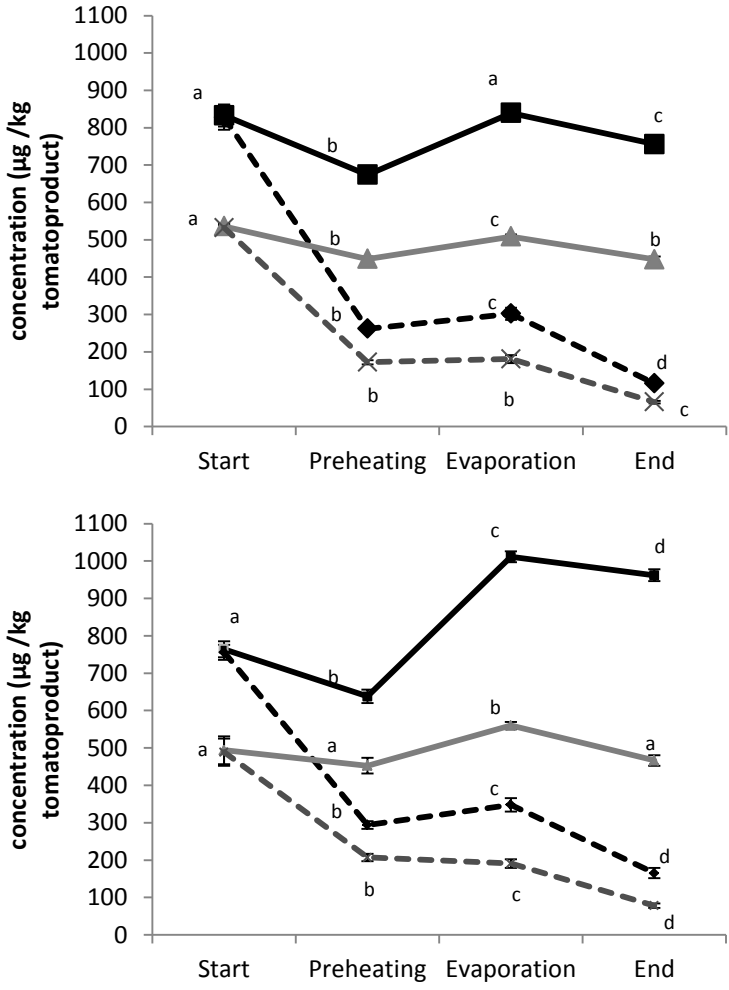

Figure 4.1: Evolution of AOH (black) and AME (grey) concentration in tomatoes throughout the production process of concentrated tomatoes, hot (left) and cold (right) break procedure. Solid line: concentration on total product (µg/kg tomato product) (mean ± SD, n = 5; a, b, and c are significantly different); Dotted line: concentration taken evaporation into account; (a*, b*, and c* are significantly different. Start, juice molded tomatoes; preheating = 65 °C, 30 min (left), 95 °C, 30 min (right), evaporation = 77 °C, 10 min, and end = final product tomato concentrate (77 °C, 10 min).

4.5. Conclusion

AOH and AME were both detected in moulded tomatoes used as raw materials for industrially prepared derived tomato products. AME was not present (anymore) in the final tomato product, probably because the starting concentration of AME in the moulded tomatoes is lower than the starting concentration of AOH. Resulting that by the dilution effect the concentration of AME will be below the detection limit in the final tomato concentrate. Out of simulated lab experiments it can be concluded that these mycotoxins are not fully destroyed when applying cold or hot break treatments. Moreover, due to the evaporation of water during the production process of tomato concentrates the final concentration of AOH increased at cold break procedure. AME and AOH have to be considered as potential hazard in derived tomato products and preventive measures are necessary to avoid to high percentages of moulded tomatoes entering tomato processing industries. Therefore, influencing parameters on mould growth and mycotoxin production need to be known and modelled. This work is presented in the next Chapter 5.

Chapter 5: Effect of temperature and fungicide concentration on the growth of *Alternaria* spp. and production of their mycotoxins alternariol and alternariol monomethylether on tomato agar and fresh tomatoes

Redrafted from: Van de Perre, E., Jacxsens, L., Deschuyfelleer, N., Lécuyer, S., Coen, A., Molina, B. G., Gianaria, A., Devlieghere, F., De Meulenaer, B., & Vermeulen, A. . Effect of temperature and fungicide concentration on the growth of *Alternaria* spp. and production of their mycotoxins (alternariol and alternariol monomethyl) on tomato agar and fresh tomatoes. *Submitted and revised*

Chapter 5: Effect of temperature and fungicide concentration on the growth of *Alternaria* spp. and production of their mycotoxins alternariol and alternariol monomethylether on tomato agar and fresh tomatoes

5.1. Abstract

This study is investigating factors affecting pre-harvest growth of *Alternaria* spp. and their mycotoxin production (alternariol (AOH) and alternariol monomethyl ether (AME)) on tomatoes used for further processing in derived tomato products. Growth experiments were conducted on tomato agar medium as simulant to establish lag phase, maximum growth rate (μ_{max}) and mycotoxin production as a function of temperature and fungicide concentration (Cu) for five strains of *Alternaria* spp. Results showed that the lag phase was very short (2-107h) at all temperatures (range between 5-35 °C). Several secondary models expressing the growth rate as a function of temperature were evaluated and based on various statistic parameters (R^2, root means square error (RSME), standard error of prediction (SEP)) and graphical evaluation), the Ratkowsky model was selected as best fitting model. The optimum temperature varied from 24-33 °C (μ_{max} range 0.21-0.29 mm/h). As a validation, the strains were inoculated on fresh tomatoes. The different statistical indices of the validation on fresh tomatoes showed a low RMSE (<0.0093) and SEP (<5.8%) indicating a good generalization capacity of the models. For two strains the bias factor (B_f) <1, which indicates that the moulds grow slower than predicted by the model (='fail safe'). Mycotoxin production seemed to be highly variable in all tested conditions and for all strains and could therefore not being quantitatively modelled. At lower temperature (5 °C) there was limited production of both AOH and AME. The highest mycotoxin production was detected at 25 °C and 30 °C,

corresponding with optimal observed temperature for growth. At all temperatures the mycotoxin production was lower in fresh tomatoes then on the tomato simulant.

5.2. Introduction

In the previous chapters it was shown that *Alternaria* mycotoxins are present in tomatoes and derived tomato products and that AOH and AME are stable in production processes of derived tomato products (Chapter 2 and 4). Therefore, it is important to have insight in the pre-harvest conditions stimulating growth of the mould and potential mycotoxin production. The use of mathematical models to predict mould growth and mycotoxin production in different conditions, such as temperature, water activity, oxygen level or applied fungicide concentrations, is therefore very relevant. Predictive models are mostly developed for bacterial growth, but after adaptation and transformation these can be used to model fungal growth (Garcia et al., 2009). Colony diameter is the most common and simple methods to measure mould growth (Gibson et al., 1997), although it does not represent the 3-dimensial growth of the mould. Primary models describe how the output (growth or mycotoxin production) changes over time at certain conditions (Marks, 2008). Secondary models describe the influence of several stress factors on the parameters (μ_{max}, lag phase) of the primary model. An important parameter that influences the growth and mycotoxin production of *Alternaria* spp. is the temperature (Magan et al., 1984). Another factor affecting growth and mycotoxin production is the water activity, but since the target products are fresh tomatoes on the field (a_w >0.99), this parameter will have no effect and was excluded in this study. Moreover, the effect of water activity is extensively discussed in a study from Pose et al. (2009). Mycotoxins are often produced when moulds are under stress, such as

less favourable temperature or the presence of fungicides (Wareing, 2014). Widely used fungicides on tomatoes are copper-based fungicides (EC, 2009b). Copper is a non-specific contact fungicide and sprayed over the tomato fruits. It is only working at direct contact, so the present spores inside the tomato will not be affected. Copper has a toxic effect on different elements of the fungi such as enzymes and transport systems (Gadd, 1994). However, it might stimulate the mycotoxin production due to the exposed stress on the mould. The effect of Cu on the growth of *Alternaria* spp. and the production of AOH and AME was not evaluated before. The effect of temperature was discussed before but no secondary models are available. The optimal temperature for *Alternaria* growth is reported between 22-28 °C (Barkai-Golan et al., 2008). Pose et al. (2009) studied the growth on tomato media and found that the optimal temperature was higher than on other media such as MEA. Additionally to studies on media, it is important to validate the results obtained from the tomato media on tomatoes used as raw materials for further processing.

In this study, the effects of temperature and copper concentration on the growth of *Alternaria* spp. and their respective mycotoxin production (AOH and AME) on pre-harvest tomatoes used for further processing in derived tomato products are evaluated. The obtained results were modelled and validated on fresh tomatoes. The outcomes of this chapter give insights in potential stress factors on the tomato fields influencing the presence of moulded tomatoes and consequent mycotoxin contamination of tomatoes.

5.3. Material and methods

5.3.1. Fungal strains isolation

In this study five *Alternaria* spp. strains were used. Strain 1 and 2 were isolated from Belgian tomatoes (fresh market). Strain 3 (*Alternaria arborescens* CBS 109730) was bought from CBS (Centraal Bureau voor Schimmel Cultuur, Utrecht, The Netherlands) and originated from tomatoes cultivated in California (USA). Strain 4 was isolated from a Spanish fresh moulded tomato and strain 5 from a Portuguese fresh moulded tomato (tomato varieties applied for further processing). Strain 1, 2 and 4 were identified as *Alternaria arborescens* by MUCL (Louvain La Neuve, Belgium) by comparison the DNA sequence with reference sequences from their database, cross-checked with the morphology and both ITS (Internal transcribed spacer) and genes coding for elongation factors were compared. Strain 5 could not be identified to species level, but showed to be related to *Alternaria alternata*, which is a complex species.

5.3.2. Preparation of the tomato agar

An agar medium with the same pH (4.4) and water activity (0.988) of fresh tomatoes used for further processing was prepared. 250 g of tomato concentrate from brand 'Delhaize' supermarket was aseptically mixed with 1 liter of mineralized sterilized water and afterwards 15 g/L bacteriological agar nº1 (Oxoid, UK) was added to the mixture. The mixture was homogenized by heating on a heating plate and later put in a warm water bath at 55 °C. The pH of the mixture was measured with a probe FC200 (Hanna Instruments, Portugal). The pH was adjusted by adding NaOH (5M). The NaOH was first filtered (Whatman® FP 30/0, 45 CA-S, VWR International,

Belgium) to avoid contamination. The pH was determined each time for each condition in 3-fold. In order to study the effect of fungicide concentration different Copper-concentrations were evaluated: 0 mg/15 mL agar, 0.3 mg/15 mL agar, 1.5 mg/15 mL agar and 3 mg/15 mL agar. Therefore, 0.4 g of copper oxychloride (Cuprex 50% W, AVEVE, Belgium) was dissolved in 10 mL of sterile demineralized water and diluted to obtain the required concentrations.

5.3.3. Inoculation of tomato agar plates

The five strains were inoculated on malt extract agar (MEA) (Oxoid, UK) and after that, the strains were incubated at 25ºC to sporulate. In order to harvest the spores Tween 80 was added to three MEA plates to dissolve the spores and then this solution was filtered over sterilized cotton in a falcon tube. After that, the solution was centrifuged (18K-3 Sigma, Sigma-Aldrich, Germany) and the supernatants was removed. Subsequently, the solution was dissolved in the same volume of PBS-tween and centrifuged. Afterwards, the supernatant was removed and the solution was dissolved in PBS and then centrifuged again. This procedure was repeated again. The final spore solution was standardized using a counting chamber under an inverted light microscope (model IX2-ILL100, Olympus, Japan). In order to obtain this standardized solution 1 mL of spore solution was transferred to an Eppendorf and then a centrifugation took place. Afterwards, the supernatant was removed and the pellet was re-dissolved in tomato broth. Tomato broth was prepared in the same way as tomato agar but without bacteriological agar nº1. Tomato broth was centrifuged to get a clear supernatant. Finally, the tomato agar plates were inoculated by adding 10 µL (μ_{max} experiments) and 20 µl (λ experiments) of solution (10^5 spores/mL).

5.3.4. Measurement of germination time

5.3.4.1. Experimental set up

Tomato agar plates with 8 mL of agar were prepared without fungicide concentration. The plates were inoculated at four points with 20 µL of spore solution in order to obtain 100 spores per spot. After inoculation, plates were sealed with parafilm to prevent moisture loss and incubated at 5, 15, 25 and 30ºC. For every strain-temperature combination growth of six colonies on an agar plate was followed. Each hour the amount of germinated spores was visually counted by use of a stereomicroscope (model SZX2-ILLB, Olympus, Japan). A spore was considered to be germinated when the length of the germ tube was equal to the highest dimension of the swollen spore (Dantigny et al., 2006).

5.3.4.2. Primary model of germination time

For the primary model, the percentage of germinated spores (P %) was evaluated as function of time for the different strain-temperature combinations. The obtained results were fitted by a logistic model (equation 5.1).

$$P = \frac{P_{max}}{1+\exp[(k*(\tau-t)]} \qquad \text{Equation 5.1}$$

With P_{max} (%) the asymptotic P value at t→+∞, k (h^{-1}) the slope term, τ (h) the inflection point where P equals half of the P_{max} and t the time (h). The lag phase was considered when 90% of the spores were germinated (Dantigny et al., 2006).

5.3.4.3. Secondary model of lag phase

The obtained lag phases from the primary models for the different temperatures were used to develop a secondary model to express the lag phase as a function of temperature for each strain. The data were fitted by using a polynomial model (Nanguy et al., 2010) by using SPSS (Statistics 22, IBM) (equation 5.2).

$$\lambda = a_0 + a_1 * T + a_2 * T^2 \qquad \text{Equation 5.2}$$

With a_i the estimated parameters, T temperature (°C) and λ the lag phase (h).

5.3.5. Determination of maximum growth rate

5.3.5.1. Experimental set up

Tomato agar plates with 15 mL of agar were prepared with different fungicide concentrations (0 0.3; 1.5 and 3 mg Cu/15 mL agar). The Cu-concentration was made by adding 0.4 g copper (Cuprex 50% W, AVEVE, Belgium) in 10 ml sterilized water. The plates were inoculated in the middle with 10µl of spore solution (10^5 spores/mL) of the five strains. After inoculation, plates were sealed with parafilm (Pechiney Plastic Packaging Company, Chicago) to prevent moisture loss and incubated in sealed PA-PE 90 bags (EuralPack, Belgium) at 5, 15, 25, 30 and 35ºC. During incubation these bags were opened every other day in order to avoid anaerobic conditions. For every strain-temperature-fungicide combination, growth of eight colonies was followed. Perpendicular diameters of the mould (mm) were measured with a 24h interval with a digital caliper (0-150 mm, Taurus impact GmbH, Germany).

5.3.5.2. Primary model to establish growth rate

The average diameter (from x and y axis of the mould) (mm) were plotted against time (h) and the maximum growth rates (μ_{max}, mm/h) were obtained from the slopes of the linear regression (Dantigny et al., 2006) in Microsoft Office Excel 2010 (Microsoft Corporation, USA). This method assumes that as soon as the lag phase is finished, the maximum growth rate is reached. μ_{max} was calculated for each of the eight replicates separately for each strain.

5.3.5.3. Secondary model of growth rate

The growth rates (n=8) obtained from the primary models for the different temperatures were used for the development of secondary models describing the influence of temperature and fungicide concentration on the growth rate. First, several functions were evaluated on their fitting capacities to describe growth rates as a function of temperature. Therefore, SPSS Statistics 22 (IBM, USA) and Microsoft Office Excel 2010 (Microsoft Corporation, USA) were used: Ratkowsky model (Ratkowsky et al., 1983), Arrhenius-Davey model (Davey, 1989), Gamma concept (Zwietering et al., 1996), Polynomial model (Nanguy et al., 2010) and Rosso model (Rosso et al., 1993). The most suitable model was selected based on the mean square error (equation 5.3), the root mean square error (RMSE) (equation 5.4), the standard error of prediction (SEP) (equation 5.5) and the determination coefficient (R^2).

$$MSE = \frac{\sum(\mu_{\max\_pred} - \mu_{max\_obs})^2}{n} \qquad \text{Equation 5.3}$$

$$RMSE = \frac{\sqrt{\sum(\mu_{\max\_pred} - \mu_{max\_obs})^2}}{n}$$ Equation 5.4

$$SEP = \frac{100}{\overline{\mu_{\max\_obs}}} \sqrt{\frac{\sum(\mu_{\max\_pred} - \mu_{max\_obs})^2}{n}}$$ Equation 5.5

With $\mu_{\max\_pred}$ the predicted value of the growth rate, μ_{max_obs} the observed value of the growth rate, n the number of data points and $\overline{\mu_{\max\_obs}}$ the mean of observed value of the growth rate.

Means were compared using T-test or analysis of variance (one-way ANOVA and ANOVA) and Post Hoc Multiple Comparison tests (Tukey when variances were equal or Games-Howell when variances were unequal). Homogeneity of variances was tested using the Levene test.

5.3.6. Validation study of mould growth on fresh tomatoes as raw materials for the tomato industry

5.3.6.1. Inoculation of fresh tomatoes

To validate model a tomato variety was applied, typically used as a raw material to be further industrially processed. The seeds of the specific cultivar were obtained from a tomato producing company in Spain and has a higher dry matter content compared to conventional tomato varieties. The tomato plants were cultivated in a greenhouse and tomatoes harvested mature. After harvesting, the ripe tomatoes were washed first with water and then with ethanol to remove potential mould spores. After the evaporation of ethanol, a small injury was made (1 mm depth) by

the back of a scalpel and 10 µL of spore solution (10^5 spores/mL) was added with a pipet. This validation study was performed for strain 2 (mycotoxin production at lower temperatures) and strain 4 (mycotoxin production at higher temperatures). The tomatoes were placed in sterile closed plastic containers (polypropylene) with an opening to avoid the creation of anaerobic conditions (IKEA 356+, Belgium). The containers were then placed in incubators at 5 (n=10), 8 (n=5), 15 (n=10), 20 (n=5), 25 (n=10) and 30 °C (n=10).

5.3.6.2. Growth measurements on fresh tomatoes

The mould diameter was measured with a digital calliper in two perpendicular directions to follow the growth (expressed in mm as a function of time). Next to this, the diameter of every tomato was measured in order to be able to calculate the relative surface of the rotten spot (S). The diameter of the curved surface infected by the moulds was calculated with equation 6 (Baert, Devlieghere, et al., 2007).

$$S = R * 2 * Arc\sin\frac{r}{R} \qquad \text{Equation 5.6}$$

With R the radius of the tomato (sum of 2 perpendicular diameters of the whole tomato/4, mm), r the radius of the moulded spot (sum of 2 perpendicular diameters of the mould/4, mm) (see Figure 5.1)

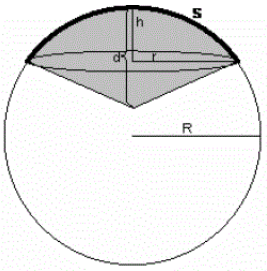

Figure 5.1 Schematic representation of tomato mould infestation (grey part)

5.3.6.3. Mathematical comparison between predictive models on tomato agar with growth evaluation on tomatoes : validation of growth

To evaluate the performance of the predictive models the accuracy (equation 5.7) and bias factor (equation 5.8) were calculated using the data of growth rate obtained on the fresh tomatoes (validation study) (te Giffel et al., 1999).

$$B_f = 10^{\sum \log(\mu_{max\_obs}/\mu_{max\_pred})/n} \quad \text{Equation 5.7}$$

$$A_f = 10^{\sum |\log(\mu_{max\_obs}/\mu_{max\_pred})|/n} \quad \text{Equation 5.8}$$

With μ_{max_pred} the predicted value of the growth rate, μ_{max_obs} the observed value of the growth rate and n the number of data points.

5.3.7. Mycotoxin analysis

The analytical method (LC-TOF-MS) used for screening of AOH and AME is explained in Chapters 2 and 4.The agar plates were transferred into a 50 mL Falcon tube and after 30 mL of water was added it was mixed with an ultraturrax for 2 min at 13000 rpm. The tomatoes were mixed in the same way, but without adding the water.

5.3.7.1. Experimental set up to measure mycotoxins in tomato agar

Samples for mycotoxin determination were taken at a mould diameter of 25, 45 and 85 mm. For every strain-diameter-temperature-fungicide combination two plates (duplicates) were analyzed for AOH and AME. A second experiment was conducted to increase the repeatability and involved only strain 2 (mycotoxin production at low temperatures) and 4 (mycotoxin production at high temperatures). Tomato agar plates with 15 mL of agar were prepared with three different fungicide concentration (0, 0.3 and 3 mg Cu/15 mL agar) at 5, 15, 25 and 30 °C. Samples were taken at a mould diameter of 45 mm. For every strain-diameter-temperature-fungicide combination, five samples were analyzed for AOH and AME. The whole agar plate was sampled for the mycotoxin analysis.

