





Le doute est désagréable, mais la certitude est ridicule  
Voltaire

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**An integrated risk assessment of patulin in apple juices  
throughout the food chain**

Thesis submitted in fulfillment of the requirements for the degree of Doctor  
(Ph.D.) in Applied Biological Sciences: chemistry

# Een geïntegreerde risicobeoordeling van patuline in appelsappen doorheen de voedselketen

Illustratie: Jonagored © Katleen Baert  
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## Objectives

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# Objectives

The availability of safe food is an essential prerequisite for public health. In this respect, risk reduction is a crucial element in food safety management today, although a complete elimination of risk in the food chain is an impossible goal. In order to evaluate which measures are necessary and efficient, a risk assessment needs to be performed.

Mycotoxins are an important hazard occurring in the food chain. Although there are geographic and climatic differences in the production and occurrence of mycotoxins, exposure to these substances is worldwide and contaminates the world food supply to some extent. Within the risk assessment framework, mycotoxins have a special position. Mycotoxins are chemical substances that have a microbiological history. In the steps hazard identification, hazard characterisation and risk characterisation mycotoxins need to be handled as chemical hazards with the distinction between threshold effects (e.g. patulin) and no threshold effect (e.g. aflatoxin). However in the exposure assessment, mycotoxins can be handled as solely a chemical hazard or as a chemical hazard with a microbiological history using a farm to fork approach. To our knowledge, exposure assessment of mycotoxins has only focused on mycotoxins as a chemical hazard without including the microbiological history. A farm to fork risk assessment has however the advantage that different measures can be tested to reduce the risk.

The main goal of this PhD was to evaluate the risk of patulin for children consuming apple juice and if necessary to evaluate different strategies to reduce the risk using a farm to fork approach. Children were selected as a specific population group and apple juice was selected as a specific matrix, since a Scientific Cooperation study has shown that children are most exposed to patulin through the consumption of apple juice (Directorate – General Health and Consumer Protection, 2002). In order to achieve this main goal, different objectives can be defined. The outline of this work is schematically presented in Figure 0.1.

A first objective of this work was to implement and validate a method for the analysis of patulin in apple products (**Chapter 2**). This method was used to determine the occurrence of patulin in apple juices marketed in Belgium (**Chapter 3**). Due to the increased popularity of organic food products and the possibility that these products are more contaminated with

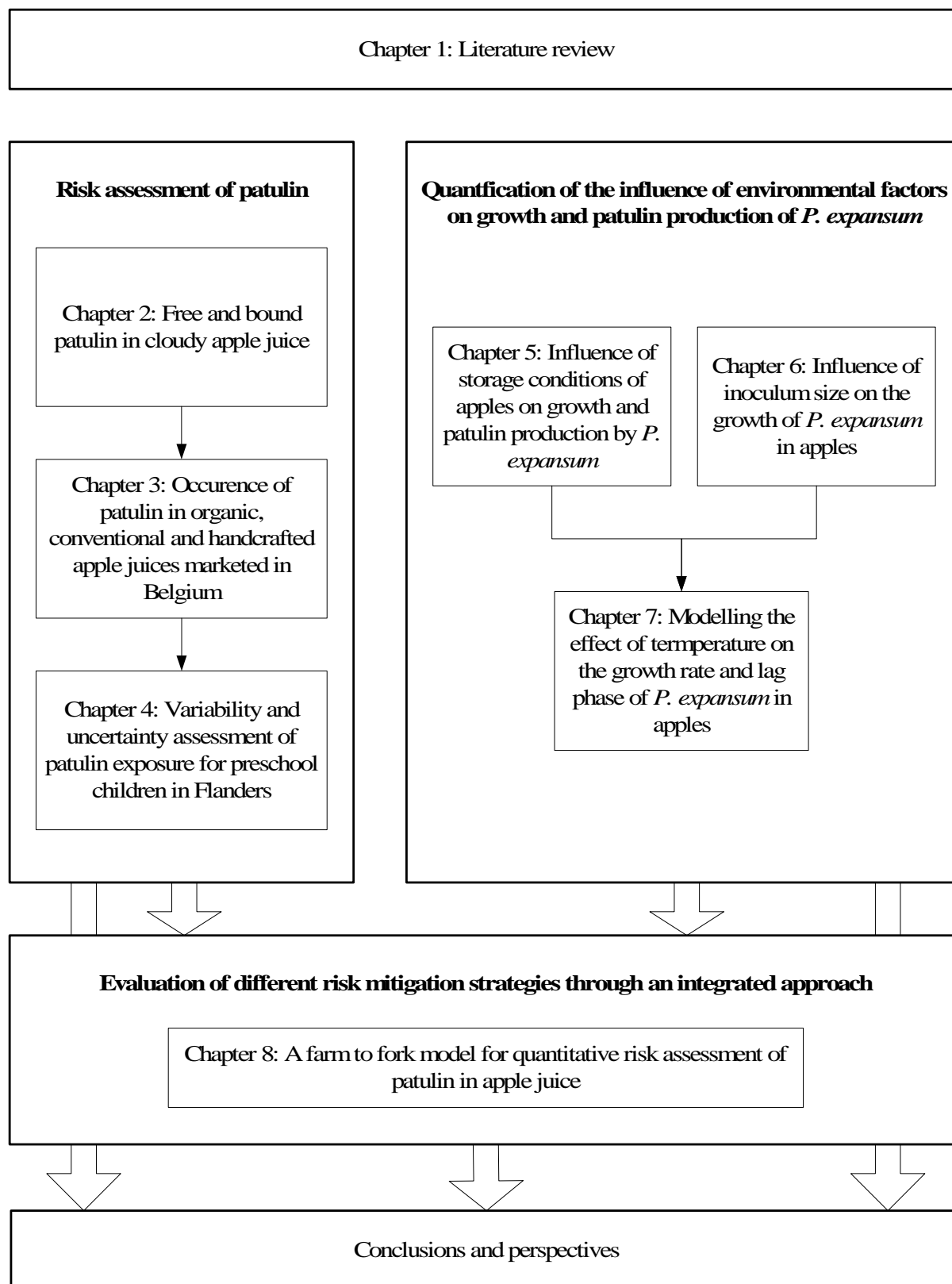
mycotoxins, an important question to be answered was: “Is organic apple juice more contaminated than conventional?” It was also the aim to evaluate whether people buying cheaper brands of apple juice are more at risk.

Once a complete dataset of contamination data was collected, it was the objective to evaluate whether the observed concentrations can cause adverse health effects through a probabilistic exposure assessment (**Chapter 4**).

Since the exposure assessment of patulin had revealed that a reduction of the contamination below 25 µg/kg is important for protecting public health, it was the objective to evaluate different risk mitigation strategies using a farm to fork risk assessment model. Therefore, the next objective was to study the influence of the inoculation level, the storage parameters (temperature and oxygen level) and strain variability on the growth parameters (lag phase and growth rate) of the patulin producing mould *Penicillium expansum* (**Chapters 5 and 6**). To incorporate the growth data of *P. expansum* in the farm to fork risk assessment model, it was the objective to develop and validate different models that predict the growth of *P. expansum* in apples (**Chapter 7**).

Also patulin production needs to be incorporated in such a farm to fork risk assessment model and therefore, the relationship between the patulin production and the growth of the mould was studied as a function of the storage conditions, temperature and oxygen level (**Chapter 5**).

The final objective of this work was to incorporate the gathered data into a farm to fork risk assessment model that allowed the evaluation of risk mitigation strategies (**Chapter 8**).



**Figure 0.1: Outline of this PhD**



## Summary

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## Summary

The first part of **Chapter 1** gives an introduction in risk assessment and the position of risk assessment within the risk analysis framework. The difference between chemical and microbiological risk assessment is discussed more in detail. It is described how risk assessment outputs are used in risk management to reduce the risk and to protect public health. The first part of this chapter ends with a brief presentation of quantitative techniques used for risk assessment. In a second part of the literature review a concise overview is given on the mycotoxin patulin with special thought for the properties of this molecule, the occurrence and the toxicity.

**Chapter 2** describes the validation of an HPLC-UV method for patulin detection. During the validation, a time dependent recovery of patulin in cloudy apple juice was observed. Recoveries were up to 20% lower on day 3 compared to day 1 following spiking on day 0. This reduction was caused by an interaction between patulin and the solid particles of cloudy apple juice and most probably with the proteins in the solid particles. Up to 20% of the present patulin is bound and not detected during HPLC-UV analysis, which can lead to an underestimation in risk assessment.

In **Chapter 3** the occurrence of patulin in organic, conventional and handcrafted apple juices marketed in Belgium was studied. In total 177 apple juices were analyzed, of which 65 juices were organic, 90 conventional and 22 handcrafted. Patulin was detected in 22 apple juices (12%) and quantification was possible in 10 (6%) of them. The patulin content exceeded the European maximum limit of 50 µg/kg in two organic apple juices. Although, the incidence of patulin in organic apple juice (12%), conventional apple juice (13%) and handcrafted apple juice (10%) was not significantly different ( $p = 0.863$ ), the mean level of patulin in contaminated samples was significantly higher in organic apple juice (43.1 µg/kg) than in conventional (10.2 µg/kg) ( $p = 0.02$ ) and handcrafted (10.5 µg/kg) ( $p = 0.037$ ). Interestingly, it was observed that the highest patulin concentrations were determined for the most expensive apple juices. This can be attributed to the higher price of organic apple juice, since this relation was not observed when only conventional apple juices were included.

**Chapter 4** evaluated the patulin exposure of children consuming organic, handcrafted or conventional apple juice through a probabilistic approach and the effectiveness of several risk management options aiming to reduce the patulin exposure. Different methods were tested to deal with the left censored data (patulin concentrations below the limit of detection (LOD)) and a uniform distribution with uncertain bounds was selected to handle this censorship. Variability and uncertainty assessment of patulin exposure showed that 0.9% [90% confidence interval (CI): 0.3-1.8%] of the children consuming organic apple juice, exceeded the tolerable daily intake (TDI) for patulin. For consumers of conventional and handcrafted apple juice, this was respectively 0.1% [90% CI: 0-0.3%] and 0% [90% CI: 0-0.2%]. Rejection of apples juices containing more patulin than 25 µg/kg reduced the percentage of the children whose patulin intake exceeds the TDI to 0% [90%CI: 0-0.2%]. Reduction of the apple juice consumption was less effective than the application of a more stringent maximum level than the current one (25 µg/kg instead of 50) and was only useful when the patulin concentration of apple juice was below 25 µg/kg.

**Chapter 5** studied the influence of storage conditions of apples (i.e. temperature and O<sub>2</sub> level) on growth and patulin production by different *P. expansum* strains on a simulation medium and on apples. Growth was strongly influenced by the temperature, while the used atmosphere (20, 3, and 1% O<sub>2</sub>; <1% CO<sub>2</sub>) had no effect. Patulin production was stimulated when the temperature decreased (from 20 to 10 or 4°C), while a further decrease of the temperature to 1°C caused a reduction in patulin production. The temperature at which the stimulation changed into suppression was strain dependent. Similar results were observed for the O<sub>2</sub> level. A reduction of the O<sub>2</sub> level from 20 to 3% O<sub>2</sub> could stimulate or suppress patulin production depending on the strain, while a clear decrease of the patulin production was observed when the O<sub>2</sub> level was reduced from 3 to 1%. These results show that a limited stress to the fungus, such as lowering the temperature or lowering the O<sub>2</sub> levels, stimulates patulin production. However, the combination of different stress conditions (e.g. low temperature and low O<sub>2</sub>) will result in a reduced formation of the toxin. The combination of stress conditions, at which the transition from stimulation to suppression is observed, is strain dependent. Moreover, patulin production is characterized by a high natural variability. The presented results show that the temperature and O<sub>2</sub> level has to be as low as possible during the storage of apples in order to suppress patulin production and to guarantee food safety.

**Chapter 6** investigated the effect of the inoculum size on the germination and growth parameters of *P. expansum* under different storage conditions in apples. Growth of *P. expansum* was observed in more than 90% of the inoculations, when the inoculum was equal or higher than  $2 \cdot 10^4$  spores. The use of a low inoculum level resulted in longer lag phases and a larger variability of the estimated lag phase, indicating that more replications are necessary to have a representative idea on the growth of the mould in the specified circumstances. At lower temperature, more inoculum was necessary to reduce the variability of the estimated lag phase, showing that this effect is temperature dependent. Moreover, the effect of the inoculum level on the lag phase is even more pronounced for a slower growing strain.

**Chapter 7** reports on the development of validated models describing the effect of storage temperature on the growth rate and lag phase of six *Penicillium expansum* strains. The growth of the selected strains was therefore studied on a simulation medium (apple puree agar medium (APAM)) at 30, 25, 16, 10, 4 and 2°C. Model validation was performed in two steps, using external, independent data. Firstly, the developed models were validated on APAM for the six strains at three intermediate temperatures (20, 12 and 7°C) and secondly on apples at 25, 20, 12, 7, 4 and 2°C for three strains. Growth rates and lag phases were estimated using linear regression. Several secondary models were evaluated on their ability to describe the change in growth rate and lag phase as a function of temperature. For the growth rate, a modification of the extended Ratkowsky model was selected, as this model was flexible enough to adequately describe the evolution of the growth rate for the complete temperature range (2-30°C). Regarding the lag phase, the Arrhenius-Davey model provided the best adjustment to the observed data. The obtained Bias factors ( $B_f$ ) ranged from 0.91 to 1.14 and the accuracy factors ( $A_f$ ) were  $<1.2$  for the validation performed on APAM, indicating that the models were good predictors of the true mean colony growth rate and lag phase. Afterwards, an external validation was carried out in apples. For the growth rate,  $B_f$  ranged from 0.64 to 0.81 and  $A_f < 1.39$ , indicating conservative predictions. On the contrary for the lag phase, a clear deviation was observed between predictions and observed values on apples ( $0.35 < B_f < 0.7$  and  $A_f > 1.6$ ). This indicates that the food matrix has a large influence on the adaptation of the spores to the matrix, but once the spores have germinated, growth occurs at a similar rate on both APAM and apples. These results highlight that the use of simulation or synthetic media for the development of predictive models for the lag phase of moulds can lead to inadequate predictions and that a validation on the real food matrix is necessary.