5.3.7.2. Experimental set up on fresh tomatoes: validation study

Also a validation study was performed on fresh tomatoes. Therefore inoculation was conducted following the procedure explained in 5.3.3. This validation study was conducted for strain 2 and 4. The tomatoes were placed in sterile closed plastic containers (polypropylene) with an opening to avoid the creation of anaerobic conditions (IKEA 356+, Belgium). The containers were then placed in incubators at 5 (n=10), 8 (n=5), 15 (n=10), 20 (n=5), 25 (n=10) and 30 °C (n=10). Samples of the moulded tomatoes were taken when the diameter of the curved surface was 45 mm. To calculate the volume of rot in the tomato, equation 5.9 was used (Figure 5.1). The whole tomato was sampled for the mycotoxin analysis according.

$$V_{rot} = \frac{\pi * h * (3r^2 + h^2)}{6} + \frac{\pi * r^2 * (d - h)}{3} \qquad \text{Equation 5.9}$$

With h (mm) the height of the segment of sphere ($= R - \sqrt{R^2 - r^2}$), r radius of moulded spot (sum of 2 perpendicular diameters of the mould/4, mm), R radius of tomato (sum of 2 perpendicular diameters of the whole tomato/4, mm) and d the depth of the rotten spot (mm). This was measured with a digital caliper (0-150 mm, Taurus impact GmbH, Germany).

5.4. Results and discussion

5.4.1. Isolation and characterization of the *Alternaria* strains

Five strains were applied in the growth modelling and potential mycotoxin production. A potential significant difference between the strains in lag phase, growth rate and mycotoxin production affected by temperature and/or Cu-concentrations was investigated in each step of the modelling. In mould research it is important to gain insight in the variability between different strains. Previous studies showed that there is variability in growth and patulin production for different strains of *Penicillium expansum* (Baert et al., 2007; Garcia et al, 2011a). Also three *Aspergillus ochraceus* isolates showed a significant difference in growth and ochratoxin A production (Pardo et al., 2004).

5.4.2. Lag phase (λ) of *Alternaria* spp.

5.4.2.1. Primary models: logistic model for germination time

The germination was followed in time for all five strains at the different temperature conditions (5 until 30 °C). R^2 obtained was >0.99 for all strain-temperature combinations. Primary modelling of the germinated spores as a function of time for the different temperature combinations is illustrated in Figure 5.2A for strain 4

Alternaria arborescens as an example *(other strains present in supplementary material)*. The lag phase (90% germination) was very short for all strains, even at lower temperatures. The longest lag phase was obtained at 5 °C (29h-107h) and the shortest between 22 and 30 °C (2-12h). This is in high contrast with the lag phase of a post-harvest mould such as *Penicillium expansum* on apples with a lag phase of 81h at 25 °C and 520h at 4 °C (Baert et al., 2007). Because of the very short lag phase it was decided not to evaluate the impact of the fungicide concentration on the lag phase.

5.4.2.2. Secondary model, expressing the lag time as a function of temperature

For the lag phase as a secondary model with temperature as the explanatory variable, the polynomial model was applied (Figure 5.2B). The obtained coefficients, RMSE, SEP and R^2 are shown in Table 5.1 for the five different strains. Low RMSE values (0.31-1.22) were obtained, indicating a small difference between the experimental data and the predicted values. Next to RMSE (depends on the magnitude of the values) also SEP was calculated since this is a relative percentage and thus not dependent on the magnitude of the dataset values (Baert et al., 2007). All SEP values were below 17.7%, indicating a good fit of the model to the experimental data. Also, high R^2 values were obtained for the five strains (>0.94), indicating that at least 94% of the observed variance can be explained by the developed model. Figure 5.2C gives a comparison of the observed and predicted lag phases and shows that the points are distributed around the line of equivalence. As discussed before and seen in the primary model, all strains show a short lag phase, even at lower temperatures. The longest lag phase was obtained at 5 °C (29h-107h) and the shortest between 22 and 30 °C (2-12h). Previous studies performed for the

species *Alternaria alternata* showed similar results. The germination time of *A. alternata* on tomato pulp agar was 36 h for 21 °C and 35 °C (from our models: 2-22 h at 21 °C and 2-48 h at 35 °C) (Pose et al., 2009). The optimum temperature reported for germination of *A. alternata* varied between 25 and 30 °C (Magan et al., 1984). The optimum temperature (lowest lag phase) in this study varied between 23 and 30 °C, confirming the previous reported results. At 5°C the highest lag phase was obtained for strain 5 and the shortest lag phase for strain 2.

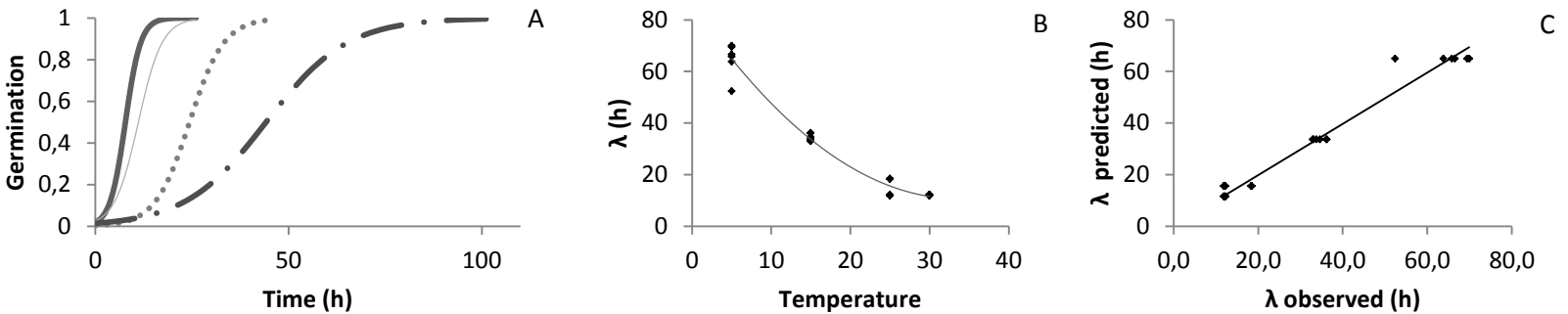

Figure 5.2: A : Primary modelling of *Alternaria* arborescens (strain 4) of the germinated spores (1=100% germinated) as function of time (h) for 5 °C (._), 15 °C (..), 25 °C (_) and 30 °C (▬). B: Secondary model: λ (h) versus temperature (°C) for strain 4-= Fitted polynomial model, ♦= observed data points. C: Comparison of observed and predicted lag phases (h)

Table 5.1: Coefficients (estimate ± standard error), root mean square error (RMSE), standard error of prediction (SEP) and determination coefficient (R^2) of the polynomial model $\lambda=a+bT+cT^2$, describing the effect of temperature (T) on the lag phase (λ) of *Alternaria* spp. on tomato agar as simulation medium

Strain	Coefficients			Statistical indices		
	a	b	c	RMSE	SEP (%)	R^2
1	45.3±1.0	-2.1±0.2	0.025±0.004	0.24	6.3	0.991
2	44.6±1.3	-3.5±0.2	0.075±0.005	0.31	12.8	0.997
3	99.3±3.2	-8.5±0.5	0.184±0.013	0.76	16.7	0.974
4	85.5±3.0	-4.4±0.4	0.066±0.012	1.19	17.7	0.973
5	170±7.9	-14.1±1.1	0.303±0.031	1.22	15.2	0.946

5.4.3. Maximum growth rate (μ_{max})

5.4.3.1. Primary model

For the five strains, the growth rate was determinated for temperature range between 5 and 30°C and 0-3 mg Copper fungicide concentration/15ml agar. Fungal growth after the lag phase was characterized by a linear growth and had a good fit for the growth curves for all strains and all temperatures with $R^2>0.9$ (Figure 5.3A as example for strain 2). Statistical analysis of variance (ANOVA) showed that the different fungicide concentrations were not for all temperatures significant ($p<0.05$) (see supplementary material). However, the μ_{max} was the highest for the moulds growing on agar plates without fungicide concentration. Therefore it was decided to exclude fungicide concentration from the model and to develop the secondary model only with the results obtained with 0 mg Cu/15mL agar (worst case scenario).

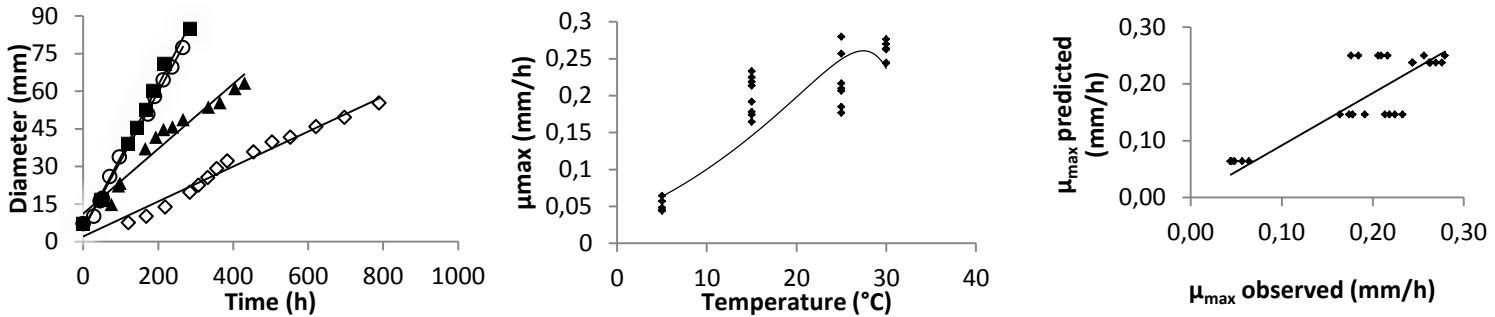

Figure 5.3: A : Primary modelling *of Alternaria arborescens* (strain 2) on tomato agar of the diameter (mm) as a function of time (h) (○=30 °C, ■=25 °C, ▲=15 °C and ◊=5 °C) without Cu; B : Secondary model of strain 2 on tomato agar : μ_{max} (mm/h) versus temperature (°C) for strain 2. - = Ratkowsky model, ♦= observed data points; C: Comparison of observed of strain 2 and predicted growth rates (mm/h) with Ratkowsky model.

5.4.3.2. Secondary model: impact of temperature on μ_{max}

Six mathematical models selected from literature for the fungal growth were evaluated and based on R^2, RSME, SEP (Table 5.2) and graphical evaluation (Figure 5.3B as example for strain 2, others available in supplementary material) the Ratkowsky model was selected as best fitting model (equation 5.10) (Table 5.3) (Ratkowsky et al., 1983).

$$\sqrt{\mu_{max}} = \mathrm{b} * (\mathrm{T} - \mathrm{Tmin}) * [1 - \exp(c * (T - Tmax))] \qquad \text{Equation 5.10}$$

With μ_{max} = maximal growth rate, T = temperature (° C), Tmin = minimum temperature (° C), Tmax = maximum temperature (° C) and b, c=constants

The estimated Tmin were very low and had a higher standard error then the Tmax. This is due to the fact that the amount of data points around Tmin was limited compared to the data points taken around the Tmax (T= 5, 15, 20 en 30 °C). Therefore, the model fits better for the higher temperatures and should not be used for temperatures below 5 °C.

The Arrhenius-Davey model showed an illogical graphical representation of the data and although the polynomial showed a good fitting results, the parameters of a polynomial model have no biological value, thus the Ratkowsky model was chosen to be used. The low RMSE values (0.0039-0.0136) indicate a small difference between the experimental data and the predicted values. SEP values were <22.4%, with for strain 2 a low value of 3.3%, indicating a good fit of the model to the experimental data. A study of *Alternaria alternata* on tomato agar showed an optimum condition for growth at 21 °C (0.34 mm/h, a_w 0.982) (Pose et al., 2009). The optimum

temperature from our models varied from 24-33 °C (0.21-0.29 mm/h). In literature, the optimum temperature growth for *A. alternata* was reported in the range of 25-30 °C (normal medium, not tomato based) (Sautour et al., 2002). Figure 5.3C gives a comparison of the observed and predicted lag phases and shows that the points are distributed around the line of equivalence.

Table 5.2: Root mean square error (RMSE), standard error of prediction (SEP) and determination coefficient (R^2) of different models tested

Model	Strain	Statistical indices		
		RMSE	SEP (%)	R^2
Arrhenius Davey	1	0.030	28.8	0.847
	2	12.700	21.2	0.891
	3	0.004	14.7	0.975
	4	0.006	22.8	0.835
	5	0.005	17.4	0.927
Ratkowsky	1	0.005	18.5	0.845
	2	0.014	3.3	0.938
	3	0.006	21.1	0.850
	4	0.005	22.4	0.759
	5	0.004	16.9	0.923
Rosso	1	0.005	32.3	0.618
	2	0.006	41.3	0.540
	3	0.003	18.6	0.862
	4	0.005	28.9	0.534
	5	0.006	45.2	0.762
Polynomial	1	0.009	29.5	0.843
	2	0.011	37.8	0.980
	3	0.003	12.0	0.981
	4	0.007	23.5	0.759
	5	0.013	44.3	0.926
Gamma concept	1	0.009	29.0	0.696
	2	0.009	49.3	0.493
	3	0.003	22.5	0.747
	4	0.003	31.0	0.445
	5	0.004	33.4	0.553

Table 5.3: Coefficients (estimate ± standard error), root mean square error (RMSE), standard error of prediction (SEP) and determination coefficient (R^2) of the Ratkowsky model $\sqrt{(\mu_{max})}=b*(T-Tmin)*[1-exp[c*(T-Tmax)\ ]\ ]$, describing the effect of temperature (T) on the growth rate (μ_{max}) of *Alternaria* spp. on tomato agar plates as simulation medium

Strain	Coefficients				Statistical indices		
	b	C	Tmin	Tmax	RMSE	SEP (%)	R^2
1*	0.01±0.001	3.354±0.019	-19.0±3.0	35.3±0.1	0.0051	18.5	0.845
2	0.13±0.002	0.371±0.074	-14.4±3.6	35.0±0.1	0.0136	3.3	0.938
3*	0.01±0.002	1.390±0.660	-19.0±3.7	35.7±1.3	0.0058	21.1	0.850
4	0.02±0.005	0.099±0.044	-11.8±4.9	39.7±1.2	0.0049	22.4	0.759
5	0.01±0.001	0.666±0.429	-20.4±2.7	36.0±0.6	0.0039	16.9	0.923

*Coefficients based on bootstrap estimates of standard errors

5.4.3.3. Validation of the secondary models of μ_{max} as a function of temperature on fresh tomatoes

To validate the developed models in real food products, a validation study was performed on fresh tomatoes which were inoculated with two strains, strain 2 and 4. The lag phase of all strains was very short (2-107h) for all temperatures and by consequence not considered in the validation study.

The different statistical indices show a low RMSE (<0.0093) and SEP (<5.8%) indicating a good generalization capacity of the models. The B_f for the two strains are

close to 1 (respectively 0.82 and 0.98 for strain 2 and 4), indicating that the models are a good predictor of the growth rate. As B_f are <1 the moulds grow slower on the real tomatoes than predicted by the model (='fail safe' approach). The A_f averages the distance between each point and the line of equivalence as a measure of how close predictions are to observations. The larger the value, the less accurate the average estimate is (Ross, 1996). The predicted growth rate differed by 29% and 36% from the observed values for strain 2 and strain 4 respectively.

5.4.4. Mycotoxin production

5.4.4.1. Mycotoxin production as function of mycelium diameter

In a first experiment, the production of AOH and AME was evaluated for all five strains at four temperatures (5, 15, 25 and 30 °C) and four fungicide concentrations (0; 0.3; 1.5 and 3 mg Cu/15 mL agar). Samples were taken at a mould mycelium diameter of 25, 45 and 85 mm. For every strain-diameter-temperature-fungicide combination two samples were analyzed for AOH and AME. These results gave insight in the potential of the mould regarding the production of AOH mycotoxin (Figure 5.4). As can be derived from the figures, a heterogeneous result was obtained for all the strains, temperatures and fungicide concentrations. At a mould diameter of 25 mm almost no mycotoxin production was observed independent of the temperature or fungicide concentration and strain. This could be related to the fact that at a mould diameter of 25 mm no sporulation was present. Most secondary metabolites, such as mycotoxins, are only produced after the mould has completed its initial growth phase and starts developing spores (Calvo et al., 2002). The results demonstrated also that even at lower temperatures, mycotoxin production is possible. The influence of the fungicide Cu on the production of mycotoxin is not

consistently clear from the results. Similar results were found for the mycotoxin AME, however the concentration of AME (0-2373 µg/kg) was much lower than the concentration found of AOH (0-4059 µg/kg).

5.4.4.2. Effect of temperature and fungicide concentration on the production of AOH and AME on tomato agar plates

Samples were taken at a mould diameter of 45 mm, because from the first experiment it could be derived that at that particular growth level mycotoxin production started to be effective. For every strain-diameter-temperature-fungicide combination, five samples were analyzed for AOH and AME. The results for AOH are presented in Figure 5.5 (A and B) showing that for the same conditions again a high variability in mycotoxin production over the five replicates was present. Consequently, the production of AOH and AME by *Alternaria arborescens* was not possible to model mathematically. However, from the experiments several qualitative conclusions could be made. At lower temperature (5 °C) there was limited production of both AOH (0-1328 µg/kg) and AME (0-121 µg/kg). For strain 4 even no mycotoxin production was observed. The highest mycotoxin production was observed at 25 °C (max 5209 µg AOH/kg, 7735 µg AME /kg) and 30 °C (max 7959 µg AOH/kg, 5082 µg AME/kg), corresponding with the optimal temperature for growth. Both between conditions and replicates there is a high variability, therefore it is difficult to conclude about differences between strains and fungicide concentrations. Overall, the mycotoxin production of AME was lower than the concentration of AOH, especially at 5 and 15 °C (Figure 5.5). Pose et al. (2010) studied the effect of a_w and temperature on the production of AOH by *Alternaria alternata* on tomato agar. The optimum AOH production was at a_w of 0.982 and 21 °C. At 6 °C no AOH was produced

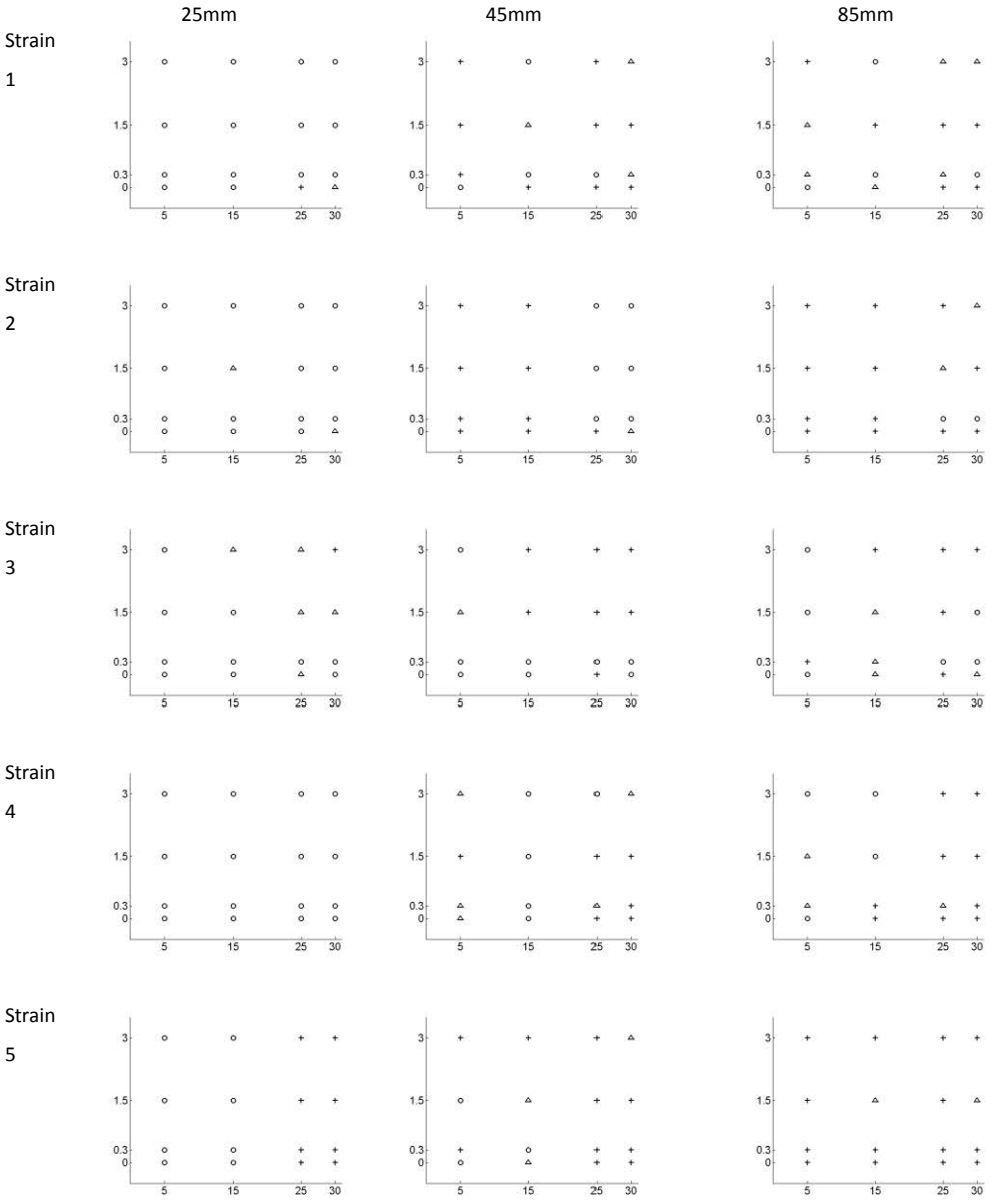

Figure 5.4: AOH production at different mould mycelium diameters for the different temperatures (x-axis, °C) and fungicide concentrations (y-axis, mg Cu/15mL agar), n=2 agar plates of tomato simulant, ○= 0/2 positive for mycotoxins, Δ= 1/2 positive, + = 2/2 positive (LOD =6.8 µg/kg)

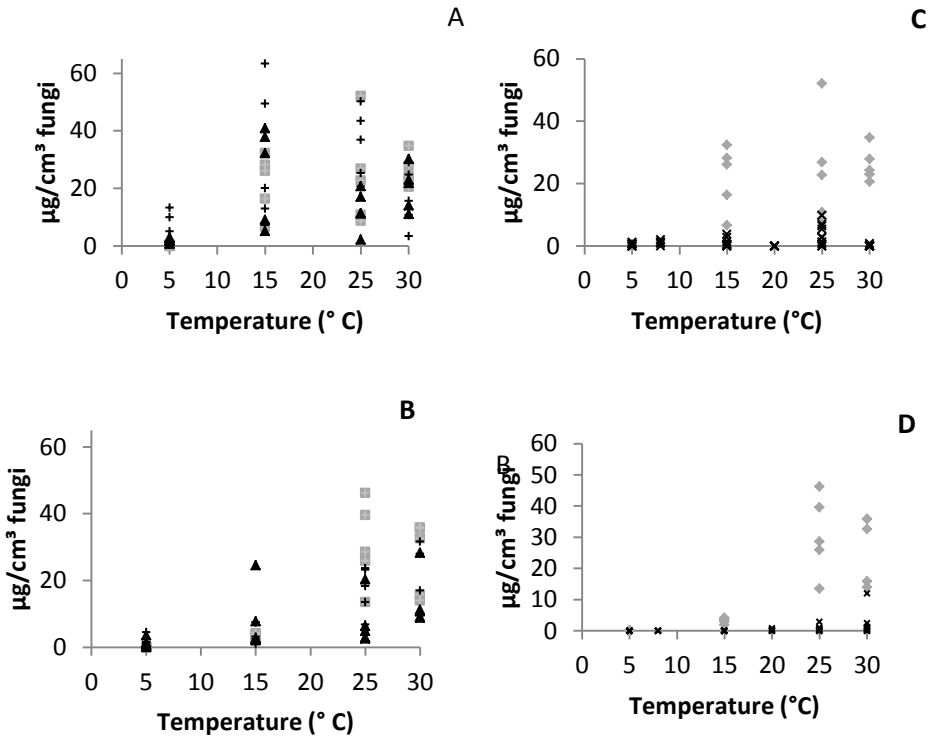

Figure 5.5: A: AOH production (µg/ cm³ fungi) as function of temperature for the different fungicide concentrations (▲=3 mg Cu/15 mL agar, +=0.3 mg Cu/15 mL agar, ■=0 mg Cu/15 mL agar) for strain 2, B: strain 4. C: Effect of temperature on the production of AOH for strain 2 and D for strain 4 (X=fresh tomatoes, ♦= tomato agar) (no Cu)

by *Alternaria alternata*. Similar results were obtained for AME production. Another study performed on tomato cultures reported an optimum temperature of 28 °C for the production of AOH and AME by *Alternaria alternata* (Hasan, 1995).