**Chapter 8** describes the development of a farm to fork risk assessment model that was used to evaluate different strategies to mitigate patulin contamination. The elaborated model described the complete chain from the picking of apples until storage of produced apple juice. Validation of the elaborated model showed that the simulated mean patulin concentration (6.8 and 3.6  $\mu\text{g}/\text{kg}$  for cloudy and clear apple juice, respectively) was in accordance with the mean patulin concentration detected in apple juice in Belgium (6.7 and 4.5  $\mu\text{g}/\text{kg}$  for cloudy and clear apple juice, respectively). However, an overestimation was observed for the higher concentrations (95<sup>th</sup> percentile). Different scenarios were evaluated in order to reduce the patulin contamination of apple juice. It was demonstrated that the concentration of patulin in apple juice can be reduced when less apples are used that were stored in controlled atmosphere. However, when apples are used that were stored in controlled atmosphere, a sorting step should be included to remove apples with an infection spot larger than 10  $\text{cm}^2$ . This measure reduces the patulin contamination in 99.7 to 99.9% of the apple juices, to levels below 25  $\mu\text{g}/\text{kg}$ . Another critical factor is the duration of deck storage (storage outside at ambient temperature) before the apples are processed.

At the end of this PhD thesis, **conclusions** of the performed research together with new **perspectives** are discussed.

## Samenvatting

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# Samenvatting

Het eerste onderdeel van **Hoofdstuk 1** geeft een inleiding tot risicobeoordeling en de plaats van risicobeoordeling binnen risicoanalyse. Het verschil tussen chemische en microbiologische risicobeoordeling wordt in detail behandeld. Er wordt beschreven hoe de resultaten van de risicobeoordeling worden gebruikt binnen het risicomanagement met als doel het risico te beperken en de volksgezondheid te beschermen. Het eerste deel van dit hoofdstuk wordt afgesloten met een korte beschrijving van kwantitatieve technieken die gebruikt worden voor risicobeoordeling. In het tweede deel van Hoofdstuk 1 wordt een beknopte bespreking gegeven van het mycotoxine patuline met bijzondere aandacht voor de eigenschappen van deze molecuule, het voorkomen en de toxiciteit.

**Hoofdstuk 2** beschrijft de validatie van een HPLC-UV methode voor patuline-analyse. Tijdens deze validatie werd een tijdsafhankelijke terugvinding (recovery) van patuline in troebel appelsap waargenomen. De terugvinding was tot 20% lager op dag 3 in vergelijking met dag 1 na dopperen op dag 0. Deze reductie werd veroorzaakt door de interactie tussen patuline en de vaste deeltjes van troebel appelsap en meer dan waarschijnlijk met de eiwitten van de vaste deeltjes. Bijgevolg is tot 20% van het aanwezige patuline gebonden en wordt niet gedetecteerd via HPLC-UV. Dit kan mogelijk leiden tot onderschattingen bij risicobeoordelingen.

In **Hoofdstuk 3** werd het voorkomen van patuline in biologisch, conventioneel en ambachtelijk appelsap onderzocht. In totaal werden 177 appelsappen geanalyseerd, waarvan 65 biologisch, 90 conventioneel en 22 ambachtelijk. Patuline werd gedetecteerd in 22 appelsappen (12%), waarbij in 10 stalen (6%) kwantificatie mogelijk was. Het patulinegehalte overschreed het Europees maximumgehalte van 50 µg/kg in twee biologische appelsappen. Alhoewel, het voorkomen van patuline in biologische (12%), conventionele (13%) en ambachtelijke appelsappen (10%) niet significant verschillend was ( $p = 0.863$ ), was het gemiddelde patulinegehalte in gecontamineerd appelsap significant hoger in biologisch appelsap (43.1 µg/kg) in vergelijking met conventioneel (10.2 µg/kg) ( $p = 0.02$ ) en ambachtelijk (10.5 µg/kg) ( $p = 0.037$ ). Er werd waargenomen dat de hoogste patulineconcentraties werden teruggevonden voor de duurste appelsappen. Dit kon worden

toegeschreven aan de hogere kostprijs van biologisch appelsap, aangezien deze relatie niet werd waargenomen wanneer enkel conventioneel appelsap werd bestudeerd.

**Hoofdstuk 4** evalueerde de patulineblootstelling van kinderen die biologisch, conventioneel of ambachtelijk appelsap consumeren via een probabilistische blootstellingsschatting en de efficiëntie van verschillende risicomanagementmaatregelen met het doel de patulineblootstelling te reduceren. Verschillende methodes werden getest om met concentraties lager dan de detectielimiet om te gaan. Een uniforme distributie met onzekerheidsbanden werd geselecteerd als beste methode aangezien het de beste weergave was van de realiteit. De risicobeoordeling van patuline toonde aan dat 0.9% [90% betrouwbaarheidsinterval: 0.3-1.8%] van de kinderen die biologisch appelsap consumeren, de tolereerbare dagelijkse inname (TDI) voor patuline overschrijdt. Voor consumenten van conventioneel en ambachtelijk appelsap was dit respectievelijk 0.1% [90% betrouwbaarheidsinterval: 0-0.3%] en 0% [90% betrouwbaarheidsinterval: 0-0.2%]. Het weren van appelsap met concentraties hoger dan 25 µg/kg reduceerde het percentage kinderen waarvan de patuline-inname de TDI overschreed tot 0% [90% betrouwbaarheidsinterval: 0-0.2%]. Een beperking van de appelsapconsumptie was minder efficiënt in vergelijking met het toepassen van een lager maximumgehalte (25 µg/kg in plaats van 50) en was enkel nuttig indien de patulineconcentratie van appelsap lager was dan 25 µg/kg.

In **Hoofdstuk 5** werd de invloed geanalyseerd van opslagcondities van appels (namelijk temperatuur en O<sub>2</sub>-gehalte) op de groei en patulineproductie van verschillende *Penicillium expansum* stammen op simulatiemedium en appels. De groei werd sterk beïnvloed door de temperatuur, terwijl de gebruikte atmosfeer (20, 3, and 1% O<sub>2</sub>; <1% CO<sub>2</sub>) geen effect had. Patulineproductie werd gestimuleerd wanneer de temperatuur afnam (van 20 tot 10 of 4°C), terwijl een verdere afname van de temperatuur tot 1°C een reductie van de patulineproductie veroorzaakte. De temperatuur waarbij de stimulatie overgaat in een onderdrukking is stamafhankelijk. Gelijkaardige resultaten werden geobserveerd voor het O<sub>2</sub>-gehalte. Een reductie van het O<sub>2</sub>-gehalte van 20 tot 3% O<sub>2</sub> stimuleerde of onderdrukte de patulineproductie afhankelijk van de onderzochte stam. Er werd echter een duidelijke afname van de patulineproductie waargenomen wanneer het O<sub>2</sub>-gehalte werd gereduceerd van 3 tot 1%. Deze resultaten tonen aan dat de aanwezigheid van beperkte stress bij de schimmel, zoals het verlagen van de temperatuur of het verlagen van het O<sub>2</sub>-gehalte, de patulineproductie stimuleert. Een combinatie van verschillende stresscondities (bv. lage temperatuur en laag O<sub>2</sub>-



gehalte) zal echter leiden tot een reductie van toxinevormig. De combinatie van stresscondities, waarbij de stimulatie overgaat in onderdrukking, is stamafhankelijk. Bovendien wordt de patulineproductie gekenmerkt door een grote natuurlijke variabiliteit. De voorgestelde resultaten tonen aan dat de temperatuur en het O<sub>2</sub>-gehalte zo laag mogelijk dienen te zijn, zodanig dat de patulineproductie onderdrukt wordt en de voedselveiligheid gegarandeerd wordt.

**Hoofdstuk 6** beschrijft het effect van de inoculumgrootte op de kieming en de groeiparameters van *P. expansum* onder verschillende opslagcondities van appels. Groei van *P. expansum* werd waargenomen in meer dan 90% van de inoculaties wanneer het inoculum groter was dan  $2 \cdot 10^4$  sporen. Het gebruik van een klein inoculum resulteert in langere lagfases en een grotere variabiliteit van de geschatte lagfase, wat aantoont dat meer herhalingen nodig zijn om een representatieve waarde te verkrijgen. Verder onderzoek heeft aangetoond dat dit effect temperatuursafhankelijk is, waarbij meer inoculum noodzakelijk is om de variabiliteit van de geschatte lagfase te reduceren bij lagere temperatuur. Bovendien, is het effect van de inoculumgrootte op de lagfase meer uitgesproken voor trager groeiende stammen.

**Hoofdstuk 7** rapporteert de ontwikkeling van gevalideerde modellen die het effect van de opslagtemperatuur op de groeisnelheid en de lagfase van zes *P. expansum* stammen beschrijven. De groei van de geselecteerde stammen werd daartoe bestudeerd op simulatiemedium (appel puree agar medium (APAM)) bij 30, 25, 16, 10, 4 en 2°C. Modelvalidatie werd uitgevoerd in twee stappen aan de hand van externe, onafhankelijke data. Eerst werden de ontwikkelde modellen gevalideerd op APAM voor de zes stammen bij drie intermediaire temperaturen (20, 12 and 7°C) en vervolgens op appels bij 25, 20, 12, 7, 4 en 2°C voor drie stammen. Groeisnelheden en lagfases werden geschat via lineaire regressie. Verschillende secundaire modellen werden geëvalueerd op hun mogelijkheid om de veranderingen in de groeisnelheid en de lagfase in functie van de temperatuur te beschrijven. Voor de groeisnelheid werd een modificatie van het uitgebreid Ratkowsky model geselecteerd, aangezien dit model voldoende flexibel was om de verandering van de groeisnelheid voor het volledige temperatuurbereik (2-30°C) te beschrijven. Met betrekking tot de lagfase, gaf het Arrhenius-Davey model de beste aanpassing tot de waargenomen data. De bekomen biasfactoren ( $B_f$ ) hadden een waarde tussen 0.91 en 1.14 en de accuraatheidsfactoren ( $A_f$ ) waren  $<1.2$ , voor de validatie uitgevoerd op APAM. Dit geeft aan dat de modellen goede voorspellers zijn van de werkelijke gemiddelde groeisnelheid en

lagfase. Naderhand werd een externe validatie uitgevoerd op appels. Voor de groeisnelheid werd een  $B_f$  bekomen tussen 0.64 en 0.81 en  $A_f < 1.39$ . Dit toont aan dat conservatieve voorspellingen bekomen werden. Voor de lagfase werd in tegenstelling tot de groeisnelheid een duidelijke afwijking waargenomen tussen de voorspellingen en de waargenomen waarden in appels ( $0.35 < B_f < 0.7$  en  $A_f > 1.6$ ). Bijgevolg heeft de matrix een belangrijke invloed heeft op de aanpassing van de sporen, maar zodra de sporen gekiemd zijn, zal groei optreden aan een gelijkaardige snelheid als in APAM. Deze resultaten tonen aan dat het gebruik van simulatie- of synthetisch medium voor de ontwikkeling van predictieve modellen voor de lagfase van schimmels kan leiden tot ontoereikende voorspellingen en dat een validatie in levensmiddelen noodzakelijk is.

**Hoofdstuk 8** beschrijft de ontwikkeling van een risicobeoordelingmodel waarbij de volledige keten werd beschouwd met als doel verschillende methoden te vergelijken die toelaten de patulinecontaminatie in appelsap te reduceren. Het uitgewerkte model beschrijft de volledige keten vanaf het plukken van de appels tot de opslag van het geproduceerde appelsap. Validatie van het model toonde aan dat de gesimuleerde gemiddelde patulineconcentratie (6.8 en 3.6  $\mu\text{g}/\text{kg}$  voor respectievelijk troebel en klaar appelsap) in overeenstemming was met de gemiddelde patulineconcentratie die werd gedetecteerd in appelsap in België (6.7 en 4.5  $\mu\text{g}/\text{kg}$  voor respectievelijk troebel en klaar appelsap). Er werd echter een overschatting waargenomen voor de hogere concentraties (95<sup>ste</sup> percentiel). Verschillende scenario's werden geëvalueerd met als doel de patulinecontaminatie in appelsap te reduceren. Er werd aangetoond dat de concentratie van patuline in appelsap gereduceerd kan worden wanneer minder appels worden gebruikt die opgeslagen werden in gecontroleerde atmosfeer. Wanneer echter appels worden gebruikt die wel opgeslagen werden in gecontroleerde atmosfeer, dient een sorteerstap uitgevoerd te worden om de appels met een geïnfecteerd oppervlak groter dan 10  $\text{cm}^2$  te verwijderen. Deze maatregel laat toe de patulinecontaminatie te verlagen tot concentraties lager dan 25  $\mu\text{g}/\text{kg}$  in 99.7 tot 99.9% van de appelsappen. Een bijkomende kritische factor is de duur van de opslag in open lucht vooraleer de appels worden verwerkt.

Dit doctoraal proefschrift wordt afgesloten met het formuleren van de **conclusies** van het uitgevoerde onderzoek en het bediscussiëren van de **perspectieven**.

Literature review



# Chapter 1

## Literature review

### 1. Risk assessment, a tool to evaluate hazards in the food chain

#### 1.1. Introduction

Food-borne diseases remain a serious problem in both developed and developing countries, causing human suffering and significant economic losses. Up to one third of the population of developed countries is affected by food-borne diseases each year, and the problem is likely to be even more widespread in developing countries (FAO/WHO, 2005). A food-borne disease is caused by a hazard which is a biological, chemical or physical agent in, or a condition of, a food product with the potential to cause an adverse health effect (European Parliament and Council, 2002). Whether a hazard has to be considered as a risk, depends on the probability of an adverse health effect to occur and the severity of that effect, after exposure to the hazard (CAC, 1999). The aim of a risk assessment is to consider this problem and to quantitatively or qualitatively estimate the risk of the hazard in consideration.

#### 1.2. Historical background

Quantitative risk assessment started as a formal process to aid in decision making for managing the uncertainties involved in determining the risk of carcinogenic hazards. During the 1960s and 1970s, procedures were elaborated for performing animal bioassays to identify carcinogens (Potter, 1996). In 1983, the National Research Council (NRC) released “Risk Assessment in the Federal Government: Managing the Process”, which forms the basis of a common understanding risk assessment (NRC, 1983). The nomenclature and definitions used in this document were sufficiently broad to be generally applicable, which allowed the use of these definitions for non-carcinogenic chemicals and microbiological and physical hazards.

The development of risk assessment was strongly stimulated when in 1995, at the GATT (general agreement on tariffs and trade) Uruguay Round, a free trade in safe food was agreed. The WTO (World Trade Organization) agreement on the application of Sanitary and Phytosanitary measures, the so-called SPS agreement, requires that countries base their laws

concerning the protection of human, animal and plant health on a risk assessment basis (WTO, 1995). Also in the European legislation it is included that food legislation is based on risk assessment (European Parliament and Council, 2002).

### **1.3. Risk assessment, a part of risk analysis**

Risk assessment is a part of risk analysis. A risk analysis framework provides a process that systematically and transparently collects, analyzes and evaluates relevant information about a chemical, biological or physical hazard possibly associated with food. The aim is to select the best option to manage that risk based on the various alternatives identified (FAO/WHO, 2005). Besides scientific information, it involves also political, social, economic and technical considerations and it needs to take into account the public's perception of risk. Risk analysis is an iterative process that is updated once new information becomes available (Kuiper-Goodman, 2004).

As a structured decision-making process, risk analysis includes three distinct but closely connected components: risk management, risk assessment and risk communication (Figure 1.1) (Benford, 2001). Although, risk management and risk communication tended to receive less attention than risk assessment in the past (FAO/WHO, 2005), it is important to stress that risk analysis will only be effective when all three components are successfully integrated. The risk analysis process normally begins with risk management, which as a first step, defines the problem, the goals and the questions to be answered by the risk assessment. During the risk assessment, an estimation of the risk is made together with answering the questions posed by the management. Risk assessment and risk management are performed within an open and transparent environment based on communication and dialogue. Risk communication encompasses an interactive exchange of information and opinions among risk managers, risk assessors, consumers and other stakeholders.



**Figure 1.1: Risk analysis framework (modified from Benford, 2001)**

### **1.3.1. Risk management**

Risk management has been defined as the process, distinct from risk assessment, of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and for the promotion of fair trade practices, and, if needed, selecting appropriate prevention and control options (CAC, 2003a). Risk management therefore plays a key role at the beginning of the risk analysis process in identifying food safety problems and considering the best ways to manage them (FAO/WHO, 2005). This requires a focus on the scientific aspects of the risk as well as any associated economic, legal, ethical, environmental, social and political factors that are important to the society.

However, also at the end of the risk analysis process, risk management has a crucial function within risk analysis. The results of the conducted risk assessment need to be weighed with related social, economical and political factors to take policy decisions (Benford, 2001). The economic evaluation of possible risk management interventions (for instance using cost-benefit analysis) enables risk managers to evaluate the health impact and feasibility of an

intervention relative to its cost, which is important for good management decisions. Risk management therefore must be carried out in consultation with interested stakeholders and in synergy with risk communication activities. (FAO/WHO, 2005). When the possibility of harmful effects on health have been identified but scientific uncertainty persists, the precautionary principle states that the risk management can elaborate measures in order to ensure that the high level of health protection chosen in the European Community, pending further scientific information for a more comprehensive risk assessment (European Parliament and Council, 2002).

### **1.3.2. Risk assessment**

Risk assessment is an independent, scientific process, consisting of the following steps: (i) hazard identification, (ii) hazard characterisation, (iii) exposure assessment, (iv) and risk characterisation (CAC, 1999). The goal is to estimate the likelihood and the extent of adverse effects occurring to humans due to possible exposure(s) to hazards (CAC, 1999). A risk assessment can be quantitative or qualitative and both are important in different circumstances (FAO/WHO, 2005).

**Qualitative risk assessment** is the process of compiling, combining and presenting evidence to support a statement about risk. While numerical data and analysis may be part of the input into a qualitative risk assessment, the final risk estimate is not a numerical value. This methodology permits risk ranking or separation into descriptive risk categories (CAC, 1999; FAO/WHO, 2005).

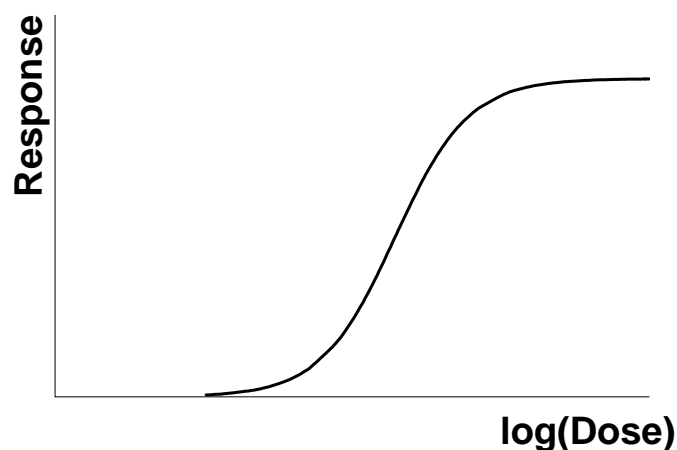
**Quantitative risk assessment** is based on numerical data and analysis and can be deterministic or probabilistic (see section 1.6 of this chapter). Quantitative risk assessments should describe uncertainty in numerical terms with uncertainty distributions determined by various statistical methods. A quantitative risk assessment can address risk management questions more in detail than a qualitative risk assessment (FAO/WHO, 2005).

During the **hazard identification**, biological, chemical, and physical agents that may cause adverse health effects and which may be present in a particular food or group of foods, are identified (CAC, 1999). Although the task of identifying a hazard is often considered as a part of risk management, risk assessors usually play an important role in hazard identification. In



particular, when possible hazards need to be analysed and prioritized on the basis of scientific evidence, risk assessors provide scientific expertise to help risk managers to select the hazard of greatest concern. In other cases, where risk managers have already identified the hazard, risk assessors provide supplementary information on the scientific nature of the hazard (FAO/WHO, 2005).

In the second step, the **hazard characterisation**, the nature of the adverse health effects associated with the hazard is evaluated in a qualitative and/or quantitative way (dose-response relationship) (CAC, 1999). The dose-response assessment is the determination of the relationship between the magnitude of exposure (dose) to a chemical, biological or physical agent and the severity and/or frequency of associated adverse health effects (response) (Benford, 2001). Figure 1.2 shows a typical example of a dose-response curve.



**Figure 1.2: A typical example of a dose-response curve**

**Exposure assessment** is defined as the qualitative and/or quantitative evaluation of the likely intake of the hazard via food as well as exposure from other sources, if relevant (CAC, 1999). It combines information on the prevalence and concentration of the hazardous material in the consumer's food supply and environment, and the likelihood that the consumer will be exposed to various quantities of this material in their food (FAO/WHO, 2005). Depending on the nature of the hazard, exposure assessment takes into account the relevant production, storage and handling steps along the food chain (see also section 1.4.3 of this chapter).

During the **risk characterisation**, all the evidence from the previous three steps is combined in order to obtain a risk estimate (i.e. an estimate of the likelihood and severity of the adverse health effects that would occur in a given population with associated uncertainties) and respond to the questions posed by the risk managers (Kroes et al., 2002; FAO/WHO, 2005). The outputs of a risk characterisation should also clearly identify important data gaps, assumptions and uncertainties in order to help risk managers judge how close the characterisation might come to describing reality (FAO/WHO, 2005).

### **1.3.3. Risk communication**

Risk communication has been defined as an interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk related factors and risk perceptions among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions (CAC, 2003a). Risk communication is a powerful, yet neglected tool in helping people to make more informed choices about risks (FAO/WHO, 2005).

### **1.3.4. Risk perception**

The general public's interest in the safety of food has increased considerably by the last years. However, the consumers perceive risks differently compared to experts. Research has shown that in a study where five food hazards (naturally occurring toxins, additives and food processing residues, microbiological contaminants, nutritional imbalance, environmental contaminants) were ranked in order of importance by the general public, additives and food processing residues were considered as the most important, while experts considered these as the least important (Feskens, 1997). In general, the difference between experts and consumers is a consequence of the fact that scientists focus on probability and severity of harm, while the general public takes also other factors into account. These factors are described as "outrage factors" and include the fairness of the risk, degree of personal control over exposure, voluntary or involuntary exposure, severity of the consequences and characteristics of the person at risk such as having children (Bruhn, 2005). However, consumers' perception has an important influence and risk managers must be aware that in the event of a crisis, consumers' perceptions about risks can have a great or greater impact on the outcome than the real food safety issues (Tennant, 2001). It is therefore important to move people towards a more accurate perception of health risks linked to food and to empower people to make informed

decisions relative to potential benefits. Risk communication has a crucial role in this process (Bruhn, 2005).

### **1.3.5. Risk-benefit analysis**

A risk-benefit analysis is an extension of risk analysis. Besides possible hazards, food contains also compounds that have a positive effect on health (e.g. vitamins, omega-3 fatty acids). When a food or food substance is associated with both potential health risks and benefits, and particularly when the levels of intake associated with risk and benefit are close, there is a need to define an intake range within which the balance of risk and benefit is acceptable (EFSA, 2006b). A risk-benefit analysis follows the same approach as risk analysis consisting of risk-benefit assessment, risk-benefit management and risk-benefit communication. Consequently, the benefit assessment part of the risk-benefit assessment should include benefit identification, benefit characterisation (dose-response assessment), exposure assessment, and benefit characterisation. In addition, the risk-benefit analysis should contain a mean, quantitative if possible, to compare the potential risk against the potential benefit (a risk-benefit comparison) (EFSA, 2006b).

## **1.4. Chemical versus microbiological risk assessment: different hazards require a different approach**

Chemical and microbiological hazards are fundamentally different and demand a different risk assessment approach. Allergens, as a special group within the chemical hazards, will not be discussed in the presented text. More information on this topic is available in Spanjersberg et al. (2007).

### **1.4.1. Hazard identification**

The emphasis of hazard identification differs for chemical and biological agents, due to the different nature of both hazards. In fact, any chemical substance is likely to produce some form of adverse effect if taken in sufficient quantity and a single chemical substance may be associated with several different adverse health effects (Benford, 2001). Therefore, the aim of chemical hazard identification is to evaluate whether the chemical has the potential to cause adverse effects in humans by reviewing all available data on toxicity and the biological mechanism that leads to toxicity (Barlow et al., 2002).

In contrast, not all micro-organisms are pathogenic, but even a very small number of pathogens has the potential to cause disease. Therefore, microbiological hazard identification aims to identify the likelihood of the presence of known pathogenic micro-organisms or microbial toxins in a certain food product, and to evaluate whether they have the potential to cause harm when present in food or water (Benford, 2001).

#### **1.4.2. Hazard characterisation**

Whereas in hazard identification, the emphasis differs for microbiological and chemical agents, the processes of chemical risk assessment (CRA) and microbiological risk assessment (MRA) are much more closely aligned for the hazard characterisation stage. The aim of both chemical and microbiological hazard characterisation is to determine the dose-response relationship.

##### **1.4.2.1. Chemical hazard characterisation**

For chemical agents, hazard characterisation is closely linked to hazard identification and is often based on the same studies. Hazard identification reveals the type(s) of toxicity associated with a particular substance, while hazard characterisation determines the relationship between dose and response and subsequent estimation of dose levels that may cause that response in humans. A distinction is made between threshold effects and non-threshold effects. Many of the non-carcinogenic adverse effects observed in animal or humans are characterised by a threshold dose, below which no adverse effects are observed (Kuiper-Goodman, 2004). For these effects, it is current practice to estimate the level of exposure without significant adverse effect and to derive a health-based guidance value such as a tolerable daily intake (TDI) or acceptable daily intake (ADI) (Barlow et al., 2006). With regard to carcinogens, it is not possible to define a dose without a potential effect, unless it can be clearly established that the mode of action involves an indirect mechanism that may have a threshold (Kuiper-Goodman, 2004; Barlow et al., 2006). Both effects are treated in a different way to extrapolate safe intake estimates as explained in the following paragraphs.

##### *Chemicals with a threshold dose*

Non-carcinogenic chemicals are usually characterised by a threshold dose, the no observed adverse effect level (NOAEL), which is the highest dose that causes no toxic effects (Dybing et al., 2002). The NOAEL is divided by a safety or uncertainty factor of 100 or 1000 in order

to calculate the amount of a chemical, expressed on a body weight basis that can be ingested daily over a lifetime without causing adverse health effects (Barlow, 2005). For food chemicals, this is expressed as the ADI or TDI. The term ADI is generally used for substances intentionally added to food, while TDI is generally used for substances appearing in food but not intentionally added (Barlow, 2005). The incorporation of a safety or uncertainty factor gives an additional margin of reassurance to take account of the possibility that humans may be more sensitive than animals (inter-species variation) and that among humans some may be more sensitive than others (intra-species variation) (Benford, 2000). For chemicals with cumulative properties (e.g. heavy metals) the tolerable weekly intake (TWI) is calculated (Benford, 2001). The TWI is similar to the TDI, but is calculated on a weekly base instead of a daily base.