5.4.4.3. Validation: effect of temperature on the production of AOH and AME on fresh tomatoes

In order to validate the mycotoxin results it is important to perform the experiments directly on the food product, because in the end the mycotoxins will be produced on fresh tomatoes. In this study we also evaluated the effect of temperature on the production of AOH and AME on fresh tomatoes for two strains (strain 2 and 4) and compared the results with those obtained on simulation media (Figure 5.5 A and B). In order to determine the correlation between AOH production in agar and tomato, the amount of toxin was recalculated per volume of infected tissue (µg/cm³ fungi) (Figure 5.5 C and D). Again a high variability was observed. The same observation was made for AME. At all temperatures the production was lower in fresh tomatoes than in agar plates. This can be explained by the natural defence mechanism of tomatoes towards the moulds, causing a lower production of both AOH and AME in comparison with the production on tomato agar. Similar results were obtained for patulin which was higher in apple puree agar medium then for apples at different atmospheres conditions (Baert et al., 2007) and for the production of ochratoxin A, which was higher on agar then on custard, jelly and marmalade (Kapetanakou et al., 2011). This could be explained by the lack of homogeneity in the matrix compared with agar plates (Garcia et al., 2011). Also a difference in nutritional value between the tomato agar medium and the tomatoes could result in a lower production of mycotoxins. Also some differences within intrinsic factors such as porosity and

viscosity could result in lower mycotoxin production (Kapetanakou et al., 2011). Other reports suggest that in food matrices local absence of oxygen and nutrients may occur (Brocklehurst et al., 1997; Noriega et al., 2008). However, decreased O_2 availability showed to develop stress and resulted in higher patulin production in apples (Baert et al., 2007).

5.5. Conclusions

All five investigated *Alternaria* strains show a short lag phase, even at lower temperatures. The longest lag phase was obtained at 5 °C (29h-107h) and the shortest between 22 and 30 °C (range between 2-12h). There seems to be no effect of copper on the growth rate or mycotoxin production. In view of the activity of copper this result could be expected, as it is a contact fungicide protecting the tomato from infestation. Once the mould is formed, copper will not intervene in the mycelium development or mycotoxin production. Therefore, the parameter copper concentration was not included in the secondary models. Several mathematical models for the fungal growth were evaluated and based on R^2, RSME, SEP and graphical evaluation, the Ratkowsky model was selected as best fitting model expressing the growth rate as a function of temperature. The optimum temperature varied from 24-33 °C (0.21-0.29 mm/h). The different statistical indices of the validation on fresh tomatoes showed a low RMSE (<0.0093) and SEP (<5.8%) indicating a good generalization capacity of the models. Both for strain 2 and strain 4 B_f <1, which indicates that the moulds grow slower than predicted by the model (='fail safe' approach). Regarding the mycotoxin production it was not possible to develop a quantitative model due to the high variability between the replicates. The optimal production of AOH and AME is reached at 25-30 °C at a mould diameter of

45 mm. At lower temperatures, the production of the mycotoxins is rather limited. At 25 mm mould diameter there is almost no production of AOH and AME. This is an important process criteria that can be used during processing of tomatoes. The risk of mycotoxin contamination can be reduced by sorting the tomatoes and remove tomatoes with a mould diameter of 45 mm or higher before the production of derived tomato products. At all temperatures the mycotoxin production was lower in fresh tomatoes then in the tomato agar plates. This shows that tomatoes have a natural defence to the mould causing a significant reduction of both AOH and AME in comparison with the production on tomato agar, which was previously reported in literature for other moulds, mycotoxins and crops also.

Chapter 6: Climate impact on *Alternaria* moulds in fresh produce: the case of the tomato chain

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Chapter 6: Climate impact on *Alternaria* moulds in fresh produce: the case of the tomato chain

6.1. Abstract

Climate change can affect the presence and concentration of mycotoxin in various foods. The objective of this study was to evaluate the effect of climate change on the growth of *Alternaria* spp. and their mycotoxin production on tomatoes in function of changing temperatures. Therefore, a climate change model 'HadGEM2-ES'' was applied and downscaling of coarse gridded data was done towards a tomato field surface. After transforming the daily temperature data towards hourly data, the growth model of the *Alternaria* mould was applied (from Chapter 5). This lead to an assessment of growth rate and actual growth for three time frames being current (1981-2000), near (2031-2050) and far future (2081-2100). The influence of the harvesting period in a growing season, RCP scenarios and time frames were evaluated and two regions, Spain and Poland were compared with each other. For Spain there were no significant differences for RCP 2.6 and 4.5. For the more extreme RCP scenarios (6.0 and 8.5) the diameter of the mould was significantly lower for the far future compared with the current time frame. This can be explained by the higher temperatures (18.2-38.2 °C) which become too high for fungal growth. For Poland, there was a significant difference in the different time frames, the diameter of the mould was for the far future>near future>current time frame. This is due to the predicted higher temperatures in the far future (14.2-28.4 °C) which becomes closer to the optimal temperature for the growth of *Alternaria* spp. compared with the colder temperatures in the present. According to the results, the situation in Poland in the far future (2081-2100) will became similar as the situation in Spain in the present time frame (1981-2000).

6.2. Introduction

Alternaria spp. growth and their mycotoxin production is occurring pre-harvest on the fields. The optimal temperature for the production of *Alternaria* mycotoxins is 21-25 °C. Therefore, a shift in environmental temperatures due to climate change may impact their growth and mycotoxin production. Climate change models are assessed by the Intergovernmental Panel on Climate Change (IPCC) for specific future scenarios. Four Representative Concentration Pathways (RCPs) are used to model climate changes: RPC 2.6 (strong mitigation), 4.5 (mitigation), 6.0 (slowdown in emissions) and 8.5 (business as usual). The different RCP levels are expected to stimulate research on the mitigation action in terms of technology change and the policy conditions (van Vuuren et al., 2011).

According to several reviews (based on hypothesis), climate change will affect the amount and presence of mycotoxin in crops (Magan et al., 2011; Paterson et al., 2010; Paterson et al., 2011). Figure 1.4 (Chapter 1) gives an overview of the potential factors that may affect mycotoxin occurrence in the tomato production chain. To have mould growth and mycotoxin production, optimal temperatures and water activities are crucial (Magan et al., 1984), so the effect of climate change on the temperature will be of particular interest with respect to the pre-harvest situation of tomato production. Many of the factors, discussed in Figure 1.4, will interact which each other, so the effect of climate change on mycotoxin production is very complex and difficult to predict completely. By using already the impact of temperature changes, however, a good indication of what could happen in the future can be made (EFSA, 2012b; Paterson et al., 2010; Van Der Fels-Klerx et al., 2012).

Some reviews address the problem of climate change, but these are only hypothetic and not based on scientific data. The objective of this study was to evaluate the effect of climate change on the mould growth in function of the changing temperature. This will lead to an assessment of current, near (2031-2050) and far future (2081-2100) time frame. Four RCP scenarios (2.6, 4.5, 6.0 and 8.5) were used in two tomato producing regions (Spain and Poland). The influence of the harvesting period, RCP scenario and time frame (current, near and far future) were evaluated and the two regions were compared with each other.

6.3. Material and methods

6.3.1. Observational data of temperature

Two regions, Badajoz, Spain and Krobia, Poland, were included in the study, since tomato fields and factories to produce tomato concentrates are located there. Observational daily data (minimum and maximum) for temperature (°C) were obtained from official weather stations in Badajoz (Spain) and Gorlitz (Germany) (closest and most representative weather station to Krobia, Poland) for the years 1981-2000.

6.3.2. Climate scenario data

Together with the observational daily data for temperature (1981-2000) from the two weather stations, climate scenario data were downscaled to be useful in the predictive growth models of the moulds as function of temperature (Chapter 5). Direct climate scenario data are inadequate for assessing local and regional food safety (Ramirez-Villegas et al., 2012), because their spatial resolution (typically 200 × 200 km) is much coarser than the detailed resolution of food safety models. Downscaling is necessary because climate change models are gridded data and these

have less local climate characteristics. Local data variability is important for the field studies (Liu et al., unpublished). The gridded data probably underestimate temperature extremes of actual field situation (Hofstra, 2011). Coarse gridded data from climate change model 'HadGEM2-ES' were selected and downscaled using the 'Delta method' with quantile-quantile correction (Liu et al., unpublished). Data were downscaled as such for the two locations (Badajoz and Gorlitz) for four RCPs for the time frames 2031-2050 (near future) and 2081-2100 (far future) resulting in a data set of daily temperatures. In this way, coarse gridded data were downscaled to point (weather station) data.

These data were further transformed in hourly data by use of a hyperbolic tangent function through the minimum and maximum temperature (equation 6.1 and 6.2) (Schaub, 1991).

For 0-9 hours:

$$T = -\frac{MAX-MIN1}{2}\tanh\left(\frac{h-4.5}{2.5}\right)+\frac{MAX+MIN1}{2} \qquad \text{Equation 6.1}$$

For 10-23h:

$$T = \frac{MAX-MIN2}{2}\tanh\left(\frac{h-16.5}{3.5}\right)+\frac{MAX+MIN2}{2} \qquad \text{Equation 6. 2}$$

Where T=hourly temperature (°C), MAX= maximum temperature of day 1, MIN1= minimum temperature of day 1, MIN2= minimum temperature of day 2, h = hour

In order to obtain the maximum temperature at 14h instead of 9h, the time is shifted by adding 5 hours. Although this simple temporal downscaling method may not strongly influence the results of the model, it strongly simplifies reality (Liu et al., 2014). At the end, temperature data (per h) were obtained for the three time

frames, two growth locations and four climate change scenarios. Out of these data, the growth and harvesting period of tomatoes were selected to be further applied (15th of June-15th of September). The growth and harvesting period in Spain and Poland tomato production regions, Badajoz and Krobia, are similar.

6.3.3. Growth of *Alternaria arborescens* on tomatoes

The growth of *Alternaria arborescens* was estimated using a predictive model for the growth rate (mm/h) developed in Chapter 5. For the evaluation of climate impact, the strain with highest maximal growth rate out of five strains was chosen: *Alternaria arborescens* (equation 6.3).

$$\sqrt{\mu_{max}} = 0.01 * (\mathrm{T} + 19) * [1 - \exp(3.4 * (T - 35.2))] \qquad \text{Equation 6.3}$$

During the ripening and harvesting period of tomatoes (15th of June-15th of September), the μ_{max} was calculated per hour using the temperature data.

A mould can start growing on a tomato as soon as the tomato starts ripening. According to agronomists the time between the start of the first tomatoes to ripen and the harvest is around 30 days. Therefore, the diameter of the mould was calculated after TTH =30, 25, 15, 10 and 5 days. Temperatures >35 °C and <35.2 °C were replaced by 35 °C (limitation of the growth model) and at temperatures >35.2 °C the μ_{max} was set to 0 mm/h (Tmax of the model).

6.3.4. Data evaluation

SPSS (Statistics 22, IBM) was used to test for significance for mould diameter:

- Between Spain and Poland (sum of all harvesting periods) in time frame 1981-2000

- Between harvesting period per locations in time frame 1981-2000
- Between different time frames per location and per RCP scenario for present, near and far future (sum of all harvesting periods)
- Between different harvesting periods per location and per RCP scenario for present, near and far future
- Between different harvesting periods per location and per RCP scenario for present, near and far future
- Between the four different RCP scenarios per location and for present, near and far future (sum of all harvesting periods)

6.4. Results and discussion

6.4.1. Current time frame (1981-2000): comparison of Spain and Poland

Tomatoes are produced in different areas and harvested during the summer months (15th of July until 15th of September). In Spain, with the current temperatures, the estimated average diameter of mould after time to harvest (TTH) =30 days, was 121.3±5.7 mm, for Poland it was significant lower with 99.6±9.9 mm (Table 6.1). For all investigated TTH the mould diameter was significantly lower in Poland compared to Spain. This is due to the colder current temperatures in the tomato cultivation season in Poland (range 12.1-22.2 °C) compared to Spain (range 16.8-33.7 °C), which are around the optimal temperature for growth and mycotoxin production of *Alternaria* species (23-25 °C) (Chapter 5). Both for Spain and Poland there were no clear trends in the difference between harvesting periods (beginning of season, mid of season and end of season). It has to be taken into account that later in the harvesting season the chance of rain does becomes higher which can lead to more cracks and thus potentially more infestation of fungi (Tirado et al., 2010).

6.4.2. Impact of climate change

6.4.2.1. Comparison of the near (2031-2050) and far future (2081-2100) with the present time frame

For Spain there were no significant differences for scenarios RCP 2.6 and 4.5. For the more extreme RCP scenarios (6.0 and 8.5) there is a significant difference between the present and the far future (Table 6.1 for RCP 8.5). The diameter of the mould is for these scenarios significantly lower for the far future compared with the present, with mean difference ranging from 1.5-11 mm (TTH 5-30 days). The decrease in growth can be explained by the higher temperatures (18.2-38.2 °C) which become too high for the fungi to grow. For Poland, there is a significant difference in the different time frames for all RCP scenarios (Table 6.1). The diameter of the mould is for the far future>near future>present. This is due to the higher temperatures in the far future (14.2-28.4 °C) which becomes closer to the optimal temperature for the growth of *Alternaria arborescens* compared with the colder temperatures in the present.

Table 6.1: Mean diameter ± stdev (mm) for the different TTH, for scenario RCP 8.5 for region Spain and Poland for the present (1981-2000), near future (2031-2050) and far future (2081-2100).

Spain RCP 8.5	TTH 30	25	20	15	10	5
Present (1981-2000)	121.3±5.6[a]	103.6±5.1[a]	83.5±4.6[a]	63.3±3.5[a]	43.1±2.7[a]	22.6±1.3[a]
Near future (2031-2050)	118.7±7.7[a]	101.6±6.9[a]	81.9±5.8[a]	62.0±4.5[a]	42.2±3.7[a]	22.2±1.8[a]
Far future (2081-2100)	110.4±9.9[b]	94.4±8.6[b]	73.3±6.8[b]	58.0±5.1[b]	39.4±4.2[b]	21.1±2.1[b]
Poland RCP 8.5	**TTH 30**	**25**	**20**	**15**	**10**	**5**
Present (1981-2000)	99.6±9.9[a]	85.7±9.2[a]	69.5±8.0[a]	53.1±6.2[a]	36.7±4.4[a]	20.5±1.8[a]
Near future (2031-2050)	108.1±9.2[b]	92.8±8.6[b]	74.9±7.7[b]	57.0±6.4[b]	39.0±4.7[b]	21.3±1.9[b]
Far future (2081-2100)	116.5±7.1[c]	99.6±6.4[c]	80.4±5.8[c]	60.1±4.6[c]	41.3±3.2[c]	22.1±1.9[c]

*a, b and c are significant within same region

These results are as suggested by Paterson et al. (2011) and now proved by scientific data, in some regions the temperature may become too high and mycotoxin production will be less prevalent. Regions with currently cooler climates will have a higher mycotoxin production as the higher temperature will become closer to the optimal temperature for growth and production. Furthermore, some regions may

become suitable for other crops to grow, and some regions may become unsuitable for existing crops to grow.

6.4.2.2. Comparison of the different harvesting periods within a season

Tomatoes for the processing industry are harvested from mid of July until mid of September. In order to study if there is a difference in mould growth on tomatoes harvested in the start, mid or end of the season, the impact of different harvesting times was investigated. For RCP 8.5 in Spain a significantly smaller diameter was obtained when harvesting at 15th of August compared with 15th of July and 15th of September both in the near and far future, explained by the higher temperatures during this period resulting in too high temperatures for the *Alternaria* species to grow.

In Poland there was a clear trend in the influence of the harvesting period on the diameter of the mould for all the RCPs. The mould diameter was significant bigger at harvest at 15th of August compared with harvesting at 15th of July and 15th of September for RCP 2.6. The same effect was observed at RCP 8.5 for 30, 25 and 20 TTH. With only 15, 10 and 5 days TTH the mould diameter was significant smaller when harvesting on the 15th of September compared with the 15th of July or August. This is due to the lower temperatures at the end of the season.

Apart from temperature, there are a number of other factors that can play a role in the growth of moulds on tomatoes and mycotoxin production during the different periods of harvesting, such as colder night temperatures which will lead to dew in the morning and consequently more cracks in the tomato, resulting in more infestation of the fungi. More rain will also increase the contamination with fungi (Cotty et al., 2007).

With the change of temperatures it is very likely that the growth period of the crops will also change. Changing weather can also have an influence on the irrigation, crop rotation and harvesting time. For example, the temperature in Spain during the summer months may become too high to grow tomatoes, and thus the harvesting period can be changed to earlier months. Therefore, studying crops produced in different seasons within one year can be useful to determine the effect of climate change in vivo. It was shown that chilies produced in summer in Pakistan had a significant higher aflatoxin contamination compared with chilies produced in winter (Iqbal et al., 2011).

6.4.2.3. Comparison of the RCP scenarios

For Spain, in the near future there was no significant difference between the different RCP scenarios for the mould diameter for the different TTH. In the far future there was no significant difference between RCP 2.6, 4.5 and 6.0 but the diameter of the mould is significant lower for RCP 8.5. This is due to the higher temperatures in this scenario which are too high for the *Alternaria* species to grow. A remark that has to be made is that with an increase of the temperature, the growing period of the crops can also change to milder temperature months in the year. A report of EFSA on the prediction modelling, predicting and mapping of aflatoxins in cereals in the EU due to climate change showed a reduction in season length, earlier flowering and harvesting dates. This will lead to an enlargement of the crop growing areas towards the northern part of EU, mainly for rice and maize (EFSA, 2012b).

For Poland, in the near future there was no significant difference between the different RCP scenario's for the mould diameter for the different TTH, as seen also for the region Spain. In the far future there was no significant difference between

RCP 2.6 and 4.5 and no significant difference between RCP 6.0 and 8.5. The diameter of the mould was significant higher for RCP 8.5 compared with RCP 2.6 and 4.5 (see Figure 6.1 and 6.2). This was due to the higher temperatures in this scenario which are closer to the optimal temperature for the *Alternaria* species to grow. No evaluation has been made in this related to the growth of tomato plants.