Another measure, useful in risk assessment and management, is the acute reference dose ( $RfD_{acute}$ ). This value is estimated to deal with situations where there is high short-term or single exposure with the possibility of subsequent adverse effects. The  $RfD_{acute}$  is derived from appropriate animal or human data and is generally 2 to 10-fold greater than the TDI/ADI. In general, it should not be used for substances for which there is no threshold for the observed toxic effects (Kuiper-Goodman, 2004).

#### *Chemicals without a threshold dose*

For carcinogenic agents, no threshold can be determined unless an indirect mechanism is involved (Kuiper-Goodman, 2004). The mathematical analysis of the dose-response data from an animal bioassay can be used to define the intake necessary to produce a given level of response, such as 10% cancer incidence. The intakes of different compounds giving the same level of response reflect the relative potencies of the compounds. Various methods are available to define a reference point on the dose-response curve, such as the benchmark dose (BMD)<sup>1</sup>, the lower confidence interval on a benchmark dose (the BMDL), the T25<sup>2</sup> (Dybing et al., 1997) or the TD50<sup>3</sup> (Peto et al., 1984). The BMD approach is being used increasingly,

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<sup>1</sup> The benchmark dose (BMD) is the dose which is associated with a low incidence of effect (e.g. 10%) (Benford, 2001)

<sup>2</sup> The T25 is defined as the chronic daily dose in mg/kg body weight which will give 25% of the animals tumours at a specific tissue site, after correction for spontaneous incidence, within the standard life span of that species (Dybing et al., 1997).

<sup>3</sup> TD<sub>50</sub>: dose equivalent to 50% of animals remaining tumour free after two years of exposure to the compound (Peto et al., 1984).

with a 10% cancer incidence being used most frequently as the benchmark response (EFSA, 2005). These reference points are then further used in the risk characterisation.

#### **1.4.2.2. Microbiological hazard characterisation**

Ingestion of a pathogen does not necessarily mean that a person will become infected, nor that illness or death will occur. The likelihood that an individual becomes ill from ingesting a micro-organism is dependent on the complex interaction among the host, the food and the pathogen. This interaction is commonly known as the ‘infectious disease triangle’ or ‘epidemiologic triad’ (Dennis et al., 2002). The components of this triangle have an influence on the dose-response relationship and it is therefore necessary to indicate which information has been used (Forsythe, 2002). In order to predict the probability of infection or illness, mathematical models are used that describe the relation between the probability of the outcome and the dose. An overview of the different models that are used to describe dose-response relationships is given by Dennis et al. (2002) and Forsythe (2002).

Until relatively recently it has been assumed that there is a threshold level of pathogens that must be ingested to cause an infection or disease (the minimum infectious dose). However, this idea has been replaced by the proposal that infection may result from the survival of a single, viable, infectious pathogenic organism (single-hit concept) (Forsythe, 2002). However, the probability that a single *Listeria monocytogenes* cell would cause a food-borne disease is estimated to be one on  $10^{14}$  (Benford, 2001).

Besides infection, pathogens can also cause intoxication. Bacterial toxins may be formed in food and may cause adverse health effect, when the dose is high enough. Risk assessment of such toxins follows the same protocol as for chemicals with a threshold effect (Benford, 2001).

#### **1.4.3. Exposure assessment**

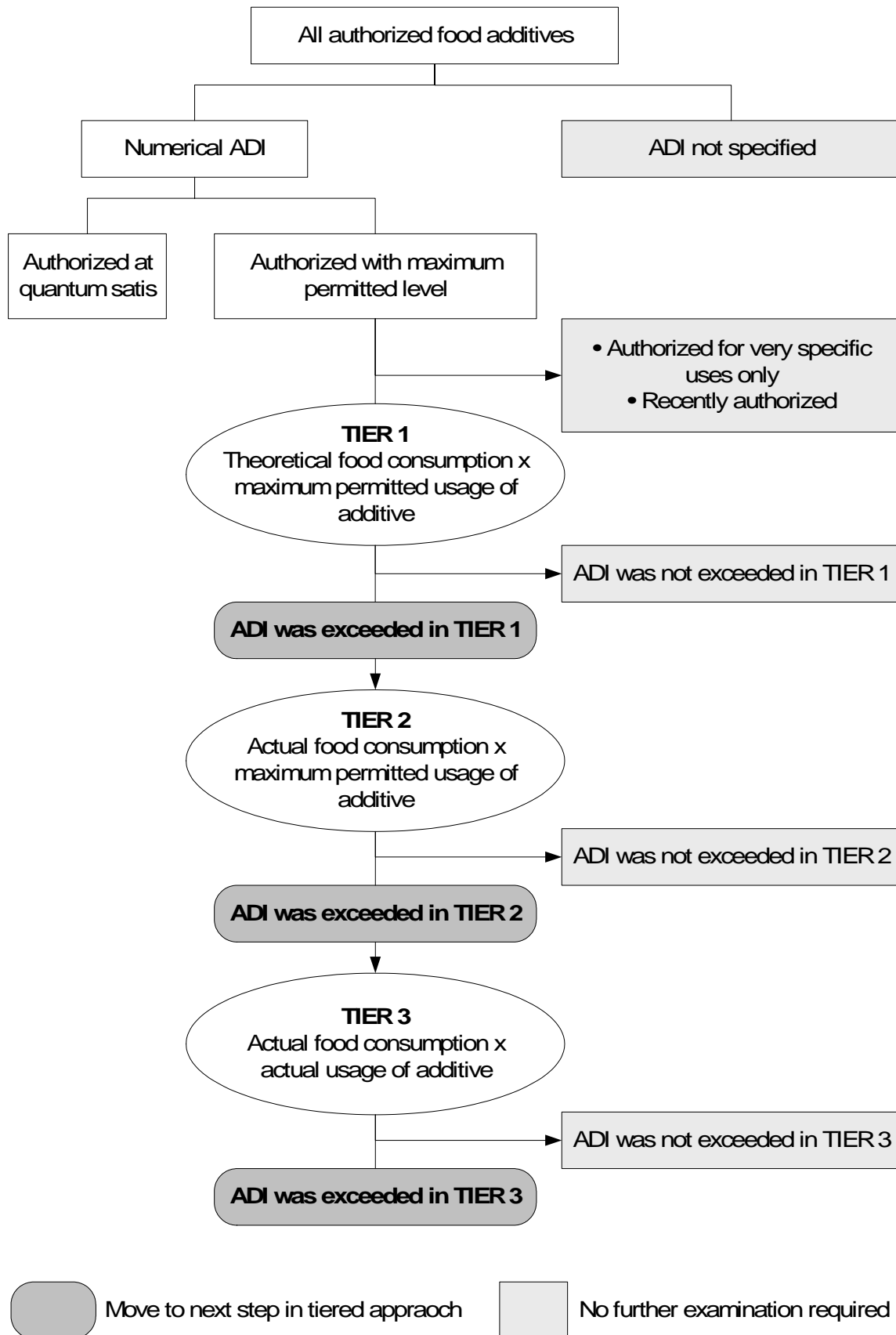
##### **1.4.3.1. Chemical exposure assessment**

In chemical exposure assessment, the level of the chemical hazard is determined and together with the consumption data, the intake of the chemical is calculated. The low levels at which chemicals can be present in food and can cause toxicological effects, have implications for the analytical performance of the analytical methods (O’Brien et al., 2006). The number of

samples below the limit of detection (LOD) and limit of quantification (LOQ) and how such 'non-detects' are treated can have an important influence on the exposure estimation (Kuiper-Goodman, 2004). In literature data below the LOD (censored data) are replaced by the corresponding LOD, by LOD divided by 2 or by 0 (e.g. Tressou et al., 2004; Govaerts et al., 2005).

In order to minimize errors in traditional exposure assessments biomarkers can be used. The level of the biomarker is measured in e.g. blood, urine, nails or hair and the exposure is estimated based on pharmacokinetic relationships (Kuiper-Goodman, 2004). The advantage of this methodology is that besides taking different exposure pathways into consideration, it also includes the bioavailability from food matrices (O'Brien et al., 2006; Slotnick & Nriagu, 2006).

For certain groups of chemical hazards (e.g. additives, pesticide residues) it can be useful to determine first if it is necessary to perform human exposure studies. In that case a tiered approach can be used, which is a logical stepwise approach that uses available information to the optimum extent while reducing unnecessary requirements for human exposure surveys. The principle of the tiered approach will be explained by the methodology elaborated by the European Commission to evaluate the dietary intake of food additives (Figure 1.3) (European Commission, 2001). The tiered approach is only executed for food additives that are authorised at a maximum permitted level and for which an ADI is specified. Additives that are authorised in one or few specific food categories and new additives are excluded from the scope. In Tier 1, the intake is calculated based on theoretical food consumption data and maximum usage levels for additives. When the ADI is not exceeded in Tier 1, the additive in question is eliminated from further considerations. Resources can then be focussed on the remaining additives for a more refined intake estimate. In the second and third tiers, the intake is calculated by combining actual national food consumption data with the maximum permitted usage levels for the additive (Tier 2) or with the actual usage levels of the additive (Tier 3). This approach enables authorities to establish priorities for monitoring and to focus on food additives for which the ADI is probably exceeded regularly (European Commission, 2001).



**Figure 1.3: Outline of the tiered approach for food additives (European Commission, 2001)**

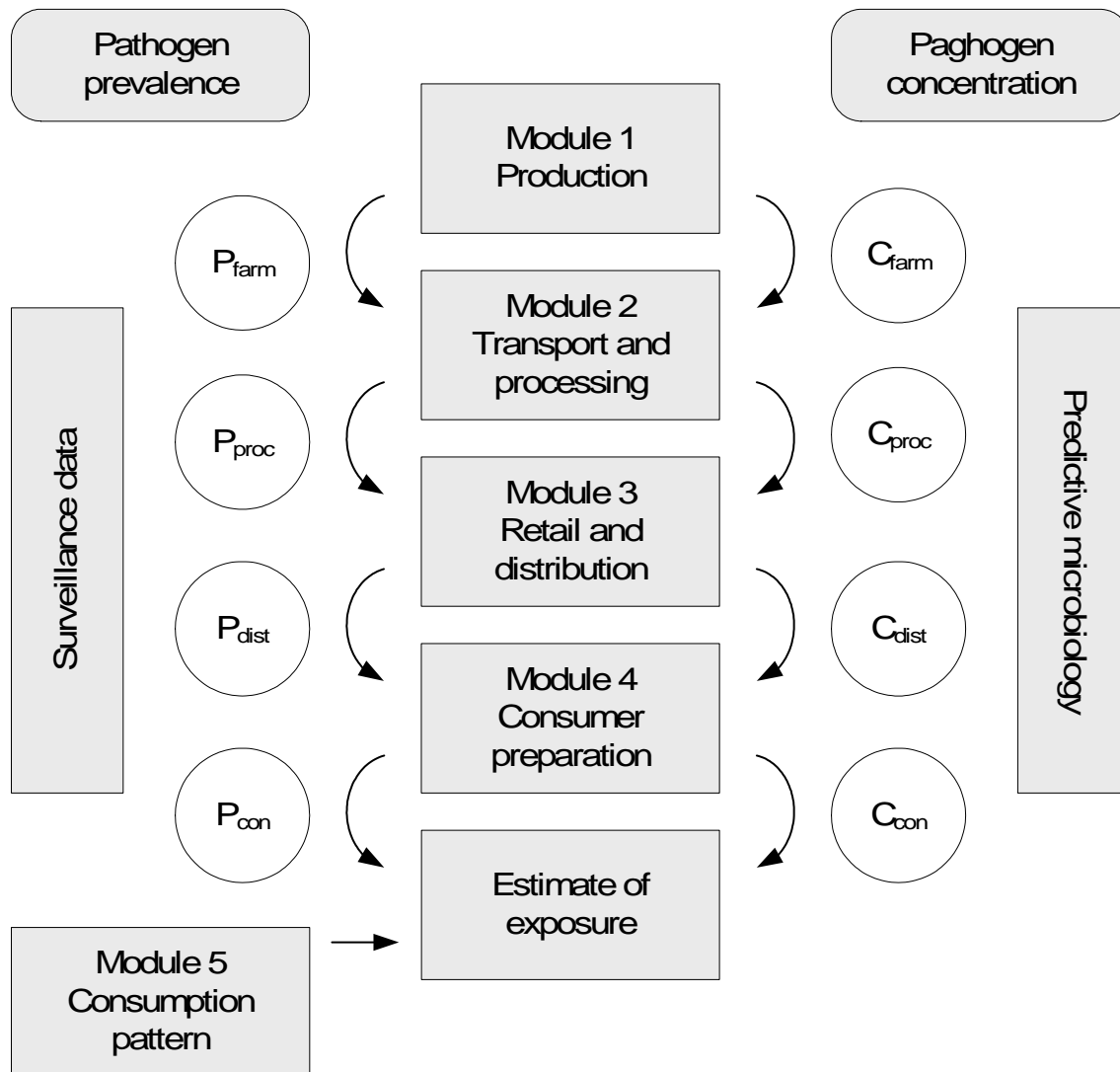


### **1.4.3.2. Microbiological exposure assessment**

In microbiological exposure assessment the hazard is alive, which reorients the focus of the exposure assessment significantly. The level of the pathogen in a food can change over time due to growth and inactivation (FAO/WHO, 2005). Also cross and post contamination will have an influence on the level and the prevalence of the pathogen. Moreover, a pathogen is able to enter the food chain at many points. Therefore, it is necessary to include the complete chain in the exposure assessment through a farm to fork approach (Figure 1.4) (Forsythe, 2002). In order to increase the transparency, the farm to fork model is divided into a series of modules, also called a Modular Process Risk Model (MPRM) (Figure 1.4) (European Commission, 2003c). Growth, survival and inactivation of micro-organisms are included in a farm to fork approach using predictive microbiology (Wijtzes, 2002; Ross & McMeekin, 2003).