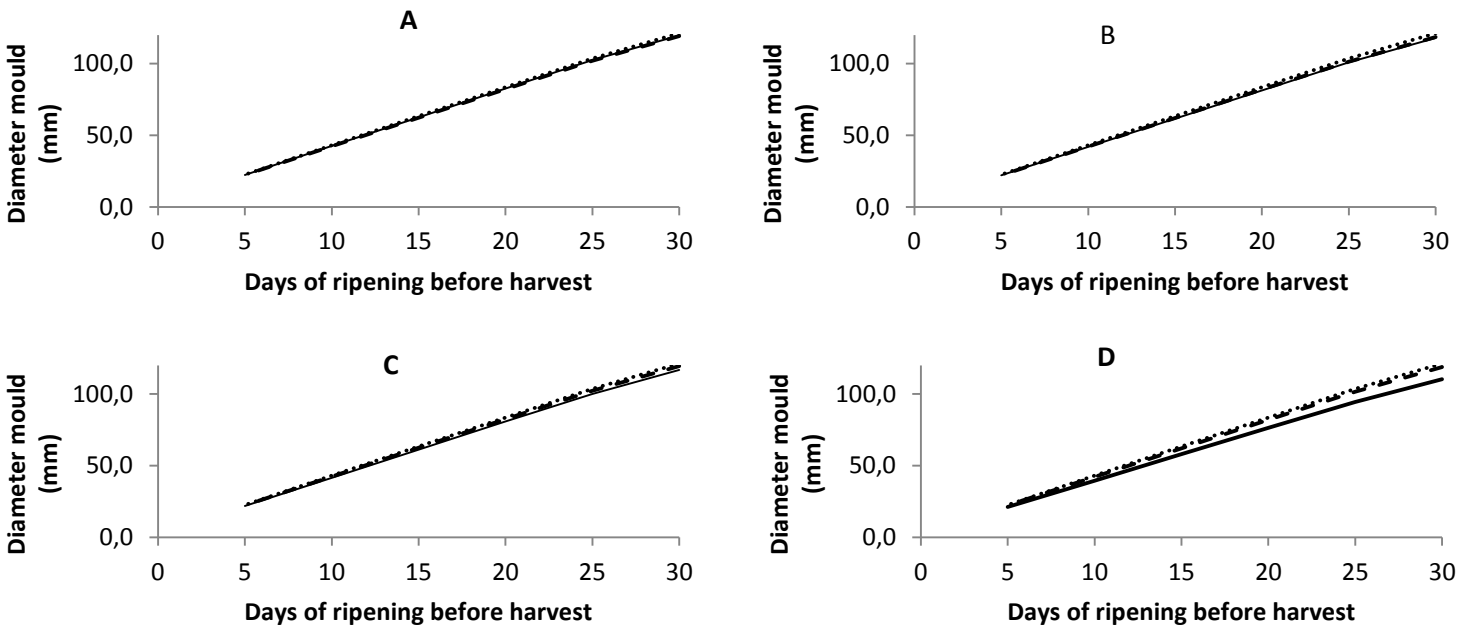

Figure 6.1: Mean diameter of the mould (mm) after 5-30 days of ripening before harvest for Spain (n=60); A: RCP 2.6, B: 4.5, C: 6.0, D: 8.5. …: 1981-2000, - - - : 2031-2050, - : 2081-2100

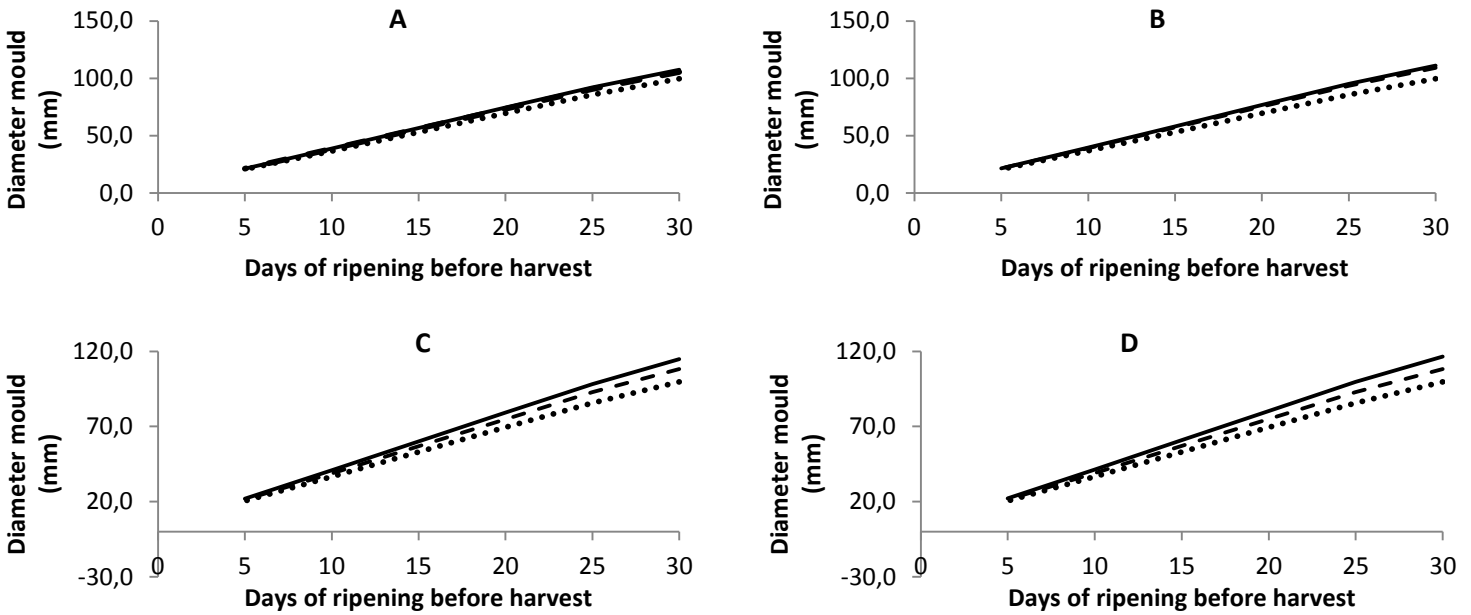

Figure 6.2: Mean diameter of the mould (mm) after 5-30 days of ripening before harvest for Poland (n=60); A: RCP 2.6, B: 4.5, C: 6.0, D: 8.5; …: 1981-2000, - - : 2031-2050, - : 2081-2100

6.4.3. Comparison of the two regions

Comparing the growth of *Alternaria arborescens* in Spain and Poland during the current time frame 1981-2000, there was a significant difference in mould diameter for TTH (5-30 days). The diameter of mould in Spain was bigger than in Poland for the same TTH, with mean difference varying from 2 mm – 21.7 mm (5-30 TTH). The same was observed for the RCP 2.6 scenario with mean differences from 1.2-13.7 mm and 1.1.-12.4 mm for the near and far future respectively. For the RCP 8.5 scenario the mean difference became smaller with 0.9-10.6 mm for the near future. For the far future however, the mould diameter of the tomatoes in Poland became bigger than the mould diameter of Spain with 1.0 and 6.1 mm mean difference. In Poland for RPC 8.5 on average 12.6, 11.7 and 10.9 days before harvest are needed to reach 45 mm of mould growth for present, near and far future, respectively. In Spain the opposite effect was observed, for RCP 8.5 on average 10.8, 11.2 and 12.0 days before harvest are needed to reach 45 mm of mould growth for present, near and far future. This means that the situation in Poland in the far future (2081-2100) is similar than the situation in Spain in the present (1981-2000).

According to the results of the EFSA report on aflatoxins presence in grains in the EU, the aflatoxin concentration is expected to increase in the +2 °C scenario, especially in center and south of Spain, south of Italy and the Balkans. With the + 5°C scenario, the results showed an overall decrease of aflatoxins, but a wider spread through whole Europe. Additionally, the harvesting dates were estimated 15-20 days earlier, resulting in a needed change in the agricultural practices management (EFSA, 2012b). Climate variability is an important factor influencing the crop production, therefore, the crop productivity and soil water balanced should be studied with crop growth models (Kang et al., 2009).

Creating awareness with respect to the change of mycotoxin issue on the level of farmers can be obtained by the developing of predictive models as presented in this paper (Kirezieva et al., 2014).

6.5. Conclusions

Climate change was exemplified in this chapter 6 as increase in temperature. For the simplification, the impact of climate change and shifts in temperature on the actual growth of tomato plants and ripening of tomatoes is not considered in this chapter. Also due to the fact that it was impossible to model the mycotoxin production of *Alternaria* moulds on tomatoes (Chapter 5), no conclusions of impact of climate change on mycotoxins can be made.

For Spain there were no significant differences for RCP 2.6 and 4.5 in mould growth. For the more extreme RCP scenarios (6.0 and 8.5) the diameter of the mould was significantly lower for the far future compared with the current time frame. This can be explained by the higher temperatures (18.2-38.2 °C) which become too high for fungal growth of *Alternaria* spp.. For Poland, there was a significant difference in the different time frames, the diameter of the mould was for the far future>near future>current time frame. This is due to the predicted higher temperatures in the far future (14.2-28.4 °C) which becomes closer to the optimal temperature for the growth of *Alternaria* spp. compared with the colder temperatures in the present. According to the results, the situations in Poland in the far future (2081-2100) will become similar as the situation in Spain in the present time frame (1981-2000).

Impact of climate change is included as one of the scenarios in the farm to fork model presented in Chapter 7.

Chapter 7: A farm to fork exposure model predicting the *Alternaria* mycotoxin exposure from derived tomato products

Chapter 7: A farm to fork exposure model predicting the *Alternaria* mycotoxin exposure from derived tomato products

7.1. Abstract

In the current chapter, a quantitative *Alternaria* mycotoxin farm to fork exposure model was developed. The model is describing the mould growth and potential concentration of mycotoxins AOH and AME during pre-harvest period in ripening tomatoes as raw material (based on predictive mould growth model of Chapter 5) and further during processing of tomatoes into derived tomato products, exemplified by tomato concentrate (including data from insights in the production process of derived tomato products, presented in Chapter 4). The expected outcome of the model gives the exposure of AOH and AME due to the consumption of derived tomato products for the Belgian population, exemplifying the European consumers. Several climate change scenarios were evaluated (based on Chapter 6). These show that with an increase in temperature, the exposure can be lower due to a too high temperature for the growth of *Alternaria* moulds. An increase in moulded tomatoes entering the production line will have a significant effect on the exposure of AOH and AME (increasing exposure). This emphasizes the importance of preventive measures at fields to avoid the prevalence of *Alternaria* moulds and sorting the moulded tomatoes out before entering the production line of derived tomato products. The hot break procedure resulted in a lower exposure compared with the cold break procedure in the production process of tomato concentrates due to other processing factors playing a role in the stability of the mycotoxins, as established in Chapter 4. The calculated mean exposure for AOH was 0.004 µg/kg BW.day while reported by EFSA ranging between 0.0036-0.026 µg/kg BW.day (LB-UB). For AME a higher

exposure was calculated compared to AOH, being 0.008 µg/kg BW.day and following EFSA calculations 0.013-0.063 µg/kg BW.day (LB-UP).

7.2. Introduction

Hazard identification on *Alternaria* spp. and their potential mycotoxins presented in the previous chapters, elucidated that they are an emerging hazard in food safety and fresh produce. However, a risk assessment is necessary to gain insight in the potential risk to human health when consumption of contaminated products is occurring. Up to now, there are no previous risk assessments of *Alternaria* toxins carried out at European or national level except the EFSA study in 2011. The estimated mean chronic dietary exposure in the adult population in food (grains, sunflower seeds and oils, fruits and vegetables juices,), was for AOH: 1.9 - 39 ng/kg BW. per day and AME 0.8 - 4.7 ng/kg BW. per day. Because of a lack of toxicological data a TTC approach was used with the TTC for AOH and AME set at 2.5 ng/ kg BW.day (EFSA, 2011b). By comparing the TTC and exposures of AOH and AME it was concluded that both mycotoxins are a potential risk towards human health and more data collection is necessary to be able to evaluate the actual risk.

In the current chapter, a quantitative *Alternaria* mycotoxin farm to fork exposure model was developed, describing the concentration of AOH and AME during pre-harvest in raw tomatoes as raw material and further during processing of tomatoes into derived tomato products, exemplified by tomato concentrate. The expected outcome of the model gives the exposure of AOH and AME level due to the consumption of derived tomato products for the Belgian population, exemplifying the European consumers. Evaluation of the potential risk was performed comparing the estimated intake with the corresponding toxicological values for each mycotoxin.

To build the model, data and information from the previous chapters were coupled with expert knowledge and industrial information on the production process of derived tomato products. Several scenarios were included to evaluate potential influences or mitigation strategies along the farm to fork chain.

7.3. Material and methods

7.3.1. Data collection

In order to obtain a correct assessment of the presence of *Alternaria* mycotoxins, a thorough analysis was made of the farm to processing chain of tomatoes. The latter analysis was performed via:

- Consultation of scientific literature on farm to fork risk assessment.
- Interviews with the tomato producing and processing industry (four companies in Spain and Portugal, where sampling was performed as explained in Chapter 3 and 4).
- Consultation of experts in this field (scientific person on pre-harvest mould infestation in Belgium, agronomist guiding the tomato farmers in Spain).
- The making of assumptions if needed.

An overview of the collected data and assumptions is described below.

7.3.2. Setup model

Table 7.1 and figure 7.1 show the developed model, its inputs and defined outputs, units and descriptions and links towards the references. This model is applied as a baseline model for further scenario analysis. @RISK version 6 (Palisade Corporation, US) was used, it performs risk analysis by using Monte Carlo simulations.

Module 1: Pre- harvest stage

- Pre-harvest prevalence and growth of *Alternaria* on tomatoes

The prevalence of moulds on tomatoes in the fields at the moment of harvest was set at 2%. This is the accepted value by the tomato processing industry at this moment (expert opinion) on their raw materials entering the processing factories. The fraction of *Alternaria* moulds on the field estimated between 0 and 1%, with most likely 0.5% (expert opinion). A Pert distribution (with min, most likely, max) is used in the model to express the prevalence of *Alternaria* moulds entering the factories for tomato products. A Pert distribution defines the minimum (in our case 0), most likely (in our case 0.5), and maximum (in our case 1) values. Values around the most likely are more likely to occur.

Alternaria moulds can start growing and developing their mycelium as soon as a tomato becomes red (expert opinion). Ripening of the tomatoes is a heterogeneous phenomenon and a discrete function can express this. After 30 days the tomatoes are harvested. According to expert opinion, the average is a 2% maturation per day, with on day 0 25% of red fruits and on day 30 85% of red fruits respectively. The maximum time for mould development is thus 30 days for 25% of the tomatoes. 15% of the tomatoes remain green at the moment of harvest and no *Alternaria* growth is presumed to be possible. This information was used in a discrete function (which defines specific values that may occur and the likelihood of each) with 30, 25, 20, 15, 10, 5 and 0 the days till harvest (= specific values that may occur) and 0.25, 0.1, 0.1, 0.1, 0.1, and 0.25 the percentages of red tomatoes (=likelihood of each).

According to previous studies, mycotoxins will be present in the tomato when the diameter of the mould becomes > 45 mm (Chapter 6). The fraction of tomatoes with moulds > 45 mm (if all tomatoes would be contaminated) was calculated by application of the mould growth predictive models of Chapter 6. The fraction of tomatoes containing an *Alternaria* mould > 45mm was defined as first output (Table 7.1) and calculated by multiplying the fraction of red tomatoes, the fraction of *Alternaria* moulds >45 mm and the prevalence of *Alternaria* moulds on tomatoes

- Pre-harvest production of mycotoxins

One day of production of tomato concentrates uses 500 ton tomatoes as raw materials (processing industry). The distribution of AOH and AME concentration in tomatoes entering the factory was calculated by using the experimental data obtained in Chapter 5. Concentration data from the agar plates were excluded since it was shown in Chapter 5 that those concentrations were higher than the ones on the inoculated tomatoes. The concentrations obtained from tomatoes inoculated with *Alternaria arborescens* in the validation study of Chapter 5 at 5 (n=20), 8 (n=10), 15 (n=20), 20 (n=10), 25 (n=20) and 30 °C (n=20) and 45 mm mould were selected to insert in @Risk and a best fit distribution was made for AOH and AME. Best fit was based on Chi-square statistics. Also, the probability/probability plots (P/P) and the quantile/quantile plots (Q/Q), resulting from the cumulative distributions, were a parameter to decide if the selected cumulative distributions corresponded to the theoretical cumulative distributions. Further, the logical test “IF” was applied, because the concentration data set had a high number of values below the LOD (De Boevre et al., 2013; Vinci et al., 2012). Concentrations below LOD were replaced with 12.2/2 µg/kg and 13.5/2 µg/kg for AOH and AME, respectively (medium bound

scenario). For AOH 60/100 data points were set at 12.2/2 µg/kg and for AME 70/100 data points were set at 13.5/2 µg/kg. The best fit distribution was made for the concentration values above these LOD/2. A Loglogistic function for the dataset of AOH could be given with location parameter 5.7, scale parameter 19.4 and shape parameter 1.4. A Pearson5 function for the dataset of AME could be given with shape parameter 0.5 and scale parameter 3.1 (Table 7.1).

Output 2 and 3 are the concentration data of AOH and AME, respectively, in the batch of 500 ton raw tomatoes entering the factory. This was calculated by multiplying the distribution of AOH and AME in the raw tomatoes with the fraction of tomatoes with moulds > 45 mm.

Module 2: Post-harvest processing

Out of lab experiments it could be concluded that AOH and AME are not completely destroyed when applying cold or hot break treatments. Moreover, due to the evaporation of water during the production process of tomato concentrates the final concentration of AOH increased at cold break procedure. The factors for the increase/decrease were obtained from Chapter 4 and are:

Factors cold break procedure (pre heating 65 °C): AOH: 1.26, AME: 0.94

Factors hot break procedure (pre heating 95 °C): AOH: 0.91 AME: 0.84

In the baseline scenario a uniform distribution is made with this observation (min, max) and applied in the model as input to express the impact of processing on the concentration of the mycotoxins. Finally, the concentration AOH and AME in the final product, being tomato concentrate, is calculated as output 4 and 5 (Table 7.1). In

scenario 8 and 9 the impact of the cold or hot break procedure on the AOH and AME exposure was evaluated (see further).

Module 3: Consumption

In this module, a preliminary risk estimate was made to describe the exposure of the population consuming a food product that contains derived tomato products. Consumption data were obtained from the 2004 Belgian National Consumption Survey (BNFCS). The food consumption database in this survey is a result of daily food intakes from two 24 hour food recalls. Aims, design and methods of the BNFCS are described by De Vriese et al. (2005). Consumption data were extracted from the database based on the food name and facet strings. The output was a combination of all derived tomato products (tomato concentrate, ketchups, sauces, peeled canned tomatoes, purees). Out of the 3200 consumers interviewed, a total of 338 ate derived tomato products during the survey. The usual food intake was further determined using the Multiple Source Method (MSM) program (German Institute of Human Nutrition) (Potsdam-Rehbrücke, 2012). The body weight was known per person. The usual dietary intake values (expressed as kg/person.day) were then entered into an excel worksheet and distribution was fitted using @RISK version 6 (Palisade Corporation, US). A Lognormal distribution gave the best fit of the consumption with mean 0.0019 and standard deviation 0.0123. The final output of the exposure to AOH is expressed as output 6 by combining the concentration of AOH in the tomato products with their consumption. Similar, the exposure to AME was calculated as output 7.

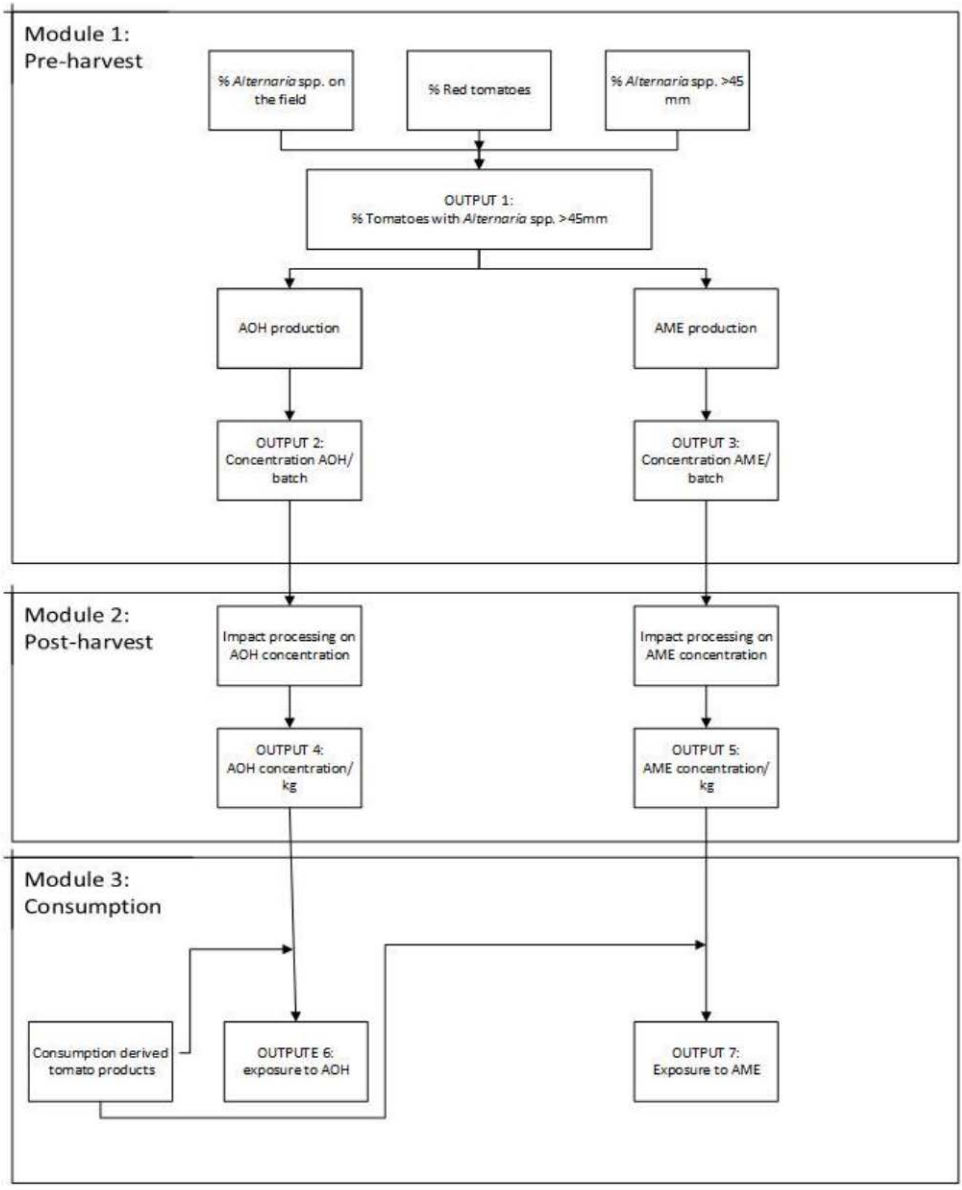

Figure 7.1: Overview of the input and output inserted in the exposure model

7.3.3. Software packages and Monte Carlo iteration.

Calculations were performed using the software package @RISK version 6 (Palisade Corporation, US). Best fit distributions were based on Chi-square statistics. Also, the probability/probability plots (P/P) and the quantile/quantile plots (Q/Q), resulting from the cumulative distributions, were a parameter if the cumulative distributions corresponded to the theoretical cumulative distributions. Further, first order Monte Carlo simulations were performed considering 90 000 iterations and three simulations (to check the stability of the simulation).

7.3.4. Scenarios

Table 7.1 shows the baseline scenario. Next to this also other scenarios are included to demonstrate potential impacts on the final exposure. Therefore, some inputs are adapted in the baseline scenario.

First, impact of different climate change scenarios are evaluated (scenario 1 until 5) on the final exposure. As demonstrated in Chapter 6, a temperature shift as a climate change result can lead to more mycelium growth and Chapter 5 showed that mycotoxin production is becoming important when the mycelium is above 45 mm. However, the impact of temperature increase is limited by the maximum growth temperature of the mould (Chapter 5 and 6).

Secondly, another initial prevalence of moulds on the fields is applied in order to evaluate other risk management strategies on company level as cut off value for accepting or rejecting incoming materials (scenario 6 and 7).

And finally, the impact of an altered production process (cold or hot break procedure) itself is evaluated on the final exposure towards these mycotoxins (scenario 8 and 9).

7.3.4.1. Influence climate change on the AOH and AME exposure

Different climate change scenarios were evaluated in order to see the effect of climate change on the mycotoxin exposure in Chapter 6. Climate change is resulting in different temperature patterns and impacting the growth of the moulds on the tomato fields and potential concentration of mycotoxins in the raw tomatoes entering the production process. The calculations from Chapter 6 were now further applied to calculate the different fractions of moulds > 45 mm at TTH (time till harvest) is 30, 25, 20, 15, 10, 5, 0 days. This was done for the current (1981-2000), near (2031-2050) and far (2081-2100) time period.

The RCP 8.5 was selected to evaluate the no mitigation case of climate change scenario. Next to the region Spain (baseline scenario and scenario 1-2), the model was also made for Poland, another important tomato processing region at this moment (scenario 3-5). The obtained results were inserted in a discrete function expressing the distribution of the fraction tomatoes with moulds >45mm as function of the tomato harvest, ranging between 30 and 0 days (Table 7.2). Per climate change scenario the input parameter fraction_alt>45 mm was changed with their respective discrete function.

Table 7.1: Overview of the input and output parameters inserted in the *Alternaria* mycotoxin exposure model.

Input	*Variable*	Description	Unit	Value/distribution	Reference
MODULE 1 : pre-harvest					
Pre-harvest prevalence and growth of Alternaria on tomatoes	*Total mould_prev_tomato*	Prevalence of moulds on tomatoes in the field at moment of harvest	%	Total accepted fraction of moulded tomatoes moment entering factory at harvest = 2%	Expert opinion
	Alt_prev_tomato	Fraction of total moulds on the field being Alternaria	%	RiskPert(0;0,5;1)	Expert opinion
	Fraction_red	Fraction of red tomatoes	%	RiskDiscrete((30\25\20\15\10\5\0);(0,25\0,1\0,1\0,1\0,1\0,1\0,25))	Expert opinion
	Fraction_alt_>45 mm	Fraction of Alternaria mould > 45 mm	%	See Table 7.2	Chapter 6
Output 1	***Alt > 45 mm_tomato***	***Fraction of tomatoes containing an Alternaria mould > 45 mm***	***%***	***=fraction_red x fraction_alt>45 mm x Alt_prev_tomato***	
Pre-harvest production of mycotoxins	Weight_batch_one day	One day batch contains 500 000 kg tomatoes	kg	500 000	Processing industry
	Weight_t	Weight of tomatoes containing >45	kg	=weight_batch_oneday x output 1	

	omatoes_45 mm	mm Alternaria moulds in a batch delivered to company (one day batch)			
	PART A : production of AOH				
	Distr_AOH	Distribution of AOH concentration detected tomatoes (not on agar)	µg/kg	IF x> 60/100: RiskLoglogistic(5,7774;19,445;1,4217;RiskName("conc AOH")), if not 12,2/2	Chapter 5
	PART B : production of AME				
	Distr_AME	Distribution of AME concentration detected tomatoes (not on agar)	µg/kg	IF x> 70/100: RiskPearson5(0,51408;3,1279;RiskShift(9,4727);RiskName("conc AME")), if not 13,5/2	
Output 2	***Conc AOH_batch***		***µg/batch***	***= distr_AOH x Weight_tomatoes_45 mm***	
Output 3	***Conc AME_batch***		***µg/batch***	***= distr_AME x Weight_tomatoes_45 mm***	

MODULE 2 : POST-HARVEST PROCESSING					
Impact of processing on mycotoxin concentration	impact_AOH	Shift in concentration due to evaporation and heating		= Riskuniform(0,91;1,26)	Chapter 4
	impact_AME	Shift in concentration due to evaporation and heating		= Riskuniform(0,84;0,94)	
	Conc_AOH_after	Shift in concentration due to evaporation and heating	µg/batch	= output 2 * impact_AOH	
	Conc_AME_after	Shift in concentration due to evaporation and heating	µg/batch	= output 3* impact_AME	
Output 4	***Conc_AOH_final product***	***Recalculation for batch towards kg***	***µg/kg***	***= output 2/weight_batch***	
Output 5	***Conc_AME_final product***	***Recalculation for batch towards kg***	***µg/kg***	***= output 3/weight_batch***	
MODULE 3 : CONSUMPTION					
	Distribution_consumption	Consumption data derived tomato products	kg/kg BW.day	=RiskLognorm(0,001974;0,012321; RiskShift(-0,0000156144);RiskName("consumption (kg/kg BW/day)"))	2004 Belgian National Consumption Survey (BNFCS)

Output 6	***Exposure_AOH***	***Exposure to AOH***	***µg/kg BW.day***	***=distribution_consumption conc_AOH_final product***	***x***
Output 7	***Exposure_AME***	***Exposure to AME***		***=distribution_consumption conc_AME_final product***	***x***

Table 7.2: Climate change scenarios and their description and inputs for the exposure model

Climate change scenarios	Description	*Fraction_alt_>45 mm*
Baseline scenario	2% mould current situation climate Spain	RiskDiscrete((1\1\1\1\0.3\0\0);(30\25\20\15\10\5\0))
Scenario 1	2% mould near future RCP 8.5 Spain	RiskDiscrete((1\1\1\1\0.3\0\0);(30\25\20\15\10\5\0))
Scenario 2	2% mould far future RCP 8.5 Spain	RiskDiscrete((1\1\1\1\0.1\0\0);(30\25\20\15\10\5\0))
Scenario 3	2% mould current situation Poland	RiskDiscrete((1\1\1\0.9\0.1\0\0);(30\25\20\15\10\5\0))
Scenario 4	2% mould near future RCP 8.5 Poland	RiskDiscrete((1\1\1\1\0.1\0\0);(30\25\20\15\10\5\0))
Scenario 5	2% mould far future RCP 8.5 Poland	RiskDiscrete((1\1\1\1\0.1\0\0);(30\25\20\15\10\5\0))

7.3.4.2. Influence amount of moulded tomatoes entering the production line on the AOH and AME exposure

Scenario 6 and 7 were included in order to see the effect of the amount of moulded tomatoes entering the production line on the AOH and AME exposure. At this moment 2% of moulded products entering the factory is acceptable (interview

tomato processing industry). It was evaluated how the exposure can be influenced when another policy by the processing industry is applied e.g. if this amount was increased to 10% moulds with min 0, max 5 and most likely 2.5% *Alternaria* moulds (Table 7.3). In scenario 6 these calculations are conducted for the situation in Spain and scenario 7 for Poland.

Table 7.3: Scenarios 6 and 7 and their description and inputs for the exposure model

Scenarios	**Description**	*Alt_prev_tomato*
Baseline scenario	2 % mould current situation climate Spain	RiskPert(0;0.5;1)
Scenario 6	10% mould current situation climate Spain	RiskPert(0;2.5;5)
Scenario 7	10% mould current situation climate Poland	RiskPert(0;2.5;5)

7.3.4.3. Influence cold/hot break procedure on the AOH and AME exposure

The impact of the cold/hot break procedure on the AOH and AME exposure was evaluated by running the model with only the cold break procedure (scenario 8) and only the hot break procedure (scenario 9) (Table 7.4). The difference in the two procedures is the temperature of the preheating, with the temperature of the cold break procedure set around 65 °C and the hot break procedure at 95 °C (Chapter 4). Although the different treatments are used for different kind of products, it is important for the industry to know what the effect of the heat treatments will be on the AOH and AME exposure. The hot break procedure is used for further processing such as the making of ketchup and different sauces (more viscous) and the cold break procedure is used mainly for tomato concentrates, resulting in cans directly for use by the consumers.

Table 7.4: Scenario 8 and 9 and their description and inputs for the model

Scenarios	Description	*Impact_AOH*	*Impact_AME*
Baseline scenario	Cold and hot break procedure	Riskuniform(0.91;1.26)	Riskuniform(0.84;0.94)
Scenario 8	Baseline scenario cold break procedure	1.26	0.94
Scenario 9	Baseline scenario hot break procedure	0.91	0.84

7.4. Results and discussion

7.4.1. Baseline scenario

Table 7.5 shows the results of the exposure in the baseline scenario (Spain). Respectively 86% and 85% of the consumers of derived tomato products is exposed to less than the set TTC value of 2.5 ng/kg BW. day for AOH and AME. The mean value of AOH (4.1±0.1 ng/kg BW.day) is comparable with the mean value reported in the EFSA study obtained (from several food products contaminated with *Alternaria* mycotoxins, such as grains, tomato products, vegetable oils and wine and beer) of 3.6-26 ng/kg BW.day (LB-UB) (EFSA, 2011b). For AME the mean value was higher than AOH (8.0±0.3 ng/kg BW. day), which was also the case in the EFSA risk exposure study (13-63 ng/kg BW.day (LB-UB)) (EFSA, 2011b).

The calculated mean values are higher than the reported TTC value, thus possible form a risk to the consumer. The P_{50} for AOH and AME is 0.2 ng/kg BW.day, meaning that 50 % of the consumers are exposed to a tenth of the TTC value. The P99 is 55

and 96 ng/kg BW.day for AOH and AME, respectively, indicating that the exposure of 1/1000 consumers is 24 and 40 times higher compared to the TTC. The P99.9 is 417 and 1021 ng/kg BW.day for AOH and AME, respectively, indicating that the exposure of 1/10 000 consumers is 160 and 408 times higher compared to the TTC. In the EFSA reports both mean and P95 exceeded the TTC value for AOH and AME, indicating a need for additional compound specific toxicity data (EFSA, 2011b).

Comparing the baseline scenario (Spain) with scenario 3 (baseline Poland) in the current time period it shows that AOH there is no difference in mean, P50, P99 and P99.9. For AME it showed that in Poland the amount of consumers below the TTC value is 1% bigger than for Spain. So, in the current time period there is no difference in exposure between Spain and Poland, whereas in Chapter 6 it was shown that the mould diameters in Poland were significant lower than the mould diameters in Spain and there was a lower fraction of *Alternaria* moulds > 45 mm for TTH 15 and 10 for Poland.

7.4.2. Influence of climate change on the AOH and AME exposure

Table 7.5 also shows the results of the different climate change scenarios on the exposure of AOH and AME. For Spain the results for the current (baseline) and near future (scenario 1) are the same due to the fact that the fraction of moulded tomatoes was the same, as can be derived from Table 7.2. For the far future period (2081-2100) (scenario 2), there is an increase of 1% of consumers who are below the TTC value for AME, but it is not significant different from scenario 1 and the baseline scenario.

For Poland there is no difference for the near (scenario 4) and far future period (scenario 5). Compared with the current time period (scenario 3) there is only a small difference in exposure results.

Overall, it can be seen in Table 7.5 that no major shifts in exposure to the mycotoxins can be expected due to climate changes with our model. Only in the calculation of the maximum some differences can be found and maybe also some inconsistencies i.e. max of AOH in scenario 2 is lower compared to baseline scenario and scenario 1. The calculation of a maximum in an exposure assessment is always a bit uncertain, due to the fact that maybe not always enough random points in the tails of a distribution are taken during the iterations.

A study on the aflatoxin exposure in grains by EFSA showed that in the + 2 °C scenario, higher levels of contamination are expected, whereas in the + 5 °C scenario, levels of contamination are predicted to be lower. Although the extend of AF exposure was not quantified, the exposure appeared to be of concern (EFSA, 2012b). This is showing the same trend in results with our findings, when the temperature is too high for the mould growth is it resulting in a lower exposure to the mycotoxins.

7.4.3. Influence amount of moulded tomatoes entering the production line on the AOH and AME exposure

Table 7.6 shows the results when 10% of the tomatoes entering the production line are moulded (>45mm) (instead of the 2% in the baseline scenario). As expected, the mean exposure increases both for AOH and AME compared with the baseline scenario with a factor 5 for Spain (scenario 6 compared with baseline scenario) and

7.5 for Poland (scenario 7 compared to scenario 3). One third of the consumers exceeds the TTC value when the amount of moulded tomatoes entering the production line increases from 2 to 10% and also the calculated concentrations are increasing clearly.

This scenario emphasizes the importance of preventive measures at fields to avoid the prevalence of *Alternaria* moulds and their development on the field. To make the tomatoes less vulnerable it is important that the stress factors are limited, which can be obtained by using the right variety for a certain region. Varieties which are more resistant to moulds and other diseases should be evaluated and used. Also, crop rotation can reduce the mould infestation. It has been for example proven that the alteration of crops which are host for *Fusarium* spp. with crops which are not, can reduce the risk of *Fusarium* contamination (Eeckhout et al., 2013). Another important practice that can decrease the amount of moulds is a good soil and crop management, such as removal of infected crops and the timely application of fungicides (Edwards, 2004). More information on the effect of these preventive measures on the prevalence of *Alternaria* moulds should be evaluated.

Also the incoming material control and eventually, criterium on sorting the moulded tomatoes out before entering the production line of derived tomato products will be important to limit the mycotoxin exposure as stated by EU recommendation 2003/598/EG to control patulin in apple products. In the Self checking guideline G014 (http://www.gidsac.be/nl/8/8.0) for the fresh produce processing industry in Belgium, the incoming raw apples in the production of apple compote is defined as a Critical Point in the company own food safety management system. Validated

process criteria are demanded on the initial cut off of moulded apples entering the production plant.

Therefore, field control and incoming material control are of upmost importance to avoid too high exposure of consumers and need to be up taken as a part of a food safety management system in tomato processing companies.

7.4.4. Influence cold/hot break procedure model on the AOH and AME exposure

Table 7.7 shows the results of the exposure for a cold (scenario 8) and hot (scenario 9) break procedure. The mean values for the hot break procedure are lower than those for the cold break procedure, both for AOH and AME. Also the percentage of consumers exceeding the TTC value is lower for the scenario with the hot break procedure. Hot break treatment is leading to higher temperatures (95°C versus 65°C in cold break) and according to our lab experiments, simulating industrial processing, it was found that impact factors are lower in hot break (0.91 – 0.84) compared to cold break (1.26-0.94) (Chapter 4). The hot break procedures leads thus to lower concentrations of AOH and AME resulting in a lower exposure.

However, the different treatments are used for different kind of products, so it may not be feasible for the industry to change all the production processes to the hot break treatment. The hot break procedure is used for further processing such as the making of ketchup and different sauces (more viscous) and the cold break procedure is used mainly for tomato concentrates, resulting in cans directly for use by the consumers.

Table 7.5: Results of the baseline scenario in Spain and climate change scenarios (scenario 1 to 5) for the exposure of AOH and AME in derived tomato products

Climate change scenario	ng AOH/ kg BW.day								ng AME/ kg BW.day							
	Min	Max	Mean	P50	P95	P99	P99.9	TTC (%)	Min	Max	Mean	P50	P95	P99	P99.9	TTC (%)
Baseline scenario	0	18105	4.1±0.1	0.2	10	55	417	86±0.5%	0	39010	8.0±0.3	0.2	10	96	1021	85±0.5%
Scenario 1	0	18105	4.0±0.1	0.2	10	55	417	86±0.5%	0	39010	8.0±0.3	0.2	10	96	1021	85±0.5%
Scenario 2	0	8388	4.0±0.1	0.2	10	55	423	86±0.8%	0	70297	8.0±0.7	0.1	10	93	950	86±0.7%
Scenario 3	0	11008	4.0±0.0	0.2	10	55	421	86±0.0%	0	69153	8.6±0.5	0.2	10	93	1149	86±0.7%
Scenario 4	0	8388	4.0±0.1	0.2	10	55	423	86±0.8%	0	70297	8.0±0.7	0.1	10	93	950	86±0.7%
Scenario 5	0	8388	4.0±0.1	0.2	10	55	423	86±0.8%	0	70297	8.0±0.7	0.1	10	93	950	86±0.7%

Table 7.6: Results of the scenarios if 10% moulded tomatoes are allowed entering the production line for the exposure of AOH and AME in derived tomato products

Scenarios	ng AOH/ kg BW.day								ng AME/ kg BW.day							
	Min	Max	Mean	P50	P95	P99	P99.9	TTC (%)	Min	Max	Mean	P50	P95	P99	P99.9	TTC (%)
Scenario 6 (Spain)	0	250000	23±3	0. 9	50	270	2170	66±0.8%	0	201400	38±2	0.8	60	460	4960	67±0.7%
Scenario 7 (Poland)	0	514100	25±10	0. 8	50	270	2220	67±0.6%	0	1526400	55±13	0.7	60	360	4050	68±1.2%

Table 7.7: Results of different heat treatments on the exposure of AOH and AME in derived tomato products, scenario 8: cold break, scenario 9: hot break.

Scenarios	ng AOH/ kg BW.day								ng AME/ kg BW.day							
	Min	Max	Mean	P50	P95	P99	P99.9	TTC (%)	Min	Max	Mean	P50	P95	P99	P99.9	TTC (%)
Scenario 8	0	28067	4.9±0.1	0.2	12	70	470	84±0.7%	0	36498	7.8±0.1	0.2	10	100	1005	85±0.3%
Scenario 9	0	8535	3.2±0.1	0.1	8.5	46	345	87±0.3%	0	23654	7.0±0.7	0.2	10	80	960	86±0.1%

7.5. Conclusions

Only one single exposure assessment is known until now which was reporting both for AOH and AME on several food products such as grains, vegetable oils, tomato products, beer and wine in Europe (EFSA, 2011b). Their intake calculation based on screening of food products relevant towards presence of *Alternaria* mycotoxins was similar as in the presented study (baseline scenario): calculated mean exposure for AOH is 4.1 ng/kg BW.day while reported by EFSA ranging between 3.6-26 ng/kg BW.day (LB-UB). For AME a higher exposure was calculated compared to AOH, being 8.0 ng/kg BW.day and following EFSA calculations 13-63 ng/kg BW.day (LB-UP). It must be stressed that our findings are limited to exposure due to consumption of derived tomato products, while EFSA report was on a broad range of food products, so if a multi-source exposure assessment could be done, higher intake of AOH and AME by Europeans consumers can be expected on the basis of our findings. The no mitigation case RCP climate change scenario 8.5 resulted only in a limited increasing impact on the exposure of near and far future both in Spain and Poland. An increase in moulded tomatoes entering the production line will have a significant increasing effect on the exposure of AOH and AME. This emphasizes the importance of preventive measures at fields to avoid the prevalence of *Alternaria* moulds and the importance of sorting the moulded tomatoes out before entering the production line of derived tomato products. The hot break procedure resulted in a lower exposure compared with the cold break procedure.

TeA was not included in this farm to fork exposure assessment, due to the focus of the PhD work on AOH and AME but it will be included in the next Chapter 8 (risk ranking). Based on the presented farm to form exposure assessment and its different

scenarios, it can be concluded that most probable the exposure to *Alternaria* mycotoxins by the European population is similar to what was earlier reported by EFSA (2011b). Therefore, it can be concluded that *Alternaria* mycotoxins are becoming a relevant food safety hazard.