A large number of factors have an influence on the microbial population. These factors include (modified from Forsythe, 2002):

- the microbial ecology of the food;
- the growth requirements (intrinsic and extrinsic parameters) and their influence on the growth/inactivation;
- the initial contamination of the raw materials;
- prevalence of infection in food animals;
- the effect of the production, processing, cooking, handling, sorting, distribution steps and preparation by the final consumer on the microbial agent (i.e. the impact of each step on the level of the pathogenic agent of concern, including the risk for cross or post contamination);
- the variability in processes involved and the level of process control;
- the level of sanitation, slaughter practices, rates of animal-animal transmission;
- the methods or conditions of packaging, distribution and storage of the food (e.g. temperature of storage, relative humidity of the environment, gaseous composition of the atmosphere) and the characteristics of the food that may influence the potential for growth of the pathogen (and/or toxin production) in the food (e.g. pH, water activity, nutrient content, presence of antimicrobial substances) under various conditions, including abuse.



**Figure 1.4: Framework of “farm to fork” modules for microbiological exposure assessment (modified from Forsythe, 2002)**

Due to the need for various information and data, MRA has to deal with a lot of data gaps (Brown & Stringer, 2002). One of the problems concerning data in MRA is the presence/absence testing of pathogens in a certain sampling size, which gives no quantitative value (concentration). This problem can be solved by using enumerations or presence/absence testing of a 10-fold serial dilution, which is however much more labour-intensive (Uyttendaele et al., 2006). A second limitation in the availability of data for MRA is data concerning consumer behaviour. As discussed above, the consumer has a large influence on the prevalence and concentration of pathogenic micro-organisms in food. However, it is very difficult to determine and investigate this behaviour (Worsfold & Griffith, 1995).

#### **1.4.4. Risk characterisation**

During the risk characterisation it is estimated how likely it is that harm will be done and how severe the effects will be. It brings together the information on exposure and health hazards defined in the previous stages of risk assessment, and outlines the sources of uncertainty in the data on which they are based (Benford, 2001).

##### **1.4.4.1. Chemical risk characterisation**

For chemical hazards, different approaches have been adopted for the risk characterisation of chemicals with a threshold and without a threshold (Renwick et al., 2003). For chemicals with a threshold effect, the results of the exposure assessment can be compared to the ADI or TDI, in order to estimate the risk (Benford, 2001). For chemicals without a threshold, four different approaches can be used (Barlow et al., 2006):

1. as low as reasonably achievable (ALARA);
2. low-dose extrapolation of data from rodent carcinogenicity bioassays;
3. threshold of toxicological concern (TTC);
4. margin of exposure (MOE).

The ALARA approach says that the intakes should be as low as reasonably achievable (Barlow et al., 2006). Although the ALARA principle is an easy to understand concept, it poses some major difficulties for the risk manager as it does not discriminate between very potent and very weak carcinogens and does not take human exposure into account (O'Brien et al., 2006). It does not give any estimate of risk and will not give risk managers sufficient information to assess the degree of urgency and extent of risk reduction measures that may be required (Barlow et al., 2006).

An estimation of the risk associated with human exposure to a low dose of a certain chemical, can be derived by extrapolation of the animal dose-response data or by the use of the TD50, T25 or BMDL10 as the point of departure for simple linear extrapolation (O'Brien et al., 2006). Estimation of the possible cancer risk at the levels of human intake is based on empirical mathematical models that however do not reflect the complexity of the underlying biology. Using the same dose-response data but different mathematical models, can result in intakes associated with very low risks (e.g. one in a million), that differ by orders of magnitude (O'Brien et al., 2006). Moreover, simple linear extrapolation methods probably

greatly overestimate the real risks (Ames & Gold, 1991). Therefore, it is not recommended to use this approach (O'Brien et al., 2006).

The TTC approach is used for risk assessment of contaminants in food in cases where the biological data are few but the chemical structure is known and good exposure data are available (Kroes et al., 2005). The TTC is the daily intake estimated to give a lifetime risk of less than one in a million. This value is derived from the dose-response data of all structurally related compounds studied in rodent cancer bioassays (Barlow, 2005). A generic threshold of 0.15 µg/person/day (or 0.0025 µg/kg bw/day) is applied for all structural alerts<sup>4</sup>, except genotoxic and high potency carcinogens (aflatoxin-like compounds, N-nitroso-compounds, azoxy-compounds) (Kroes et al., 2004). It is advised to use this approach when no good hazard characterisation data are available (Barlow et al., 2006; O'Brien et al., 2006).

A fourth approach is the margin of exposure (MOE): the ratio between a dose leading to tumours in experimental animals and the human intake. The magnitude of the MOE reflects, but does not attempt to define the possible magnitude of the risk: the larger the MOE, the smaller the risk posed by exposure to the compound under consideration. Calculation of the MOE requires two decisions; defining the point on the dose-response curve and the human intake (O'Brien et al., 2006). The TD50, T25 or BMD are often taken as comparative estimates of the potency of genotoxic carcinogens (Sanner et al., 2001). For the human intake, different values can be used, providing risk managers with different information, for example the median intake provides a general picture, while the intake by the 90<sup>th</sup> or 97.5<sup>th</sup> percentile provides information about the high consumer (O'Brien et al., 2006). The European Food Safety Authority (EFSA) Scientific Committee considered that an MOE of 10 000 or more, based on animal cancer bioassay data, “would be of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions” (EFSA, 2005). The MOE is considered as the preferred approach to characterise risks without a threshold, because it is based on the available animal dose-response data, without extrapolation, and on human exposures. The MOE can be used for prioritisation of risk management actions but it is recognised that the MOE is difficult to interpret in terms of public health (Barlow et al., 2006).

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<sup>4</sup> Structural alert: a particular chemical grouping within a chemical structure which is known to be associated with a particular type of toxic effect, e.g. genotoxicity (Barlow, 2005).

#### **1.4.4.2. Microbiological risk characterisation**

In the microbiological risk characterisation, the output of the exposure assessment is used as an input for the dose-response relationship and the result is the risk estimate, namely the probability of an adverse effect (Forsythe, 2002).

### **1.5. The use of risk assessment outputs in risk management**

#### **1.5.1. Chemical risks**

When the risk assessment indicates that the proportion of consumers with the potential to exceed the TDI/ADI is zero, there is no need to take action in order to control the risk. However, even in these circumstances other activities, such as risk communication, might be appropriate (Tennant, 2001).

If during the risk characterisation, it is observed that the exposure poses a possible health risk, recommendations for risk reduction need to be formulated. Priorities for risk reduction depend on the extent and frequency that the TDI/ADI is exceeded, or on the size of the MOE (Kuiper-Goodman, 2004). When it concerns a chemical for which a threshold dose can be defined, the simplest approach is to identify the level of contamination that would cause a high-level consumer to exceed the TDI/ADI and to use this value as maximum level in legislation (Tennant, 2001). The maximum level is also called MRL (maximum residue level), typically used for phytosanitary products and residues of medicines for veterinary use (Benford, 2001; CAC, 2003b)

For non-threshold carcinogens, some jurisdictions prefer to use an approach that keeps residue levels ‘as low as reasonably achievable’ (ALARA), but it is then difficult to derive an appropriate maximum permitted concentration (Kuiper-Goodman, 2004). Other jurisdictions try to ensure that maximum levels for unavoidable non-threshold carcinogens pose a ‘negligible risk’, which corresponds to a MOE of 10 000 (EFSA, 2005).

## **1.5.2. Microbiological risks**

### **1.5.2.1. *Appropriate level of protection***

The aim of a risk assessment is to provide risk managers with answers to one or more questions that may enable them to make better informed decisions (Van Schothorst, 2002). Government risk managers need to decide what in the WTO-SPS agreement (WTO, 1995) is called an appropriate level of protection (ALOP). ALOP is defined as the level of protection deemed appropriate by the member (state) establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory (WTO, 1995).

ALOP is a way to express, on a population level, what level of risk a society is prepared to tolerate or is considering to be achievable (Gorris, 2005). ALOP is also called the ‘acceptable level of risk’(ALR) or ‘tolerable level of risk’ (TLR). The latter expression is preferred because consumers may tolerate food safety risks but they are reluctant to accept them (Van Schothorst, 2002). Discussion is actively ongoing whether in addition to scientific insights, other factors can be considered in the decision of an ALOP. Such factors could be for instance technological and economical (e.g. the potential damage in terms of loss of production, the cost of control) (de Swarte & Donker, 2005).

In comparison to microbial hazards, it is more difficult to set a meaningful ALOP for chemical hazards. With chemical hazards, often there is no proof of causality between a chemical hazard and an individual case of food-borne disease because impacts of chemical hazards may be more chronic in nature. A second complication regarding ALOPs for chemicals is that most chemical hazards can be found in a variety of products, both food and non-food, and in our direct living environment (de Swarte & Donker, 2005).

### **1.5.2.2. *Food safety objective***

An ALOP, expressed for instance as a number of illnesses in a population per annum, is not a measure that is meaningful for food safety management in practice. The food safety professionals responsible for controlling the specific hazards need more specific guidance. To that end, and within the current risk analysis framework, competent authorities can formulate a so-called food safety objective (FSO) (Gorris, 2005). FSO is defined by the Codex Alimentarius Commission (CAC) as the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate

level of protection (CAC, 2004). Some hypothetical examples of FSO values are given in Table 1.1. Although the CAC considers FSOs only for microbial hazards, the concept could also be applied to other types of hazards as well (de Swarte & Donker, 2005).

**Table 1.1: Hypothetical examples of concepts used in food safety control (Gorris, 2005; Stringer, 2005)**

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**Food safety objectives**

- *Listeria monocytogenes* in a ready-to-eat food product shall not exceed 3.5 log<sub>10</sub> CFU/serving size of food when eaten
- The concentration of aflatoxin in shelled, roasted peanuts shall not exceed 15 µg/kg when consumed
- The frequency of *Salmonella* Enteritidis in eggs may not exceed 1 egg per 100 000

**Performance objectives**

- Salmonellae and pathogenic *E. coli* shall not exceed 1 CFU/10 L when fruit juice is packaged for distribution
- *Clostridium perfringens* shall not exceed 100/g in cooked meat or poultry products when ready for distribution

**Control measures**

- A product requirement, e.g. pH below 4.6 (product criterion)
- Educate catering staff about proper hygiene

**Performance criteria**

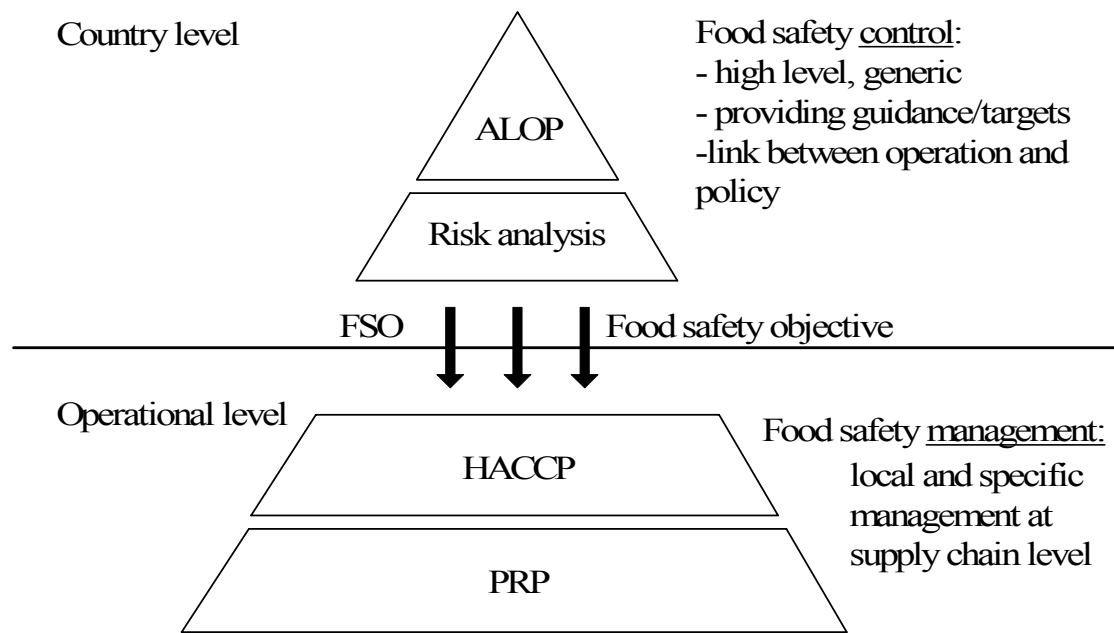
- Assure a 12 log reduction of *Clostridium botulinum* in low acid canned foods
- Heat juice to achieve a 5 log reduction of enteric pathogens
- Avoid more than 3 log<sub>10</sub> CFU increase in *Staphylococcus aureus* during the manufacture of cheese and fermented meats

**Process criteria**

- Three minutes at 121°C for 12-D inactivation of spores of proteolytic *C. botulinum*
  - Ten minutes at 90°C for 6-D inactivation of spores of non-proteolytic *C. botulinum*
- 

Food safety on an operational level is primarily the responsibility of the food industry. The national authorities however, are responsible for controlling the process of setting, achieving and evaluating the level of a hazard that can be tolerated (de Swarte & Donker, 2005). Within this framework, the FSO value is an important communication tool for the overall management of the chain as it gives the expected level of control in the food chains in order to

make a product that can be considered as safe. It is a concept that bridges from a population's generic requirements for safe food to specific operational measures (Figure 1.5) (Gorris, 2005). The implementation of an FSO as a target at the end of the food chain, leaves the food industry more freedom and flexibility to organize their quality management tools (e.g. hazard analysis critical control points (HACCP), Prerequisite programs (PRP)) on the condition that this target is achieved (de Swarte & Donker, 2005; Gorris, 2005).