Chapter 8: Comparison of emerging mycotoxins (*Alternaria* mycotoxins) with established mycotoxins (aflatoxins and ochratoxin A in nuts and dried fruits)

Redrafted from:

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Chapter 8: Comparison of emerging mycotoxins (Alternaria mycotoxins) with established mycotoxins (aflatoxins and ochratoxin A in nuts and dried fruits)

8.1. Abstract

The risk ranking is divided in two parts: on the one hand the risk ranking of **established mycotoxins** (aflatoxin B1 and ochratoxin A in nuts and dried fruits) and on the other hand the risk ranking of **emerging mycotoxins** (alternariol, alternariol monomethyl ether and tenuazonic acid) in processed tomato products. Well-established mycotoxins were subjected to extensive scientific research and are also well known from toxicological perspective. Moreover, for most associated commodities, legal limits and mitigation strategies are set.

In the first study the impact of setting European legal maximum criteria in relevant foods on the exposure to aflatoxin B1 (AFB1) (via nuts and figs) and ochratoxin A (OTA) (via dried fruits) is evaluated for the Belgian population, as an example for the European population. Focus was made on dried plant products, which can be consumed as such and not on staple foods as cereals. Two different scenarios were evaluated. In scenario 1 all collected literature data were considered, assuming that there are no border controls or legal limits in Europe. In the second scenario, contamination levels above the maximum limits are excluded for exposure calculation. The results from scenario 1 demonstrated that if no regulation nor border controls are in place, AFB1 and OTA concentrations reported in the considered foods can have a potential health risk to the population consuming these products. The estimated exposures of OTA for scenario 2 are below the TDI of 0.35 µg/person.day, indicating that OTA concentrations accepted by EU legislation pose a low risk to the Belgian (European) population. For AFB1, the MOE values of scenario 2 are above 10 000 and can be considered to be of low health concern, based on

$BDML_{10}$ for humans, except for figs (MOE = 5782 for mean). This means that for all matrices, with the exception of figs, the maximum values of AFB1 in the European legislation are sufficient to be of a low health concern for consumers.

In a second study, a probabilistic exposure assessment was conducted with the analytical data obtained from *Alternaria* mycotoxins (AOH, AME and TeA) in derived tomato products (Chapter 2) and consumption data of the Belgian population of derived tomato products such as tomato concentrate, juice, canned tomatoes etc., as proxy for the European population (also applied in Chapter 7 for the farm to fork calculations). Both for AOH and AME the dietary exposure is higher than the set threshold of toxicological concern (TTC) value of 2.5 ng/kg BW.day. These results reflect the conclusion EFSA made in their report of 2011 (EFSA, 2011b), that there is a need for additional information and research on the toxicology of AOH and AME to be able to really evaluate their impact on human health. Also for TeA, the obtained mean value (4230 ng/kg BW/day) was higher than the TTC value of 1500 ng/kg BW/day set by EFSA, whereas in the EFSA study it was below the TTC value (EFSA, 2011b).

8.2. Introduction

Due to the global trade of food products, the European Commission protects its consumers by increasing border controls on mycotoxins in specific crops imported from certain regions (EU Regulation 669/2009) (EC, 2009a). However, no systematic border controls are conducted, so there is a potential risk that food products with higher mycotoxin levels are present on the European market.

In this chapter the impact of setting European criteria on exposure to AFB1 via consumption of nuts and dried fruits and OTA via consumption of dried fruits is evaluated. Mycotoxin data are collected from reported literature and combined with consumption data of nuts and dried fruits from Belgian consumers, exemplifying the European population, to be able to calculate the exposure to mycotoxins by consuming these commodities imported from International Cooperation Partner Countries (ICPC) (see Chapter 1). Two different scenarios will be evaluated. In scenario 1 all collected data are considered, assuming that there is no border control nor legal limits in Europe. In the second scenario, the legal limits on mycotoxins are considered. The mycotoxin concentration database will be adapted: data points with higher concentrations than legal limits will be removed from it. The exposure assessment will be conducted again with the same consumption data as in scenario 1. By comparing the exposure in scenario 1 and 2 the impact of the border controls and setting legal limits on EU level on the mycotoxin exposure and potential risk to European consumers will be evaluated.

In a second study the exposure to *Alternaria* mycotoxin AOH, AME and TEA in derived tomato products was evaluated. A probabilistic exposure assessment was made in order to get inside of the actual risk towards the population and to risk rank

the *Alternaria* mycotoxins in derived tomato products. The recent report of EFSA on *Alternaria* mycotoxins is the only risk assessment on *Alternaria* mycotoxins performed on European and international level today (EFSA, 2011b).

8.3. Materials and methods

8.3.1. Nuts and dried fruits

8.3.1.1. Developing database with mycotoxin concentrations

As explained in Chapter 1, a database file was developed in Microsoft Excel (2007) and sent to the different involved partners (UFRGS (Brazil), RIIL (Egypt), UGent (Belgium)) to collect the world-wide available data on mycotoxins in fresh produce and on (dried) derived products (i.e. nuts, dried fruits, spices and herbs, fermented olives). Data were received from different partners from own research projects : UFRGS (Brazil) (7 samples grapes, nuts, apples, tomatoes, patulin), RILL (Egypt) (2508 samples of groundnuts, aflatoxins) and UGent (Belgium) (170 samples of apple juice, patulin). Additionally, 826 data points from scientific literature data from over the world were collected: dried fruits (186), herbs (64), nuts (250), olives (97) and spices (229). Also, data from RASFF reporting (border rejections 2008-2010) were included: nuts (1360 cases), dried fruits (292 cases) and spices (136 cases).

Information collected for the data points were product, matrix, preservation technique, date of the analysis, method used, equipment used, LOD, LOQ and concentration (expressed as µg/kg). Based on the collected data points, distributions were fitted for the expsoure assessment (see 8.3.1.3).

8.3.1.2. Consumption data

Consumption data for nuts, nut products and dried fruits were obtained from the 2004 Belgian National Consumption Survey (BNFCS). The food consumption database in this survey is a result of daily food intakes from two 24 hour food recalls. Aims, design and methods of the BNFCS are described by De Vriese et al. (2005). Similar data analysis was performed as explained in Chapter 7: consumption data for the different matrices (hazelnut, groundnut, almonds, pistachio, figs and raisins) were extracted from the database based on the food name and facet strings. The usual food intake for nuts, dried fruits was then determined using the Multiple Source Method (MSM) program (German Institute of Human Nutrition) (Potsdam-Rehbrücke, 2012). The body weight was known per person. The usual dietary intake values (expressed as kg/person.day) were then entered into excel worksheet and distribution was fitted using @RISK version 6 (Palisade Corporation, US) for each food matrix.

8.3.1.3. Exposure assessment

Dietary exposure for the establisdhed mycotoxins included the analysis of two different scenarios: In scenario 1 all collected data were considered, assuming that there is no border control nor legal limits to be respected in Europe.

In the second scenario, the legal limits on mycotoxins and border controls were included. Therefore, data points with higher concentrations than European legal limits were removed before conducting the exposure assessment i.e. for AFB1: 12 µg/kg for almonds and pistachio, 8 µg/kg for hazelnuts and 2 µg/kg for groundnuts and figs, for OTA: 10 µg/kg for raisins (EC, 2006). Since there is no limit for OTA in figs the same maximum limit as for raisins was applied (i.e. 10 µg/kg).

Best fit distributions were applied for the different matrices and mycotoxins. Best fit was based on Chi-square statistics. Also, the probability/probability plots (P/P) and the quantile/quantile plots (Q/Q), resulting from the cumulative distributions, were a parameter if the cumulative distributions corresponded to the theoretical cumulative distributions.

8.3.1.4. Probabilistic analysis

Calculations were performed using the software package @RISK version 6 (Palisade Corporation, US). The logical test "IF" was applied when the concentration data set had a high number of reported values below the reported LOD/LOQ. First order Monte Carlo simulations were performed considering 100 000 iterations and three simulations. Estimated mycotoxin intakes (min, mean, max and percentiles) were determined for each matrix/mycotoxin combination.

The MOE values were calculated using the following equation: MOE=BMDL/exposure (EFSA, 2005). The BMDL values derived from dose-response curves for rodents and humans. $BMDL_{10}$ (rat)=0.340 µg/kg BW.day, $BMDL_{10}$ (human)=0.87 µg/kg BW.day, $BMDL_1$ (rat and human)=0.078 µg/kg BW.day, T25=0.39µg/kg (Benford et al., 2010; JECFA, 2007). The exposure was based on the mean and 95th percentile. Average body weight to recalculate the BMDL values was 70 kg.

8.3.2. Derived tomato products

8.3.3.1. Mycotoxin concentrations

The information on concentration data was gathered in Chapter 2 and 4. For AOH and AME 153 tomato samples (tomato concentrates) were analysed (with 21 samples >LOD).

For TeA two scenarios were calculated, a first scenario (TeA 1) with only the positive concentrations (n=11), a second scenario (TeA 2) assuming that all the other products were negative for TeA (although not analyzed) (n=119). Both lower bound (LB) and upper bound (UB) scenarios were calculated. The LB is obtained by giving a zero to all mycotoxin concentrations lower than the LOQ, the UB is obtained by giving the value of LOD (=0.1 µg/kg for TeA) to samples below LOD.

For AOH and AME the LB scenario contained not enough samples >0 to perform a probabilistic study, so a deterministic approach was used. For the UB all the samples < LOD were given the value of LOD (=12.2 and 13.5 µg/kg, AOH and AME respectively) and thus it was possible to perform a probabilistic exposure assessment. As a further consequence it is possible that the values for mean and P95 for UB are lower than for LB because of a different calculating approach. Data were fitted to distributions with @Risk software (Version 6, Palisade). Best fitting distribution was selected by using the P-P plot and statistical information provided by @Risk.

8.3.2.1. Consumption data

Consumption data were obtained from the 2004 Belgian National Consumption Survey (BNFCS). The food consumption database in this survey is a result of daily

food intakes from two 24 hour food recalls. Aims, design and methods of the BNFCS are described by De Vriese et al. (2005). Consumption data were extracted from the database based on the food name and facet strings. The output was a combination of all derived tomato products (tomato concentrate, ketchups, sauces, peeled canned tomatoes, purees). The usual food intake was further determined using the Multiple Source Method (MSM) program (German Institute of Human Nutrition) (Potsdam-Rehbrücke, 2012). The body weight was known per person. The usual dietary intake values (expressed as kg/person.day) were then entered into excel worksheet and distribution was fitted using @RISK version 6 (Palisade Corporation, US).

8.3.2.2. Probabilistic analysis

The software program @Risk version 6 (Palisade Corporation, US) was used to perform probabilistic analysis and to calculate the Monte Carlo simulations (iterations=100 000, three simulations). The outcome of the analysis was compared to the TTC (threshold of toxicological concern) set by the EFSA panel on Contaminants in the Food Chain (EFSA, 2011b). TTC for AOH and AME was set at 2.5 ng/kg BW.day and for TeA 1500 ng/kg BW.day.

8.4. Results and discussion

8.4.1. Selection of products and mycotoxins

For the selection of cases to perform a probabilistic exposure assessment study a minimum number of five data points of which three positive points was needed in order to perform probabilistic analysis (De Boevre et al., 2013; Vinci et al., 2012). It was decided to evaluate AFB1 and OTA, since enough data points for a specific

commodity were available. Patulin was not evaluated in this study, because this risk assessment was earlier performed by Baert et al. (2007). Therefore, following combinations could be performed out of the developed database : AFB1 in almond, groundnut, pistachio, hazelnut and figs, OTA in figs and raisins. The selection of cases with the total number of data points is given in Table 8.1.

8.4.2. Mycotoxin exposure assessment nuts and figs

8.4.2.1. AFB1 and OTA exposure estimates

Concentration and consumption data were fitted to best fit distributions using the @Risk software. When a high number of data points was <LOD the distribution was only developed for the positive concentrations (> LOD) and the IF function was used to distinguish between positive and negative values (Table 8.2 e.g. ALB1 in almond). The logical test "IF" was also applied for concentration of AB1 in groundnuts and OTA in figs to divide the dataset in a PERT distribution for the low concentrations and the best fitted distribution for the other concentration values (using @RISK software). The best fit distributions for all matrices and scenario's are listed in Table 8.2. Table 8.3 gives an overview of the probabilistic estimates of AFB1 and OTA exposure in the different food categories. For scenario 1, the highest AFB1 exposure (mean) were 0.528 and 0.344 µg/kg person.day, respectively for groundnuts and pistachio nuts. When shifting to scenario 2 (considering only datapoints below legal limit), the exposure for AFB1 for these commodities shifted towards 0.00041 and 0.00131 (mean) µg/kg person.day. By excluding the concentrations above the legal limits, a 99.9% and 99.6% reduction of the exposure was obtained for groundnuts and pistachios respectively. For scenario 2, where all data above EU limit were removed, the highest AFB1 exposure was found for figs being, 0.01053 (mean) µg/kg

Table 8.1: Selection of cases for probabilistic analysis with the total number of data points, quantifiable data points (> LOQ), <LOQ (NQ) and <LOD (ND)

Matrix	Number of data points		
	n	AFB1	OTA
Almonds	Total	120	-
	>LOQ	100	-
	NQ	7	-
	ND	13	-
Groundnut	Total	3239	-
	>LOQ	2932	-
	NQ	289	-
	ND	18	-
Hazelnut	Total	126	-
	>LOQ	113	-
	NQ	3	-
	ND	10	-
Pistachio	Total	429	-
	>LOQ	354	-
	NQ	38	-
	ND	37	-
Figs	Total	293	33
	>LOQ	254	15
	NQ	20	18
	ND	19	0
Raisins	Total	-	87
	>LOQ	-	83
	NQ	-	0
	ND	-	4
Chili	Total	111	37
	>LOQ	110	37
	NQ	1	0
	ND	0	0
Curry powder	Total	31	-
	>LOQ	31	-
	NQ	0	-
	ND	0	-
Nutmeg	Total	26	-
	>LOQ	26	-
	NQ	0	-
	ND	0	-
Olives	Total	34	97
	>LOQ	7	78
	NQ	0	4
	ND	27	15

person.day. Risk assessment studies on mycotoxins have been performed in several countries, both in Europe and non-European countries. From these studies, the highest mean values were also obtained in pistachios and groundnuts for aflatoxins (AFB1, AFT) (Awuah, 2000; Park et al., 2004; Thuvander et al., 2001).For OTA, a reduction of respectively 96.2% for figs and 94.6% for raisins could be calculated in exposure due to elimination of concentrations above legal EU limits (scenario 2, Table 8.3).

Table 8.2: Best fit distributions on food consumption and mycotoxin concentration for scenario 1 (all data) and scenario 2 (only data below EU limit) in the different food categories

		Scenario 1			Scenario 2	
	Product	**Food consumption**	**Mycotoxin content**	**IF-function**	**Mycotoxin content**	**IF-function**
AFB1	**Almond**	RiskBetaGeneral(0,27081;0,39677;0,0024648;0,08642)	RiskBetaGeneral(0,57566;7,7398;0,1;221,57)	IF(L5>0,16;riskbeta;0)	RiskLogistic(2,8903;2,0429)	IF(L5>0,25;risklogistic;0)
	Groundnut	RiskLoglogistic(-0,013777;0,034696;4,6493)	RiskPert(0;1;1)		RiskPert(0;1;2)	
			RiskLognorm(42,761;162,24;RiskShift(1,029))	IF(N9>0,213;risklog;riskpert)		
	Hazelnut	RiskExpon(0,025943;RiskShift(-0,0018376))	RiskInvgauss(7,9855;4,6355;RiskShift(-0,8345))	IF(O9>0,103;invgaus;0)	RiskNormal(3,1685;2,2795)	IF(O10>0,13;risknorm;0)
	Pistachio	RiskLogistic(0,016948;0,0072228)	RiskPert(0;0;1)		RiskPert(0;0;1)	IF(N9>0,43;ext;pert)
			RiskInvgauss(41,908;12,138;RiskShift(-1,0111))	IF(N9>0,235;pert;invgaus)	RiskExtvalue(4,2187;2,3745)	

	Figs	RiskExtvalue(0,0148714;0,0099673)	RiskPearson5(1,6643;8,4742;RiskShift(-1,3242))	IF(L3>0,13;pearson;0)	RiskUniform(0,0076087;2,0424)	IF(L3>0,45;RiskUniform;0)
OTA	**Figs**	RiskExtvalue(0,0148714;0,0099673)	RiskPert(0;0;1)	IF(J7>0,73;betageneral;Pert)	RiskPert(0;0;7,2)	
	Raisins	RiskInvgauss(0,029929;0,044849;RiskShift(-0,0034895))	RiskPearson5(0,91797;1,5265;RiskShift(-0,33719))	IF(J5>0,15;pearson;0)	RiskExpon(2,1996;RiskShift(0,062076)))	IF(J5>0,18;RiskExpon;0)

Table 8.3: Probabilistic estimated intake of AFB1 and OTA (mean, STD and percentiles, ng/kg person.day) for scenario 1 (all data) and scenario 2 (data below EU limit) by Belgian adult population from the different food categories.

		Scenario 1						Scenario 2					
AFB1	**product**	**mean**	**STD**	**P50**	**P75**	**P95**	**P99**	**mean**	**std**	**P50**	**P75**	**P95**	**P99**
	almonds	1.8E+01	1.7E-01	8.0E-02	3.0E+00	1.1E+02	3.1E+02	3.4E+00	4.0E-02	0.0E+00	4.1E-01	2.7E+01	5.4E+01
	groundnut	5.3E+02	9.2E+00	3.5E+01	2.4E+02	2.1E+08	8.0E+08	4.1E-01	0.0E+00	0.0E+00	3.9E-01	1.8E+00	4.6E+00
	hazelnut	4.5E+00	1.1E-01	0.0E+00	7.4E+00	1.9E+01	8.2E+01	2.0E+00	3.0E-02	0.0E+00	5.2E-01	1.0E+01	3.6E+01
	pistachio	3.4E+02	1.5E+00	7.3E+00	2.2E+02	1.8E+08	5.6E+08	1.3E+00	2.0E-02	1.5E-01	1.3E+00	6.3E+00	1.3E+01
	figs	1.2E+02	2.9E+00	0.0E+00	8.6E+01	4.6E+02	1.5E+08	1.1E+01	6.0E-02	0.0E+00	1.6E+01	4.7E+01	7.5E+01
OTA	**figs**	6.7E+02	6.9E+00	1.1E+01	1.3E+08	3.6E+08	5.9E+08	2.5E+01	8.0E-02	1.5E+01	3.3E+01	8.2E+01	1.4E+02
	raisins	5.6E+02	2.7E+01	0.0E+00	4.0E+01	5.6E+02	2.9E+08	3.0E+01	1.0E-01	4.0E-01	2.8E+01	1.5E+02	3.5E+02

8.4.2.2. AFB1 and OTA risk assessment

In order to evaluate the risk to the Belgian population, as an example for the European population, a MOE value was determined for AFB1. Table 8.4 gives an overview of the obtained MOE values derived from estimates of mean exposure to AFB1 for different food categories and considering both scenario 1 (all data) and scenario 2 (date below EU limit). Bold values are MOE values which are higher than 10 000, which is considered of low concern (EFSA, 2005). The Scientific Committee from EFSA recommended the use of the $BMDL_{10}$. The calculated MOEs obtained ranged from 3-148,295 for scenario 1, which are generally higher than previous estimated results by Shephard (2008) (1-621.4, AFB1 in Africa), Benford et al. (2010) (100-600, AFB1 in different regions) and by Ding et al. (2012) (24.1-1272, AFB1 in post-harvest groundnuts in China). By removing the data above EU limit, the MOE values are all above 10 000 ($BDML_{10}$ human), except for AFB1 in figs. This means that for all matrices, with the exception of figs, the maximum values in the European legislation are sufficient to be of a low health concern for consumers. However, for figs, still after removing the data above the maximum limit, a MOE value of 5782 (mean, $BDML_{10}$ human) was obtained. Thus it could be argued to lower the maximum limits for AFB1 in figs. A MOE value above 10000 was obtained by removing all data for figs contamination above 1.3 µg/kg (EU legislation 1881/2006: 2µg/kg for dried fruits). However, lowering the maximum limit is something that should take into account the balance between food safety and economical losses (Dohlman, 2003).

Comparing the obtained estimated intake of OTA (mean and 95th percentiles) for scenario 1 (all data) and scenario 2 (data below EU limit) (Table 8.3) with the TDI of

0.35µg/person.day set by SCF (SCF, 2002) shows that the values from scenario 2 are below the TDI (both for the mean and the 95th percentile). These results indicate that the maximum OTA concentrations accepted by EU legislation are sufficient to protect the Belgian population. However, limitations of these calculations are the fact that no systematic screening is conducted from products actually present on the European market and that only Belgian consumption data are applied.