**Figure 1.5: Illustration of how food safety control at a country level can link into food safety management at the operational level through a food safety objective set by a governmental competent authority on the basis of a public health goal (ALOP) established following the risk analysis framework (modified from Gorris, 2005)**

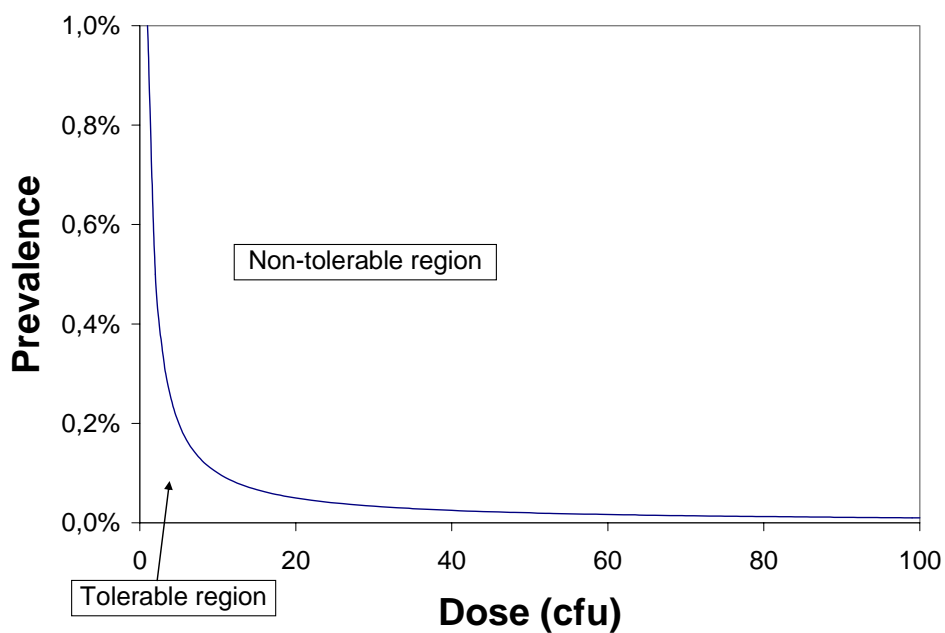
Achieving the given FSO depends to a large extent on the efficiency of the control measures along the food chain. It is necessary to establish whether PRP and HACCP systems can provide the level of technical control needed to achieve the FSO. If not, the procedures should be re-evaluated and adapted until the FSO is achieved (Stringer, 2005). For this reason, the International Commission on Microbiological Specifications for Foods (ICMSF) has proposed a “conceptual equation” that expresses the relation between the initial level, reduction, increase and the FSO (ICMSF, 2002):

$$H_0 - \sum R + \sum I \leq FSO \quad (1.1)$$



where  $H_0$  is the initial level of the hazard,  $\sum R$  is the sum of the hazard reductions,  $\sum I$  is the sum of the hazard increases (growth or recontamination), and FSO,  $H_0$ ,  $R$  and  $I$  are expressed in  $\log_{10}$  units.

However, some scientists do not agree with the current definition of FSOs, since variability and uncertainty about the contamination of food is not included. Therefore, Havelaar et al. (2004), propose a P-D equivalence curve that separates “tolerable” from “non-tolerable” combinations of prevalence (P) and dose (D) (Figure 1.6).



**Figure 1.6: P-D equivalence curve for *E. coli* O157 in raw steak tartare patties. The curve describes the boundary between acceptable and non-acceptable combinations of prevalence (P) and numbers (D) of the pathogen in a food product (Havelaar et al., 2004)**

### 1.5.2.3. Other food safety management targets

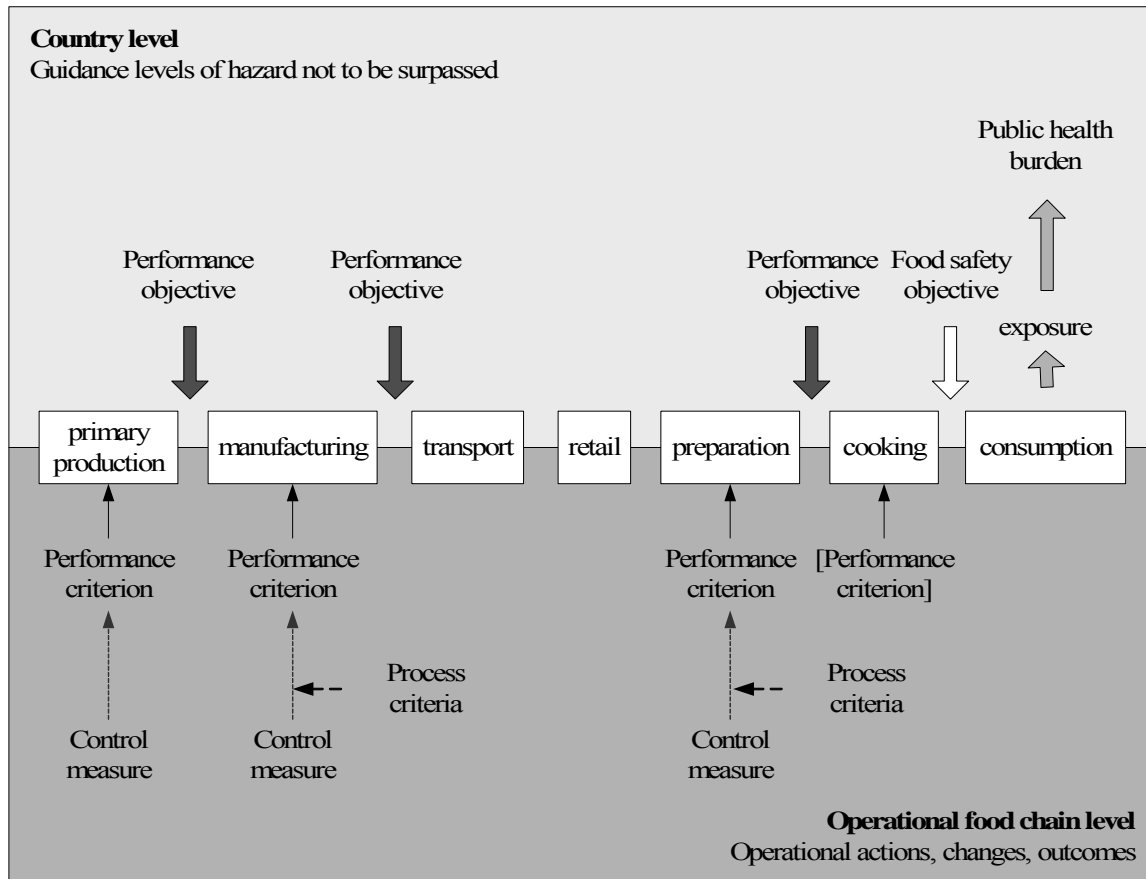
In order to assure that an FSO is met at consumption, it can be relevant to specify one or more targets earlier in the food chain. These targets are called performance objectives (PO) and are defined as the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to an FSO or ALOP, as applicable (CAC, 2004). POs are linked to the FSO and, when proposed by governments, can be viewed as a kind of milestones that governments provide as guidance in

order to help to meet the FSO (Gorris, 2005). However, POs can also be established by operational food safety managers as an integral part of the design of a food safety management system.

To comply with a PO or an FSO, at the operational level, control measures (CM) need to be established. A control measure is any action and activity that can be used to prevent or eliminate a food safety hazard or to reduce it to an acceptable level (it can be microbiological specifications, guidelines on pathogen control, hygiene codes, microbiological criteria, specific information (e.g. labelling), training, education, and others) (ICMSF, 2002). Examples of control measures are given in Table 1.1. At a particular step in the chain, one or more control measures can be implemented as part of the product and process design to control a hazard.

The overall effect of control measures on the hazard level at a particular step, is called performance criterion (PC). The PC is defined as the effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a PO or an FSO (CAC, 2004). PCs are the specific operational, supply chain measures at (a) specific step(s) that result in meeting the objective for that step, the PO or FSO. The control parameters (e.g. time, temperature, flow rate) at a step or a combination of steps that are applied to achieve a PC are called the process criteria (Van Schothorst, 2002). Product criteria on the other hand are the parameters of the food that are used to prevent unacceptable growth of micro-organisms (e.g. pH,  $a_w$ ) (Van Schothorst, 2002). PCs in general will be decided on by food safety managers as key points in the design of a production flow that allows the production of safe foods (Gorris, 2005). Some hypothetical examples of PC values are given in Table 1.1.

Although PO and PC like the FSO are not intended to be enforced, these concepts on occasion could lend themselves to be verified by specific testing or could be linked to specific microbiological criteria. Figure 1.7 gives an overview of how various guidance milestones and control measures relate to each other in an imaginary food supply chain (Gorris, 2005).



**Figure 1.7: Schematic representation of how governmental or country guidance along an imaginary food chain links in with operational level measures at relevant points. The guidance is given in the form of FSO or PO values stipulated by the appropriate food control function. The operational level measures are embedded in the food safety management systems operated in the chain, such as PRP and HACCP (Gorris, 2005)**

#### 1.5.2.4. Microbiological criteria

Establishing microbiological criteria is another risk management option to guarantee food safety. A microbiological criteria (MC) is defined as the acceptability of a product or a food lot, based on the absence or presence, or number of micro-organisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot (CAC, 1997).

In the establishment of MC's, FSOs or PSs can be useful and an MC for a food should be related to its FSO. An MC that is excessively stringent relative to an FSO may result in rejection of food even though it has been produced under conditions that provide an appropriate level of protection (Van Schothorst, 2002). However, microbiological criteria differ in function and content from FSOs (Table 1.2). An FSO will normally not prescribe a

sampling plan, while for MCs it is essential that such a plan is developed, because it will assist in achieving transparency and equivalence as mentioned in the WTO/SPS agreement (Van Schothorst, 2002). A two-class attribute sampling plan is used, which is characterized by the number of samples to be tested ( $n$ ), the number of samples ( $c$ ) that exceed the test criteria (in microbial testing associated with pathogenic micro-organisms,  $c$  is usually zero), the lower limit of detection for the test ( $m$ ), the upper limit of detection for the test ( $M$ ) and a confidence level (e.g., 95%) that the test will identify an unacceptable lot (Whiting et al., 2006). The European Commission has published in 2005 a new regulation concerning microbiological criteria (European Commission, 2005).

**Table 1.2: Characteristics of FSOs and microbiological criteria (Van Schothorst, 2002)**

Food safety objective	Microbiological criterion
A goal upon which food processes can be designed so the resulting food will be safe	A statement that defines acceptability of a food product or lot of a food
Aimed at consumer protection	Confirmation that effective PRP and HACCP plans are applied
Applied to food at the moment of consumption	Applied to individual lots or consignments of food
Components: <ul style="list-style-type: none"> <li>• Maximum frequency and/or concentration of a microbiological hazard</li> </ul>	Components: <ul style="list-style-type: none"> <li>• Micro-organisms of concern and/or their toxins/metabolites</li> <li>• Sampling plan</li> <li>• Analytical unit</li> <li>• Analytical method</li> <li>• Microbiological limits</li> <li>• Number of analytical units that must be conform to the limits</li> </ul>
Used only for food safety	Used for food safety or quality characteristics

## 1.6. Risk assessment: a quantitative approach

*This section is a modified version from Devlieghere, F., Francois, K., De Meulenaer, B., Baert, K., 2005. Modelling food safety. In: Luning, P.A., Devlieghere, F., Verhé, R. (Eds.), Safety in the agri-food chain. Wageningen Academic Publishers, Wageningen, 684 p.*

Quantitative risk assessment (QRA) is characterised by assigning a numerical value to the risk, in contrast with qualitative risk analysis, that is typified by risk ranking or separation into descriptive categories of risk (CAC, 1999). During QRA, a model (QRAM) is developed that estimates the risk based on the exposure and the dose-response. Several methods can be used to obtain a numerical value for the risk: (i) point estimates or deterministic QRA; (ii) simple distributions and (iii) probabilistic QRA (Kroes et al., 2002).

### 1.6.1. Deterministic risk assessment

Deterministic modelling (point estimates) uses a single input value for each variable within the model to determine the model's outcome(s) (Vose, 2000). The point estimate which is used can be the mean or the 95<sup>th</sup> or 97.5<sup>th</sup> percentile depending on the questions that need to be answered. For contaminants also the maximum level according to the legislation can be used.

Point estimates are commonly used as a first step in exposure assessments because they are relatively quick, simple and inexpensive to carry out. Inherent to the point estimates models are the assumptions that all individuals consume the specified food(s) at the same level, that the hazard is always present in the food(s) and that it is always present at an average or high concentration. As a consequence, this approach does not provide an insight into the range of possible exposures that may occur within a population or the main factors influencing the results of the assessment. It provides limited information for risk managers and public. The use of this method also tends to significantly over- or under-estimate the actual exposure (Finley & Paustenbach, 1994). Using high-level values to represent either the food consumption or hazard level may lead to high and often implausible overestimates of the intake. Point estimates are generally considered to be most appropriate for screening purposes (Parmar et al., 1997). If they demonstrate that the intake is very low in relation to the accepted safe level for the hazard or below a general threshold value, even when assuming high

concentrations in the food and high consumption of the food, it may be sufficient to decide that no further exposure assessments are required (Kroes et al., 2000).

### **1.6.2. Probabilistic risk assessment**

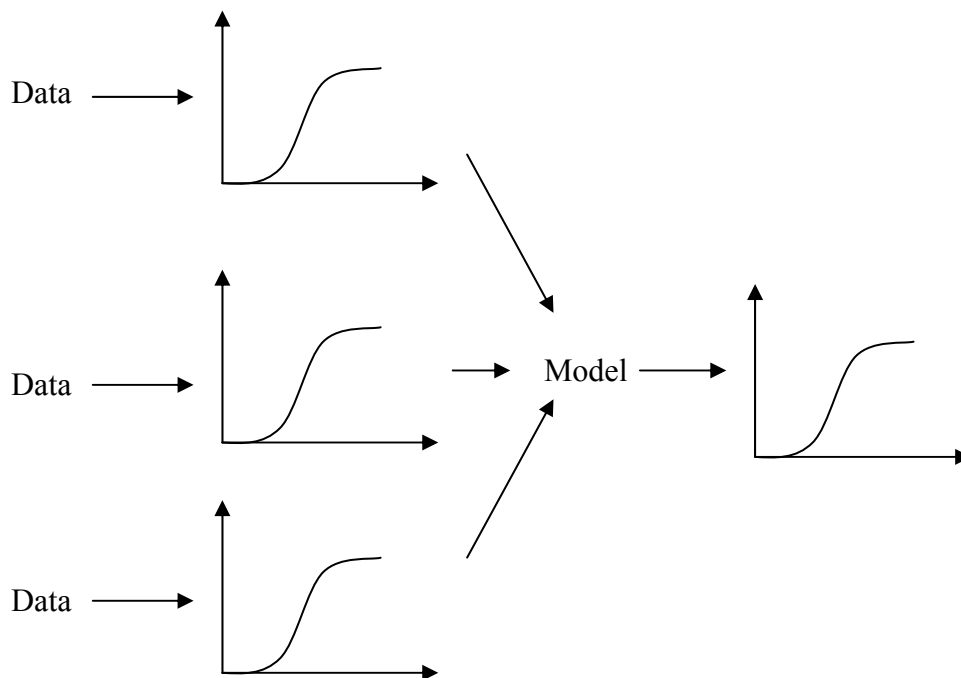
In probabilistic analysis, the variables are described in terms of distributions instead of point estimates. In this way all possible values for each variable are taken into account and each possible outcome is weighted by the probability of its occurrence. Different techniques are available to calculate the outcome distribution (Vose, 2000). However, this text will only discuss Monte Carlo simulation.

Monte Carlo simulation has been extensively described in literature (Cullen & Frey, 1999; Vose, 2000) and will be discussed briefly in this text. Monte Carlo simulation involves the random sampling of each probability distribution within the model. In this way one value is obtained for each distribution and entered in the model. The model is then solved, as it would be for any deterministic analysis and the result is stored. This process, iteration or trial is repeated several (hundreds or thousands) times until the specified number of iterations is completed. Instead of obtaining a discrete number for model outputs (as in a deterministic simulation) a set of output samples is obtained (Figure 1.8) (Cullen & Frey, 1999; Vose, 2000; Rousseau et al., 2001). Each probability distribution is sampled in a manner that reproduces the distribution's shape (Vose, 2000). This technique is named first order Monte Carlo simulation or one-dimensional Monte Carlo simulation.

The process of setting up and running the models requires appropriate modelling software and a high level of computer processing power. There are a variety of risk analysis software products on the market, which include software for modelling and distribution fitting. Examples of software products are @RISK (Palisade, UK), Crystal ball (Decisioneering, UK) and Fare Microbial (Exponent, USA - free software).

An important advantage of probabilistic risk assessments is that it permits to consider the whole distribution of exposure. In this way, more meaningful information is provided to risk managers and public. A second important advantage is the possibility to carry out a sensitivity analysis (see section 1.6.5 of this chapter) (Finley & Paustenbach, 1994). However, an important disadvantage of the current probabilistic risk assessment procedures is the need for

accurate prediction of the tails in a distribution (e.g. the 5<sup>th</sup> and 95<sup>th</sup> percentile). These tails are very important in performing a QRA because they represent the highest exposures (e.g. 95<sup>th</sup> percentile) and the most sensitive population (e.g. 5<sup>th</sup> percentile) (Verdonck et al., 2001). Also the high degree of complication and time-consumption are a disadvantage (Finley & Paustenbach, 1994). The reliability of the results of a probabilistic analysis depends on the validity of the model and the quality of the model inputs. The latter reflects both the quality of the data on which the input will be based and the selection of the distribution to represent the data in the model (Kroes et al., 2002).



**Figure 1.8: The principle of a first order Monte Carlo simulation (based on Verdonck, 2003)**

The distributions that are used as a model input can be parametric or non-parametric. In a parametric approach, a theoretical distribution function is used to describe the data instead of the collected data itself. In order to have a good representation of the dataset, the parameters (e.g. mean, variance) of the probability distribution are to be estimated (Cullen & Frey, 1999). In a non-parametric approach, the collected data points themselves are considered to form the distribution function (no fitting to a pre-defined model) (Vose, 2000).

### **1.6.3. Simple distributions**

In the context of exposure assessments, ‘simple distributions’ is a term used to describe a method that is a combination of the deterministic and probabilistic approach. It employs a distribution for the food consumption, but it uses a fixed value for the level of the hazard. The results are more informative than those of the point estimates, because they take account of the variability that exists in food consumption patterns. Nonetheless, they usually retain several conservative assumptions (e.g. all soft drinks that an individual consumes contain a particular sweetener at the maximum permitted level; 100% of a crop has been treated with a particular pesticide, etc.) and can therefore only be considered to give an upper bound estimate of exposure (Kroes et al., 2002).

Whether a probabilistic or deterministic risk assessment will be performed depends on a number of factors, including the degree of required accuracy and the availability of data (Parmar et al., 1997). No single method can meet all the choice criteria that refer to cost, accuracy, time frame, etc. Therefore, the methods have to be selected and combined on a case-by-case basis (Kroes et al., 2002).

### **1.6.4. Variability versus uncertainty**

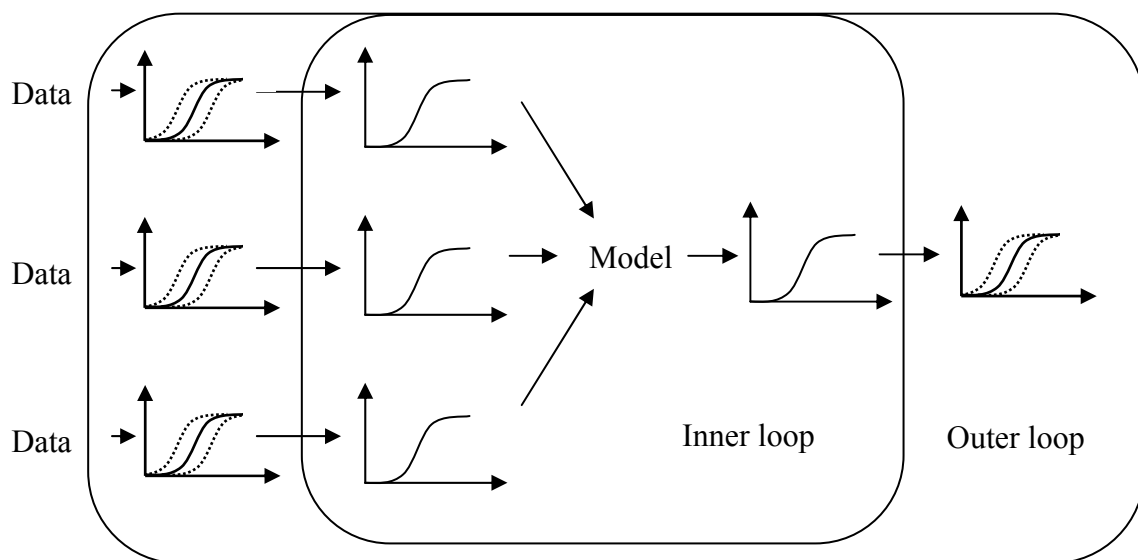
The inability to precisely predict what will happen in the future is due to two components: variability and uncertainty. Variability represents inherent heterogeneity or diversity in a well-characterised population. Fundamentally a property of nature, variability is usually not reducible through further measurement or study (e.g. biological variability of species sensitivity or the variability of the consumed amount of food). As explained in section 1.6.1. and 1.6.2. of this chapter, deterministic risk assessment does not take the variability into account, while probabilistic risk assessment includes variability in the calculations (Verdonck et al., 2001).

Uncertainty (epistemic uncertainty) represents the lack of perfect knowledge of a parameter value (e.g. measurement error) that can be partly reduced through further research (Vose, 2000). Different sources and types of uncertainty can be defined as explained by EFSA (2006a). For each percentile of the variability distribution, an uncertainty or confidence interval can be calculated (i.e. the uncertainty distribution) (Verdonck et al., 2001). Techniques such as bootstrapping can be used to get a measure of the uncertainty associated



with parameters of a distribution due to sample size (Cullen & Frey, 1999). The more limited the data set, the wider the confidence intervals will be.

The separation of uncertainty and variability as sources of variation of the model parameters is an important issue, since they are two different concepts (Nauta, 2000). Until now, models that make the separation between variability and uncertainty are still scarce. This is caused by the fact that it is often very difficult to separate uncertainty and variability on the basis of the available data and that more sophisticated simulation techniques are required (Nauta, 2000). To separate variability and uncertainty in a QRA, a second order or 2-dimensional Monte Carlo simulation is needed (Burmaster & Wilson, 1996; Cullen & Frey, 1999). It simply consists of two Monte Carlo loops, one nested inside the other (Figure 1.9). The inner loop deals with the variability of the input variables, while the outer one deals with uncertainty (Verdonck, 2003). First a distribution is selected randomly from every uncertainty band (outer loop). Then this distribution is used in the inner loop and a first order Monte Carlo simulation is carried out. The result of this process is a distribution. When this process is repeated a large number of times, a large number of output distributions is obtained and an uncertainty band can be constructed.



**Figure 1.9: The principle of a second order Monte Carlo simulation (based on Verdonck, 2003); the full line represents the best estimated variability distribution and the dashed line represents the uncertainty band**

### **1.6.5. Sensitivity analysis**

A sensitivity analysis is performed to determine which variables in the model have the largest influence on the results. This is achieved by measuring the variation in its outputs resulting from changes to its inputs (CAC, 1999).

The results of a sensitivity analysis permit risk managers to consider the relative merits of different strategies for reducing exposure in cases where levels of exposure are deemed unacceptably high. Because the probabilistic analysis provides information on the full distribution of exposures, the exposure assessor can determine how different scenarios will affect different sections of the distribution. For examples, different scenarios of modelling nutrient supplementation or fortification can be applied to evaluate the impact at the lower tail of the distribution (possible inadequate nutrient intake) and the upper tail of the distribution (possible nutrient toxicity) (Kroes et al., 2002).

A sensitivity analysis method should be able to: deal with simultaneous variation in inputs; address specific model characteristics such as nonlinearity, interactions, and thresholds; handle alternative input types (continuous versus categorical); discriminate among sensitive inputs; and provide quantitative measures of sensitivity (Mokhtari & Frey, 2005). A detailed comparison of the capabilities of many sensitivity analysis methods with regard to these characteristics is given by Frey et al. (2004).

## **2. Patulin, a mycotoxin of potential concern**

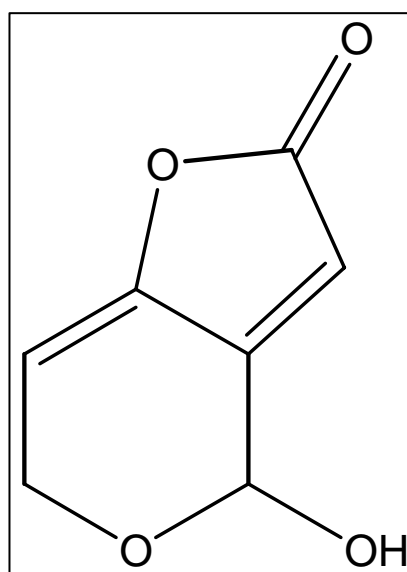
### **2.1. Introduction**

In the 1940's, patulin was isolated as an antibiotic during the search for new antibiotics after the discovery of penicillin by Fleming in 1929 (Stott & Bullerman, 1975). Initially, patulin was isolated as a broad-spectrum antifungal antibiotic, but was later found to inhibit more than 75 different bacterial species, including Gram-positive and Gram-negative bacteria (Moake et al., 2005). Although, a clinical trial suggested the application of this compound for treatment of nasal congestion and common cold (Raistrick et al., 1943), it was not used for this purpose due to numerous negative health effects that were attributed to it (Moake et al., 2005). Later on, patulin was found in foods and was classified as a mycotoxin.

Patulin is produced as a secondary metabolite by more than 60 fungi species (Lai et al., 2000), including the genera of *Penicillium*, *Aspergillus*, *Byssochlamys* and *Alternaria*. *Penicillium expansum* is the most important producer of this toxin and the cause of 'blue mould rot', a major post-harvest disease of apples worldwide (Rosenberger, 2003). Patulin biosynthesis is well understood and involves a series of condensation and redox reactions, of which a complete overview is given by Moake et al. (2005).

## 2.2. Chemical properties and stability

Patulin or 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one is an  $\alpha,\beta$ -unsaturated, heterocyclic  $\gamma$ -lactone (Figure 1.10). It is stable in an acidic environment (pH 3.3-6.3) (Lovett & Peeler, 1973), but unstable in an alkaline solution due to hydrolysis of the lactone ring (Dombrink-Kurtzman & Blackburn, 2005). *P. expansum* is capable of producing organic acids during sugar metabolism, by which the stability of patulin is improved (McCallum et al., 2002). Patulin is relatively stable to thermal degradation in the pH range of 3.5 to 5.5, with a lower pH leading to greater stability (Lovett & Peeler, 1973; Heatley & Philpot, 1947). A heat treatment of apple juice containing patulin at 90 and 100°C during 5 minutes results in a limited reduction of 6 and 13% respectively (Kadalkal & Nas, 2003).