In this research, it is assumed that all samples above the legal EU limits are excluded from the market and consequently only products with mycotoxin concentrations below the legal limit are consumed. However, no systematic borders controls are conducted for these commodities in Europe, but a risk based increased border control can be imposed (EC, 2009a). Therefore, it can be assumed that the actual intake of the European consumers to AFB1 and OTA via consumption of groundnuts, pistachio nuts and dried fruits will be higher compared to scenario 2 calculations. A study on the dietary intake of some important mycotoxins by the Swedish population showed that the mean intake for AFT was up to 1750 µg/person.day for groundnuts and 3.5 µg/ person.day for almonds. 10% of the analysed nut samples in the study exceeded the maximum limits set by the EU, hence resulting in a high intake for AFT. The estimated intake for OTA in raisins was 0.28 µg/person.day, so below the TDI of 0.35 µg/person.day (Thuvander et al., 2001). EFSA evaluated the effect on the dietary exposure of an increase of the existing EU maximum levels for AFT from 4 to 10 µg/kg in nuts and figs. They concluded that for almonds, hazelnuts and pistachios the average total dietary exposure would increase with 1% (EFSA, 2007b). An increase for dried figs specifically, would result in a AFT increase for the adult population by 0.15 to 0.26% (EFSA, 2012a).

The results from scenario 1 demonstrate that in sourcing countries, where little or no regulation is in place, AFB1 and OTA concentrations found in the analysed food can have potential health risk to the population consuming these products. Next to this, sourcing countries can also suffer from lost export opportunities to developed countries, due to the more stricter mycotoxin regulations (Dohlman, 2003). It is up to the importer's responsibility to bring in commodities with mycotoxin concentrations below legal limits. Therefore, supplier selection and establishing a food safety management system backed with a sampling plan in trading and importing companies is of upmost importance. At the side of the exporter it is important to increase awareness among the producers by implementing good agricultural and manufacturing practices to minimize the mycotoxin contamination.

Table 8.4 : MOEs derived from estimates of mean and 95th percentile exposure to AFB1 for different food categories and for scenario 1 (all data) and scenario 2 (date below EU limit), bold values are MOE values which are higher than 10,000, which is considered of low concern (EFSA, 2005).

	Exposure for AFB1 (µg/person.day)		BMDL$_{10}$				BMDL$_1$				T25	
			Rat		Human		Rat		Human			
	Mean	P95	mean	P95	mean	P95	mean	P95	mean	P95	mean	P95
Almond scenario 1	**0.0182**	0.1128	927	87	3346	540	302	49	300	48	1500	242
Almond scenario 2	**0.0034**	0.0273	4972	360	**17947**	2234	1621	202	1609	200	8045	1002
Groundnut scenario 1	**0.5277**	2.1100	32	5	115	29	10	3	10	3	52	13
Groundnut scenario 2	**0.0004**	0.0018	**41080**	5444	**148295**	**33833**	**13398**	3057	**13295**	3033	**66477**	**15167**
Hazelnut scenario 1	**0.0045**	0.0019	3744	5158	**1517**	**32053**	1221	2896	1212	2874	6059	**14368**
Hazelnut scenario 2	**0.0020**	0.0100	8550	980	**30867**	6090	2789	550	2767	546	**13837**	2730
Pistachio scenario 1	**0.3443**	1.7000	49	6	177	36	16	3	16	3	79	16
Pistachio scenario 2	**0.0013**	0.0060	**12845**	1633	**46371**	**10150**	4189	917	4157	910	**20787**	4550
Figs scenario 1	**0.1183**	0.4600	143	21	515	132	46	12	46	12	231	59
Figs scenario 2	**0.0105**	0.0460	1602	213	5782	1324	522	120	518	119	2592	593

8.4.3. Mycotoxin exposure assessment for derived tomato products

Table 8.5 gives an overview of the best fitting distribution functions of mycotoxin concentrations analyzed in derived tomato products (Chapter 2 and 4). Table 8.6 gives an overview of the mean and high dietary exposure to AOH, AME and TeA for adult population both for LB and UB scenarios, for risk assessment performed by our calculations and by EFSA (EFSA, 2011b). It should be emphasized that the risk assessment by EFSA is not only done for tomato products, but also for other products containing *Alternaria* mycotoxins such as grains and grain-based products, sunflower seeds and sunflower oil, fruits and fruit products, and beer and wine. Moreover, it was carried out for the European consumers, so not specifically dedicated to Belgian consumers.

Table 8.5: Distribution functions of mycotoxin concentration in derived tomato products for AOH, AME, TeA (TeA1: n=11, only samples analyzed, TeA2: n=119, including negative values for samples not analyzed) (LB= lower bound, UB= upper bound)

	Food consumption	Mycotoxin content	IF-function
AOH - LB	RiskLognorm(0,001974;0,012321;RiskShift(-0,0000156144))	Deterministic distribution (n=3) for positive values	=IF(L3<0.0196;38.7;0)
AOH - UB	RiskLognorm(0,001974;0,012321;RiskShift(-0,0000156144))	RiskPert(12,2; 12,2; 57,3)	
AME - LB	RiskLognorm(0,001974;0,012321;RiskShift(-0,0000156144))	Deterministic distribution (n=1) for positive values	=IF(L3<0.0065;41.2;0)
AME - UB	RiskLognorm(0,001974;0,012321;RiskShift(-0,0000156144))	RiskPert(13;13;41.2)	
TeA1 – LB	RiskLognorm(0,001974;0,012321;RiskShift(-0,0000156144))	RiskBetaGeneral(0,27885;0,41357;700;4800)	RiskTriang(12,2;12,2;49,76)
TeA2 - UB	RiskLognorm(0,001974;0,012321;RiskShift(-0,0000156144))	RiskTriang(0;0;4846,2)	RiskTriang(0,1;0,1;4846,2)

Both for AOH and AME the dietary exposure is considerably higher than the reported TTC value of 2.5 ng/kg BW.day (Table 8.6). The results reflect the conclusion EFSA made in their report, that there is a need for additional information and research on the toxicology of AOH and AME. Comparing the two exposure assessments (this PhD and EFSA) it can be derived that for AOH and AME we found similar exposure, but for TeA we found much higher outcomes even higher than the set TTC value of 1500 ng/kg BW.day. In order to get a clear picture more analyzes should be performed for TeA (since we only tested 11 samples). However, it should also be remarked that in the EFSA study more food items were included and we only focused on derived tomato products. Therefore, the exposure towards the emerging mycotoxins is most probably more problematic than earlier reported.

Table 8.6.: Mean and high dietary exposure to AOH, AME and TeA (ng/kg BW/day) for adult population in LB and UB scenarios performed in this PhD (left) via derived tomato products and EFSA (right) (EFSA, 2011b) via grains and grain-based products, tomato and tomato products, sunflower seeds and sunflower oil, fruits and fruit products, and beer and wine.

Emerging mycotoxins	Chapter 2/4 Mean (ng/kg BW.day) LB-UB	Chapter 2/4 P95 (ng/kg BW.day) LB-UB	TTC	EFSA Mean (ng/kg BW.day) LB-UB	EFSA P95 (ng/kg BW.day) LB-UB
AOH	38-39*	64-54*	2.5	3.6-26	13-63
AME	41-35*	67-49*	2.5	1.3-3.1	7.5-11
TeA 1	4230	10820	1,500	41-94	127-262
TeA 2	2900	2900			

*Different distribution used for LB and UB (deterministic ⇔ Pert) causes the lower value for UB compared to LB

At the moment there is limited information available on the toxicity of these *Alternaria* mycotoxins. The report from EFSA (EFSA, 2011b) concluded that the effects of *Alternaria* toxins are limited and are not sufficient to establish a NOAEL, thus making it difficult to estimate the potential hazard of these mycotoxins in humans.

8.5. Conclusion: Risk ranking

At the end of all the research performed and calculations made, a risk ranking was made to compare the well-established mycotoxins occurring in dried fruits and nuts with the emerging mycotoxins in derived tomato products. Because different mycotoxins are compared, multiple toxicological thresholds and concepts have to be applied.

Table 8.7 gives an overview of the results of the performed exposure assessment in this study.

Table 8.7: Overview of the results of the performed exposure assessments. For AFB1 and OTA only scenario 2 is given (= EU limits). Values in bold are of concern compared to the most appropriated toxicological value (1 person =70 kg BW).

	Exposure (µg/person.day)		MOE	
	mean	**P95**	**mean**	**P95**
Almond AFB1	0.0034	**0.0273**	17947	**2234**
Groundnut AFB1	0.0004	0.0046	148295	13116
Hazelnut AFB1	0.0020	**0.0482**	30867	**1265**
Pistachio AFB1	0.0013	**0.0586**	46371	**1040**
Figs AFB1	**0.0105**	**0.0425**	**5782**	**1433**
			TDI	
Figs OTA	0.02476	0.02648	0.35	
Raisins OTA	0.03000	0.01047	0.35	
			TTC	
Derived tomato products AOH	**2.66-2.73**	**3.78-4.48**	0.175	
Derived tomato products AME	**2.45-2.87**	**3.43-4.69**	0.175	
Derived tomato products TeA	**296**	**757**	105	

Figure 8.1 gives an overview on the final conclusions of the risk ranking based on the previous table. For OTA we can conclude that there is no pressure for human health

by consuming figs or raisins. For AFB1, the MOE values at mean level are above 10 000 and can be considered to be of low health concern, based on $BDML_{10}$ for humans, except for figs (MOE = 5782, mean). However, for P95 also the MOE values of almonds, hazelnut and pistachios are below 10 000.

In view of the fact that no clear toxicological thresholds are defined for the *Alternaria* toxins, it is difficult to compare the potential impact of their dietary exposure on human health compared to the impact linked to the dietary exposure of aflatoxin B1. However, reported TTCs are exceeding in our calculations for derived tomato products.

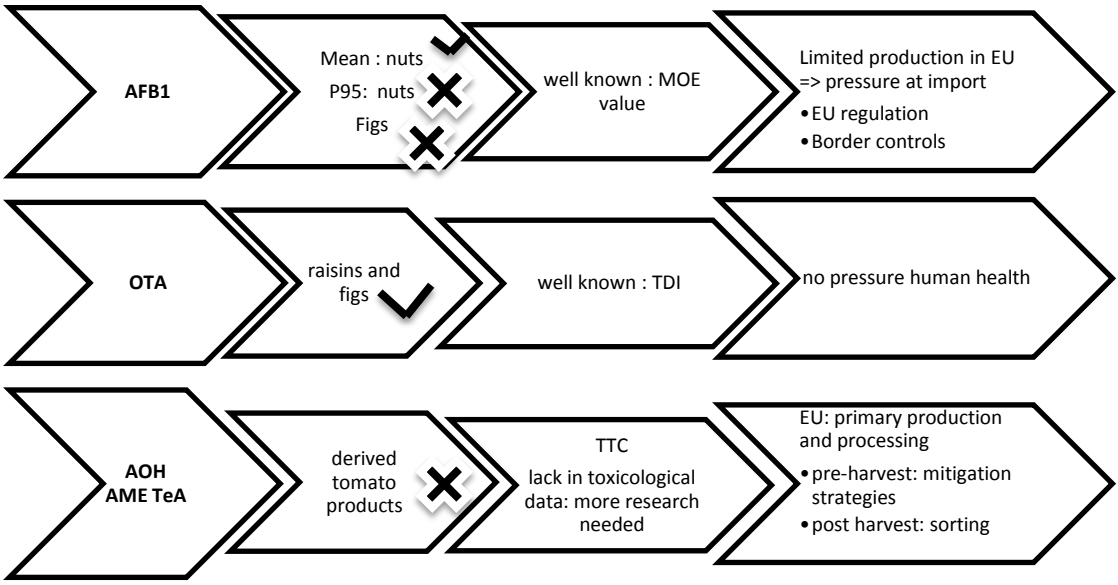

Figure 8.1: Overview of risk ranking results of AFB1, OTA, AOH, AME and TeA; V: below toxicological threshold; X: higher than toxicological threshold

Chapter 9: Conclusions and perspectives

Chapter 9: Conclusions and perspectives

In the presented work, a hazard identification on *Alternaria* mycotoxins in tomatoes and their derived products is conducted (Chapter 2 and 4), followed by further in-depth analysis on *Alternaria* spp. growth and mycotoxins (Chapter 5) and the influence of climate change on the growth of *Alternaria* spp. (Chapter 6). In Chapter 7 and 8, exposure and risk assessment calculations and considerations are made related to the farm to fork exposure of *Alternaria* mycotoxins and established mycotoxins occurring in dries nuts and fruit products. Finally, general conclusions and further perspectives beyond the presented work are discussed in this Chapter 9.

Selection of crops and mycotoxins

In this work it was decided to focus on tomatoes and their derived products because of three reasons. (1) The rather high prevalence of *Alternaria* mycotoxins in tomatoes. Out of the screening conducted in Chapter 2 and 3 it could be concluded that the *Alternaria* mycotoxins seemed to be the major problem compared to OTA and fumonisins in the selected fresh produce. (2) The fact that tomatoes are used frequently in further processing. (3) The high consumption of the derived tomato products. However, as showed in Chapter 2, other fresh produce, such as the soft red fruits, which are also often used for further processing, should also be evaluated on the presence of mycotoxins in the derived products. It was shown that the moulded soft red fruits contained *Alternaria* mycotoxins and patulin. Soft red fruits are highly used in concentrates, juices and jams. Additionally, soft red fruits have a soft and thin epidermis, resulting in a high vulnerability for cracks and wounds and thus for mould growth and mycotoxin production.

In this work, TeA was not further considered, because the EFSA report of 2011 (EFSA, 2011b) considered it as probably not of concern to public health. However, limited screening of samples demonstrated high concentrations also of this mycotoxin and it was again up taken in our final risk ranking.

Modified mycotoxins

From the screening it was concluded that the presence of AOH and AME in derived tomato products should be further evaluated. However, next to AOH and AME, also modified forms of these mycotoxins can be of interest in the tomato products and should be further investigated. This can be done by screening for the conjugated mycotoxins as such or by transforming the conjugated form back to its original mycotoxin (by treating with acid and heat for example). There are two types of conjugates of mycotoxins: masked mycotoxins (soluble conjugates) and bound mycotoxins (insoluble conjugates). The formation of conjugated mycotoxins can be excreted directly by the fungi, although there are not many examples present at the moment (Berthiller et al., 2009). Another source of conjugated mycotoxins are plant conjugates, where the plant converts mycotoxins in a more polar metabolite to protect themselves. A last source is food processing, especially heating or fermentation steps, which can potentially alter mycotoxins. The most important types of conjugates and their characteristic mass shifts can be found in Table 9.1. Rychlik et al. (Rychlik et al., 2014) suggested a new definition of modified and other form of mycotoxins. Modified mycotoxins were divided in free mycotoxins, matrix-associated mycotoxins (complexes and covalently bound) and modified mycotoxins. The modified mycotoxins were further differentiated in biologically modified (conjugated by plants, animals or fungi and functionalized) and chemically modified (thermally and non-thermally formed). In this new definition proposal the term

'modified' describes any modification of the basic chemical structure of the mycotoxins (chemical or biological).

Modified mycotoxins can be less, equal or more toxic then the normal mycotoxins and is important that further studies investigate them. Moreover, it is important to understand what happens with the modified mycotoxins during food processes and digestion in the body. Therefore, it is important to assess the bioavailability and toxic potential of modified mycotoxins (Rychlik et al., 2014).

Table 9.1: The most important types of conjugates and their characteristic mass shifts (based on Berthiller et al. (2009))

Conjugation	Nominal mass shift	AOH + conjugate(H-)	AME+conjugate (H-)
Methylation	14	271	285
Acetylation	42	299	313
S-methylation	46	303	317
Glycine	57	314	328
Sulfatation	80	337	351
Cysteine	119	376	390
N-Acetlycysteine	161	418	432
Glucose	162	419	433
Cysteine-glycine	176	433	447
Glucuronidation	176	433	447
N-acetylglucosamine	203	460	474
Glucose sulphate	242	499	513
γ-glucose-cysteine	248	505	519
Malonyl-glucose	248	505	519
Glucose-xylose	294	551	565
Gluthathione	305	562	576
Glucose-glucose	324	581	595
Acetyl-glucose-glucose	366	623	637
Malonyl-glucose-glucose	410	667	681
Glucose-glucose-glucose	486	743	757

Stability of mycotoxins

As shown in Chapter 4, AOH and AME are very heat stable. This means that preventive measures should be performed on the field and before entering the production line. Once the tomatoes are entering the production line, the mycotoxins are not destroyed/removed. A first mitigation strategy that can be performed is the measures taken on the field. To make the tomatoes less vulnerable it is important that the stress factors are limited. This can be obtained by using the right tomato variety for a certain region. Varieties which are more resistant to moulds and other diseases should be evaluated and used. Also, crop rotation can reduce the mould infestation, which is often not feasible in practice because the growing fields are located close to the processing factories. Another important practice that can decrease the amount of moulds is a good soil and crop management, such as removal of infected crops and the timely application of fungicides. Also the control and sorting of the incoming raw material used for industrial processing of derived tomato products is crucial to limit the mycotoxin exposure as stated by EU recommendation 2003/598/EG to control patulin in apple products. In the self-checking guideline G014 (http://www.gidsac.be/nl/8/8.0) for the fresh produce processing industry in Belgium, the incoming raw materials apples in the production of apple compote is defined as a control point in the companies food safety management system. Validated process criteria are demanded on the initial cut off of moulded apples entering the production plant. This modus operandus could also be applied in the tomato processing industry.

Climate change

Climate change can have an effect on several factors which are influencing the pre-harvest mould development and mycotoxin production as discussed in Chapter 1 and 6. The effect of climate change is very complex, and in this study we only evaluated the influence of temperature. The results show that an increase in temperature could lead to a decrease in mycotoxin concentration, because of the too high temperatures for the moulds to grow and produce mycotoxins. However, too high temperatures can also result in a shift in season growth, because at 35°C and higher the growth of tomatoes might be not possible anymore with the current varieties. The report of EFSA on aflatoxins in grains showed that the harvesting dates were estimated to be 15-20 days earlier. Therefore, more complex studies investigating this shift in season growth should be evaluated. Next to temperature, also the humidity can play an important role in the development of moulds. More humidity will lead to more cracks in the tomatoes, resulting in a higher vulnerability for the contamination by moulds.

Risk assessment

When an exposure assessment study is performed using the screening results obtained from Chapter 2 (concentration of AOH and AME on tomato products in combination with the consumption data of Belgian consumers), a higher exposure is calculated compared with the results of the farm to fork exposure model from Chapter 7. Table 9.2 is summarizing the outcomes of exposure assessment calculations based on the screening results of Chapter 2 and 4 (see also Chapter 8), and also the obtained exposure via the farm to fork calculations presented in Chapter 7.

Table 9.2: Summary of the outcomes of the exposure assessment calculations based on the results from chapter 7 and 8

Emerging mycotoxins	Screening Mean (ng/kg BW.day) LB-UB	Screening P95 (ng/kg BW.day) LB-UB	Model Mean (ng/ kg BW.day)	Model P95 (ng/kg BW. day)	TTC
AOH	38-39*	64-54*	4	10	2.5
AME	41-35*	67-49*	8	10	2.5
TeA 1	4,230	10,820			1,500
TeA 2	2,900	2,900			

The difference between both studies can be explained by the difference in approach. The farm to fork model includes in each step an uncertainty, whereas the screening of the final derived products focuses directly on the concentrations towards which consumers are exposed to. Additionally, the farm to fork model in baseline scenario estimates 2% of infected tomatoes, with a prevalence of up to 1% *Alternaria* moulds. In practice however, it is up to the tomato processing industry to decide the limit of infected tomatoes which are entering the production line. At this moment, no recommendations nor legal documents are available, which can also explain the higher detected concentrations in the screening of tomato products, present on the (global) market. So, most probably a high amount of infected tomatoes are currently processed into derived tomato products. From the scenarios 8 and 9 of the farm to fork model it was clear that the incoming quality of the tomatoes is of upmost importance.

The results of the exposure assessment calculations (Chapter 8) can be applied for the real estimation of risk towards the consumers at this moment, based on the screening of tomato products available on the market while the farm to fork

exposure model can be used in order to evaluate which mitigation strategies are most effective, to create awareness to various stakeholders and to evaluate the impact of different scenarios. Exposure assessments of final products are important to have an insight in the real actual exposure of consumers to the mycotoxins.

Risk ranking: toxicology

For the investigated emerging mycotoxins (AOH, AME and TeA) not many studies are conducted and more information is needed on the toxicological effect in order to evaluate the risk towards consumers concerning *Alternaria* toxins and derived tomato products.

Overall conclusions

The overall objective of the presented work was to establish a farm to fork risk assessment of emerging mycotoxins in fresh produce and derived products in view of the pressure of potential climate change scenarios and increasing import across European borders.

A first objective of this work was gaining insights in the potential presence and characterization of emerging mycotoxins in fresh produce and their derived products. On moulded tomatoes, onions and soft red fruits, *Alternaria* spp. and their associated mycotoxins (AOH, AME and TeA) were detected. AOH, AME and TeA were also found in derived tomato products. Patulin was present on moulded sweet bell peppers, tomatoes and soft red fruits, but not on onions. Patulin was not a problem in derived tomato products. Based on this first screening and characterization it was decided to work further on the tomato case. From the stability experiment during the production process of derived tomato products it could be concluded that AOH

and AME can be considered very heat stable and are contributing to the fact that it is mainly a pre-harvest problem. Preventive measures are necessary to avoid the entrances of high percentages of moulded tomatoes in tomato processing industries.

A second objective of the presented work was the evaluation of pre-harvest conditions (temperature and copper concentration) on mould growth and mycotoxin production during tomato cultivation. Models were developed for lag phase and maximum growth rate. All strains showed a short lag phase, even at lower temperatures (2-107h). There was no effect of Cu-fungicides on the growth rate or mycotoxin production. The optimum growth temperature varied from 24-33°C (0.21-0.29 mm/h). Regarding the mycotoxin production it was not possible to develop a quantitative model to describe mycotoxin production as function of temperature and time due to the high variability between the replicates. The optimal production of AOH and AME was reached at 25-30 °C at a mould diameter of 45 mm. At lower temperatures, the production of the mycotoxins was rather limited. At a mould diameter of 25 mm there was almost no production of AOH and AME. This is an important criterium which can be used during processing of tomatoes. The risk of mycotoxin contamination can be reduced by sorting out the tomatoes and remove tomatoes with a mould diameter of 45 mm before the production of derived tomato products. The influence of climate change on mould growth was evaluated for two productions regions: Spain and Poland. In Spain, for the more extreme climate change scenarios, the temperatures are becoming too high for fungal growth. For Poland, the diameter of the mould was for the far future>near future>current time frame. This is due to the predicted higher temperatures in the far future (14-28°C) which becomes closer to the optimal temperature for the growth of *Alternaria* spp. compared with the colder temperatures in the present. According to the results, the

situation in Poland in the far future (2081-2100) will became similar as the situation in Spain in the present time frame (1981-2000).

The third objective of the presented study was a risk assessment calculation on the emerging mycotoxins and their discussion in view of established mycotoxins in plant products. For the emerging mycotoxins a farm to fork model was developed and different scenarios were evaluated. The different climate change scenarios showed that with an increase in temperature the exposure can be lower due to a too high temperature for the growth of *Alternaria* moulds.

Finally, the risk of established mycotoxins occurring in dried fruits and nuts were compared with the emerging mycotoxins in derived tomato products. Because different mycotoxins are considered, multiple toxicological thresholds and concepts had to be applied. For OTA it could be concluded that there is no pressure for human health by consuming figs or raisins. The mean exposure to AFB1 by consuming nuts can be considered to be of low health concern. The *Alternaria* mycotoxins in derived tomato products are exceeding the set TTC-value both for mean and P95. More information on toxicology is thus needed to evaluate the exposure to AOH and AME. The presented work established a farm to fork risk assessment for *Alternaria* mycotoxins in tomatoes and derived tomato products and also included climate change scenarios in the risk assessment.

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Supplementary material

Lag phase - Primary model

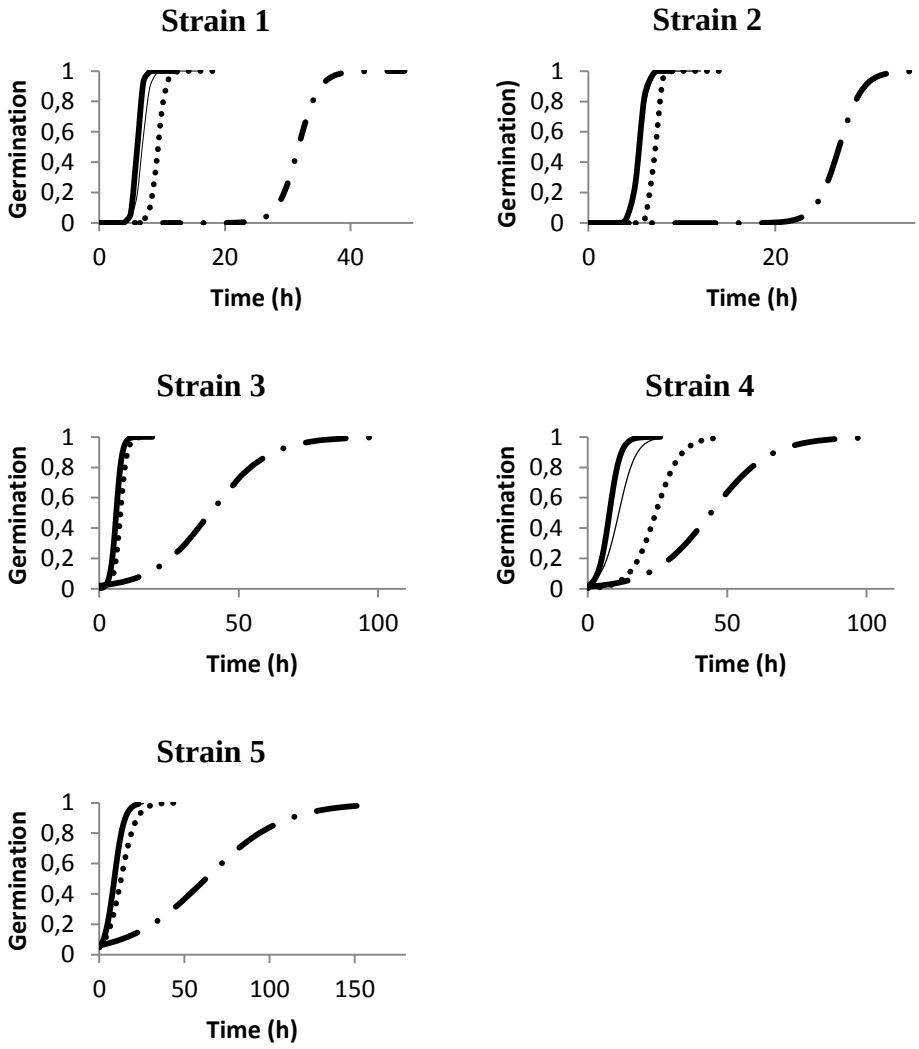

Figure S.1: Primary modelling of *Alternaria* spp (five strains) of the germinated spores (1=100% germinated) as function of time (h) for 5 °C (._), 15 °C (..), 25 °C (_)and 30 °C (▬)

Lag phase - Secondary model

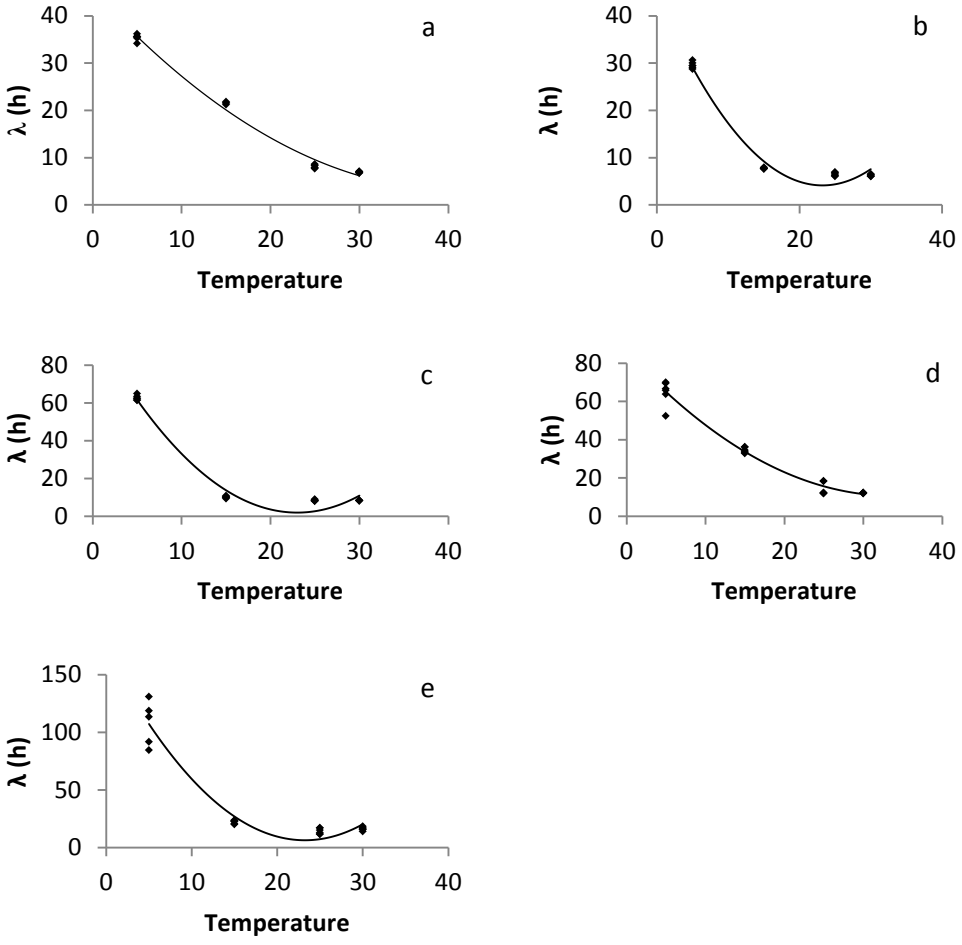

Figure S.2: λ (h) versus temperature (°C) for strain 1 (a), strain 2 (b), strain 3 (c), strain 4 (d) and strain 5 (e). -= Fitted polynomial model, ♦= observed data points

Effect of fungicide concentration on μ_{max}

Strain	Temperature	Fungicide concentration	Fungicide concentration	Significant difference between fungicide concentrations at same temperature (+= significant different)
1	5	0	0,3	
		0	1,5	+
		0	3	+
		0,3	1,5	+
		0,3	3	+
		1,5	3	
	15	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	25	0	0,3	
		0	1,5	+
		0	3	
		0,3	1,5	+
		0,3	3	
		1,5	3	+
	30	0	0,3	+
		0	1,5	
		0	3	+
		0,3	1,5	+
		0,3	3	
		1,5	3	

Strain	Temperature	Fungicide concentration	Fungicide concentration	Significant difference between fungicide concentrations at same temperature (+= significant different)
2	5	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	15	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	+
		0,3	3	+
		1,5	3	
	25	0	0,3	+
		0	1,5	+
		0	3	+
		0,3	1,5	
		0,3	3	
		1,5	3	
	30	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	

Strain	Temperature	Fungicide concentration	Fungicide concentration	Significant difference between fungicide concentrations at same temperature (+= significant different)
3	5	0	0,3	+
		0	1,5	+
		0	3	
		0,3	1,5	+
		0,3	3	+
		1,5	3	+
	15	0	0,3	
		0	1,5	+
		0	3	+
		0,3	1,5	
		0,3	3	+
		1,5	3	
	25	0	0,3	+
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	+
		1,5	3	
	30	0	0,3	+
		0	1,5	+
		0	3	
		0,3	1,5	
		0,3	3	+
		1,5	3	+

Strain	Temperature	Fungicide concentration	Fungicide concentration	Significant difference between fungicide concentrations at same temperature (+= significant different)
4	5	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	15	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	25	0	0,3	
		0	1,5	+
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	30	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	

Strain	Temperature	Fungicide concentration	Fungicide concentration	Significant difference between fungicide concentrations at same temperature (+= significant different)
5	5	0	0,3	+
		0	1,5	+
		0	3	+
		0,3	1,5	
		0,3	3	
		1,5	3	
	15	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	25	0	0,3	
		0	1,5	
		0	3	
		0,3	1,5	
		0,3	3	
		1,5	3	
	30	0	0,3	+
		0	1,5	+
		0	3	+
		0,3	1,5	
		0,3	3	
		1,5	3	

Growth rate -Secondary model

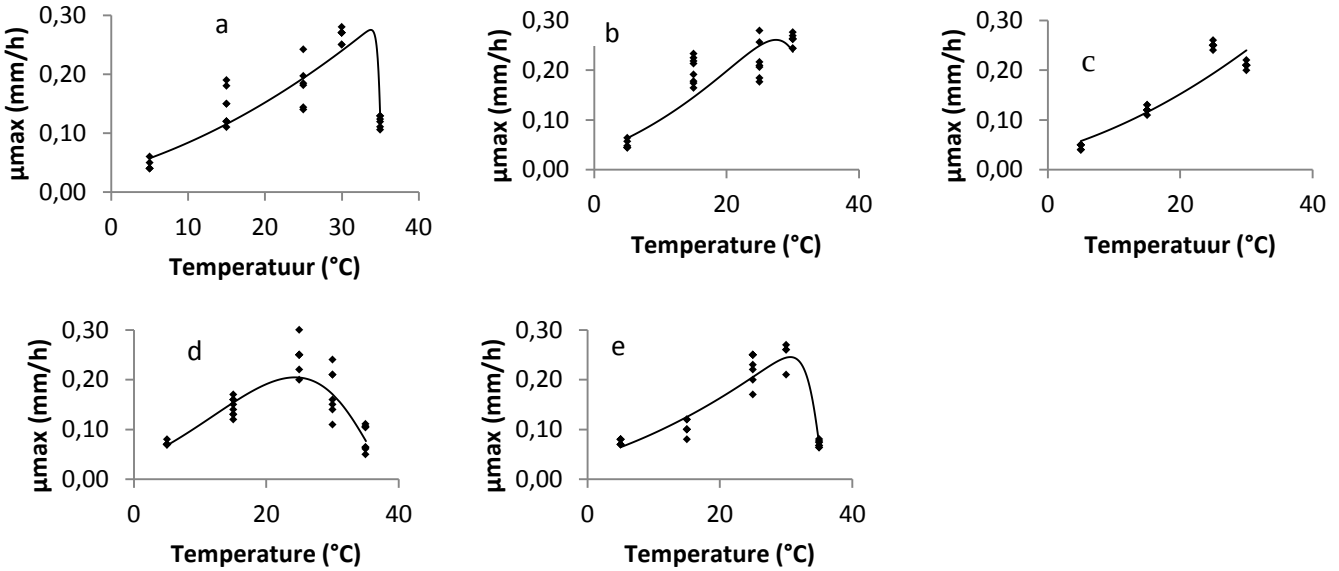

Figure S.3: μ_{max} (mm/h) versus temperature (°C) for strain 1 (a), strain 2 (b), strain 3 (c), strain 4 (d) and strain 5 (e). - = Ratkowsky model, ♦= observed data points

Mycotoxins Alternariol monomethyl ether (AME)

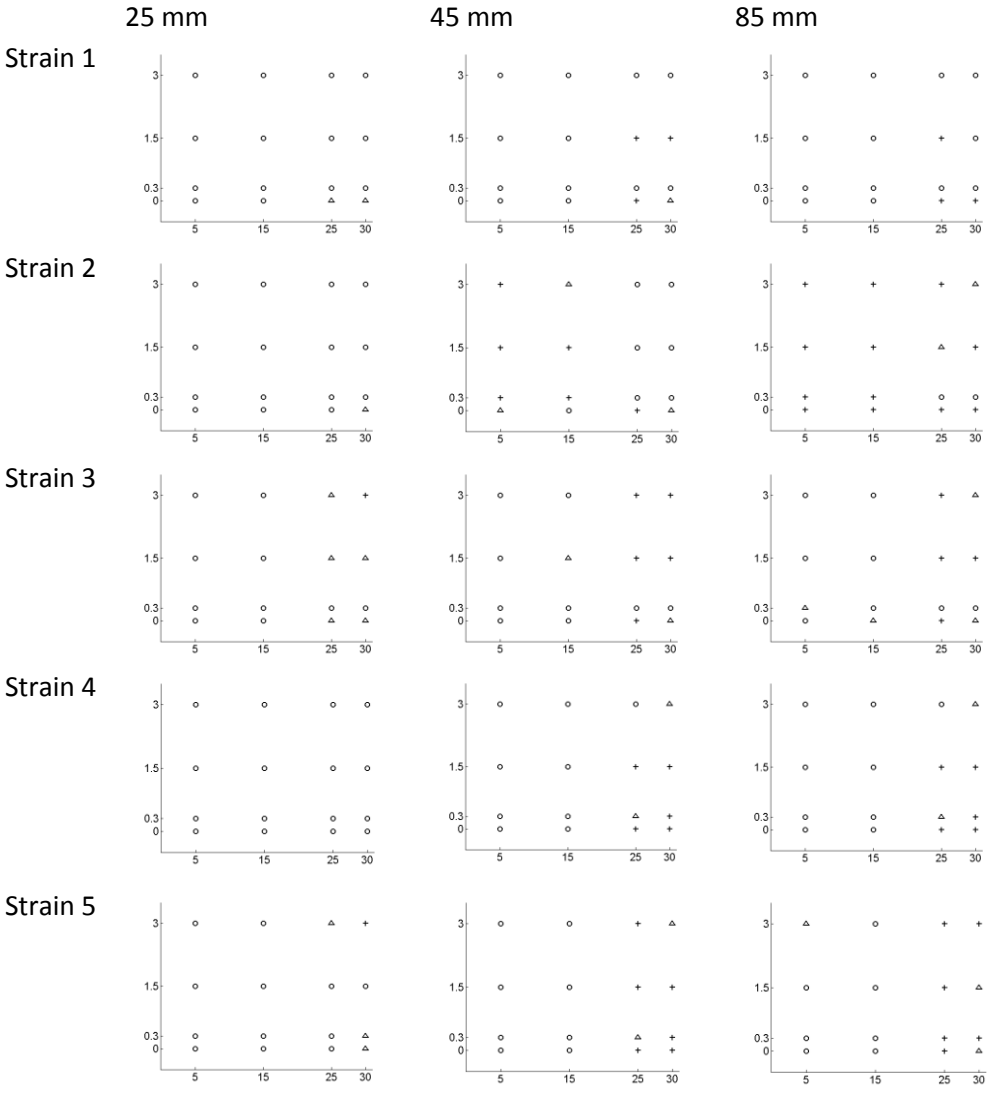

Figure S.4: Results of the mycotoxin production AME at different mold mycelium diameters for the different temperatures (x-axis, °C) and fungicide concentration (y-axis, mg Cu/15mL agar), n=2 agar plates of simulant, ○= 0/2 positive for mycotoxins, Δ= 1/2 positive, + = 2/2 positive (LOD =8.1 µg/kg)

Curriculum vitae

Evelien Van de Perre was born on 1st of January, 1987 in Asse, Belgium. In 2005, she started the studies Bio-science engineering and in 2010 she graduated in Bio-science engineering: Food technology and nutrition. Since August 2010, she has been working on her PhD project entitled “Farm to fork exposure assessment of mycotoxins in derived tomato products” with the support the European Community’s Seventh Framework Programme (FP7) under grant agreement no. 244994 (project VEG-i-TRADE). The research has been carried out in the NutriFOODchem laboratory, Department of Food Safety and Food Quality (Faculty of Bioscience Engineering, Ghent University) under the guidance of Prof. Bruno De Meulenaer, Prof. Frank Devlieghere and Prof. Liesbeth Jacxsens. The results of this research project were published in a number of peer-reviewed scientific journals and presented during several national and international symposia. During this period, she also supervised number of students for the fulfillment of their Master thesis and internships and has given lectures to Master students regarding mycotoxins.

List of publications

Papers in internationally distributed journals with peer review

Kerkaert, B., Jacxsens, L., Van de Perre, E., & De Meulenaer, B. (2012). Use of lysozyme as an indicator of protein cross-contact in fresh-cut vegetables via wash waters. *Food Research International, 45*(1), 39-44.

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Van de Perre, E., Jacxsens, L., Deschuyfelleer, N., Lécuyer, S., Coen, A., Molina, B. G., Gianaria, A., Devlieghere, F., De Meulenaer, B., & Vermeulen, A. Effect of temperature and fungicide concentration on the growth of *Alternaria* spp. and production of their mycotoxins (alternariol and alternariol monomethyl) on tomato agar and fresh tomatoes. *Under revision*

Van de Perre, E., Jacxsens, L., Haesaert, I., Gianaria, A., & De Meulenaer, B. (2014). Evaluation of the stability of alternariol and alternariol monomethyl ether throughout the production of derived tomato products. S*ubmitted*

Conferences: presentations and posters

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Integrated risk assessment of selected mycotoxins in fresh produce and derived food products throughout the food chain, affected by climate changes and globalization. Poster presentation 6th World Mycotoxin Forum, 2010, Noordwijkerhout, The Netherlands

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Integrated risk assessment of selected mycotoxins in fresh produce and derived food products throughout the food chain, affected by climate changes and globalization. Poster presentation 16th Symposium Applied Biological Sciences, 2010, Ghent, Belgium

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Integrated risk assessment of selected mycotoxins in fresh produce and derived food products throughout the food chain, affected by climate changes and globalization. Poster presentation MS in food and feed, 2011, Merelbeke, Belgium

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Integrated risk assessment of selected mycotoxins in fresh produce and derived food products throughout the food chain, affected by climate changes and globalization. Poster presentation 4th International Symposium Mycotoxins: Challenges and Perspectives, 2011, Ghent, Belgium

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Screening of mycotoxins in tomatoes, onions, bell peppers, soft red fruits and derived tomato products with LC-

TOF-MS. Oral presentation 34th Mycotoxin Workshop, 2012, Braunschweig, Germany

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Sreening of mycotoxins in tomatoes, onions, bell peppers, soft red fruits and derived tomato products with LC-TOF-MS. Poster presentation 7th World Mycotoxin Forum and XIIIth International IUPAC Symposium on Mycotoxins & Phycotoxins, 2012, Rotterdam, The Netherlands

Van de Perre, E., Jacxsens L. & De Meulenaer, B. Screening of mycotoxins in tomatoes, onions, bell peppers, soft red fruits and derived tomato products with LC-TOF-MS. Poster 18th Symposium Applied Biological Sciences, 2013, Leuven, Belgium

Van de Perre, E., Jacxsens, L., Deschuyffeleer, N., Vermeulen, A., Devlieghere, F., De Meulenaer, B. Modelling the growth of *Alternaria* spp. on tomatoes used for derived tomato products as function of temperature and fungicide concentration. Poster 2014 European Symposium on Food Safety, IAFP, 2014 Budapest, Hungary

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“We learn by practice. Whether it means to learn to dance by practicing dancing or to learn to live by practicing living, the principles are the same." – Martha Graham

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