**Figure 1.10: Structural formula of patulin**

Due to its electrophilic character, patulin is able to bind with thiol and amino groups of for example glutathione, cysteine, thioglycolate and proteins (Lieu & Bullerman, 1978; Fliege & Metzler 1999a; Fliege & Metzler 2000). The low levels of sulphhydryl groups in apple juice compared to other fruit juices like orange juice, explain at least partially the stability of patulin in apple juices (Doores, 1983; Moake et al, 2005). Presence of ascorbic acid or ascorbate causes a reduction of patulin in apple juice in a concentration-dependent manner (Brackett & Marth, 1979a; Aytac & Acar, 1994). Patulin is decomposed by free radicals that are generated by the reaction of ascorbic acid to dehydroascorbic acid (Drusch et al., 2007). Treatments with sulphur dioxide have been tested for the decontamination of apple juice, but no consistent results were obtained. Most studies agree that patulin is unstable in the presence

of sulphur dioxide (Pohland & Allen, 1970), but they vary on its degree of effectiveness (Moake et al., 2005).

Patulin is almost completely degraded during yeast fermentation. Approximately 90% of patulin can be removed during yeast fermentation (Burroughs, 1977). However, breakdown products such as ascadiol are produced and the toxicity of these compounds is not fully understood. A study with *Saccharomyces cerevisiae* showed that patulin decrease is dependent on the growth conditions of the yeast and occurs only during fermentative growth and not during aerobic growth (Moss & Long, 2002).

### **2.3. Occurrence of patulin**

Patulin producing fungi have been isolated from a variety of foods such as apples (Frank et al., 1976; Hasan, 2000), grapes (Sommer et al., 1974), pears, apricots (Buchanan et al., 1974; Sommer et al., 1974), tomatoes (Harwig et al., 1979), dried beans (Mislivec et al., 1975; Trucksess et al., 1987) and macaroni (Trucksess et al., 1987). However, the presence of patulin producing fungi does not necessarily mean that patulin will be present in the food product. Growth of *P. expansum* and patulin production is influenced to a large extent by environmental and intrinsic factors typical for the substrate (Drusch & Ragab, 2003). The most important ones are water activity ( $a_w$ ) and temperature, although atmosphere, pH and other chemical characteristics intrinsic to fruits have also a large influence (Northolt et al., 1978; McCallum et al., 2002). Table 1.3 gives an overview of the temperature and  $a_w$  range that allows growth and patulin production by *P. expansum*. This overview shows that patulin production occurs over a narrower  $a_w$  and temperature range than growth (Roland & Beuchat, 1984).

Despite the fact that patulin occurs mainly in apples and derived products like apple juice, also other foods can contain patulin (Table 1.4). A Scientific Cooperation (SCOOP) study conducted by the Directorate – General Health and Consumer Protection of the European Union (EU) on the assessment of the dietary intake of patulin by the population of EU member states showed that apple juice and apple nectar is the main source of patulin intake in most countries, particularly for young children (Directorate – General Health and Consumer Protection, 2002).

**Table 1.3: Temperature and water activity ( $a_w$ ) range for growth and patulin production by *P. expansum***

	Minimum	Reference	Maximum	Reference
<b>Growth</b>				
$a_w$	[0.83;0.85]	Drusch & Ragab, 2003		
	0.85	Northolt & Bullerman, 1982		
Temperature	[-6;-2°C]	Pitt & Hocking, 1997	35°C	Pitt & Hocking, 1997
	0°C	Sommer et al., 1974	33°C	Northolt & Bullerman, 1982
<b>Patulin production</b>				
$a_w$	0.97	Northolt & Bullerman, 1982		
	0.99	Drusch & Ragab, 2003		
	0.95	Pitt & Hocking, 1997		
Temperature	0°C	Sommer et al., 1974; Moake et al., 2005	30°C	Moake et al., 2005

**Table 1.4: Foods contaminated with patulin**

Food	Reference
Apple	Hasan, 2000; Martins et al., 2002; Piemontese et al., 2005
Apple cider	Wheeler et al., 1987; Tangni et al., 2003
Apple juice	Lindroth & Niskanen, 1978; Brackett & Marth, 1979b; Prieta et al., 1994; Gökmen & Acar, 1998; Rychlik & Schieberle, 1999; Yurdun et al., 2001; Tangni et al., 2003; Cheraghali et al., 2005; Piemontese et al., 2005; Watanabe & Shimizu, 2005
Apple nectar	Directorate – General Health and Consumer Protection, 2002
Apple puree	Ritieni, 2003
Baby food	Beretta et al., 2000; Directorate – General Health and Consumer Protection, 2002; Ritieni, 2003;
Blackcurrant juice	Rychlik & Schieberle, 1999
Blueberry	Weidenbörner, 2001
Bread	Rychlik & Schieberle, 1999; Moake et al., 2005
Cheese	Weidenbörner, 2001; Moake et al., 2005
Cereals and corn	Dutton & Westlake, 1985; Lin et al., 1993; Weidenbörner, 2001
Cranberry	Weidenbörner, 2001
Dried apple	Directorate – General Health and Consumer Protection, 2002
Dried figs	Karaka & Nas, 2006
Grape juice	Rychlik & Schieberle, 1999; Directorate – General Health and Consumer Protection, 2002
Jam	Lindroth & Niskanen, 1978; Moake et al., 2005
Kokonte <sup>5</sup>	Wareing et al., 2001
Oil seeds	Dutton & Westlake, 1985
Orange juice	Rychlik & Schieberle, 1999
Passion fruit juice	Moake et al., 2005
Peach	de Sylos & Rodriguez-Amaya, 1999
Pear	de Sylos & Rodriguez-Amaya, 1999
Pear juice	Lehmann & Wald, 1990; Piemontese et al., 2005
Pineapple juice	Moake et al., 2005
Plum	Weidenbörner, 2001
Raspberry syrup	Rychlik & Schieberle, 1999
Sour cherry	Rychlik & Schieberle, 1999
Tomato puree	Directorate – General Health and Consumer Protection, 2002

<sup>5</sup> dried cassava product

Removal of the moulded part from a food product is not sufficient to remove all patulin due to the migration of the toxin. In apples, patulin can be detected up to a distance of 2 cm from the occurring lesion. In tomatoes however, the mycotoxin is able to penetrate through the whole fruit and this is a consequence of the fact that tomatoes contain less structural polysaccharides and much more water which makes them less viscous and easily permeable for patulin (Taniwaki et al, 1992; Rychlik & Schieberle, 2001; Marin et al., 2006a).

## **2.4. Toxicology**

During the last 60 years, several adverse health effects resulting from exposure to patulin have been described. Briefly, patulin is reported to be acutely toxic (Escoula et al., 1977; McKinley & Carlton, 1980a and 1980b), genotoxic (Alves et al., 2000; Liu et al., 2003; Schumacher et al., 2003), cytotoxic (Riley & Showker, 1991), immunosuppressive (Escoula et al., 1988a and 1988b), teratogenic (Ciegler et al., 1976; Sugiyanto et al., 1993) and possible neurotoxic (Devaraj et al., 1982). As described above, patulin has a strong affinity for sulphhydryl groups, which causes the inactivation of enzymes (Arafat et al., 1985). Although no specific studies on the mode of action of patulin have been performed, it is becoming clear that this ability of patulin to react with sulphhydryl groups would explain the cytotoxic and some of the genotoxic effects (Speijers, 2004). However, it is unlikely that the toxicity is systemic since patulin is degraded quickly after penetrating the gastric wall. This degradation is caused partly by the reaction with glutathione and probably also by the reaction with proteins. However, the significant depletion of glutathione in gastric tissue can lead to local toxic effects (Rychlik, 2003; Rychlik et al., 2004). For many years patulin was believed to be carcinogenic based on a study of Dickens and Jones (1961), however the International Agency for Research on Cancer (IARC) concluded that there is inadequate evidence for carcinogenicity of patulin in experimental animals (IARC, 1987).

A study on the combined effects of patulin on reproduction and long-term toxicity pointed to a safe intake of 43 µg/kg body weight/day. On the basis of this work and using a safety factor of 100, the Joint FAO/WHO Expert Committee on Food Additives set a provisional maximum tolerable daily intake of 0.4 µg/kg body weight (JECFA, 1995). Based on this TDI, patulin is regulated in the European Union at levels of 50 µg/kg in fruit juices and fruit nectar, 25 µg/kg in solid apple products and 10 µg/kg in apple-based products for infants and young

children (European Commission, 2003b). A complete and detailed overview of the health effects ascribed to patulin is given by Speijers (2004).



### Free and bound patulin in cloudy apple juice<sup>1</sup>

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<sup>1</sup> Based on: Baert, K., De Meulenaer, B., Kasase, C., Huyghebaert, A., Ooghe, W., Devlieghere, F., 2007. Free and bound patulin in cloudy apple juice. *Food Chemistry* 100, 1278-1282.



## Chapter 2

### Free and bound patulin in cloudy apple juice

#### Summary

During validation of an HPLC-UV method for patulin analysis, a time and concentration dependent recovery of patulin was observed. Spiked cloudy apple juice was analysed on successive days, which resulted in recoveries on day 3 which were up to 20% lower than on day 1. This reduction was caused by an interaction between patulin and the solid particles of cloudy apple juice. Since these solid particles are richer in proteins compared to the liquid phase of cloudy apple juice, and the binding of patulin to proteins has been described in literature, patulin will most probably interact with the proteins in the solid particles. As a consequence, up to 20% of the present patulin is bound and not detected during HPLC-UV analysis, which can lead to an underestimation on toxicological level.

## **1. Introduction**

Many different methods have been developed for the detection of patulin (Scott & Kennedy, 1973; Ware et al., 1974; Subramanian, 1982; Brause et al., 1996; Sheu & Shyu, 1999; Rupp & Turnipseed, 2000; Aktas et al., 2004; Ito et al., 2004; Gökmen et al., 2005; Llovera et al., 2005; Ha & Sabino, 2006; de Champdoré et al., 2007). At the moment, high performance liquid chromatography with ultraviolet light detection (HPLC-UV) is the most frequently used method, since patulin is relatively polar and exhibits a specific absorption wavelength at 276 nm (Gökmen & Acar, 1996; Yurdun et al., 2001). In this chapter the analytical procedure described by MacDonald et al. (2000) based on HPLC-UV was implemented. The method was adapted to improve the performance and was validated. During the validation of the method, it was observed that the recovery of spiked patulin decreased during storage. Therefore, a study was conducted to investigate the observed phenomenon.

## **2. Materials and methods**

### **2.1. Patulin analysis**

#### **2.1.1. Reagents and chemicals**

Ethanol (pa), ethyl acetate (pa), anhydrous sodium carbonate (pa), ultra pure water and acetic acid 99-100% (pa) were purchased from Chemlab, Belgium. Acetonitrile (HPLC-grade) was obtained from Fisher scientific, UK. Perchloric acid (pa) and 5-(hydroxymethyl) furfural (HMF) 98% were purchased from Acros Organics, Belgium. Anhydrous sodium carbonate (pa) was obtained from VWR, Belgium. Patulin was purchased from Sigma, Belgium. Pectinex 1X L was obtained from Novozymes, Switzerland.

#### **2.1.2. Preparation of stock and working standard solutions of patulin**

A stock standard solution of patulin was prepared by dissolving 5 mg of patulin in 25 ml of ethyl acetate. A 1000 µl volume of this solution was transferred to a 20 ml volumetric flask and evaporated until dryness under a stream of nitrogen at room temperature. The residue was immediately dissolved in 20 ml of ethanol. A stock standard solution of HMF was prepared by dissolving 5 mg HMF in 25 ml ethyl acetate and further diluting 1152 µl of this solution to a final volume of 20 ml with ethyl acetate. The working standard solutions were prepared by

evaporating 500  $\mu$ l of the patulin stock standard solution and 500  $\mu$ l of the HMF stock standard solution until dryness under a stream of nitrogen at room temperature and the residue was immediately dissolved in 2.5 ml water acidified with acetic acid to pH 4.0 (water at pH 4.0). The working standard solutions were prepared by making appropriate dilutions of this solution with water at pH 4.0. The stock standard solution was stable at  $-20^{\circ}\text{C}$  for several months and the working solution was stable at  $7^{\circ}\text{C}$  for one week.

### **2.1.3. Sample preparation for patulin analysis**

Since patulin is unstable in the presence of alkaline compounds, all glassware was rinsed with ethyl acetate or water at pH 4.0 to remove alkaline residues that can be present due to the washing of the glassware. Cloudy apple juices (20 ml) were pre-treated with pectinase (10 drops) and incubated overnight in the dark at room temperature. For the solid particles of cloudy apple juice 10 ml of water and 10 drops of pectinase were added to 10 g of the solid particles and the mixture was incubated under the same conditions as cloudy apple juice. The samples were centrifuged at 2560 g for 5 minutes. No pre-treatment was required for clear apple juice. The centrifugate or the clear apple juice (10 ml) was extracted three times with ethyl acetate (20 ml) by shaking vigorously for 1 minute. The organic phases were combined and extracted with 4 ml of 1.5% (w/v) sodium carbonate solution by shaking vigorously for 30 seconds to remove interfering acidic compounds. The organic phase was dried by pouring it into a round-bottomed flask through a funnel with filter paper containing 15 g anhydrous sodium sulphate. The sodium carbonate solution was extracted immediately with 10 ml ethyl acetate by shaking for 30 seconds. The organic phase was also dried with anhydrous sodium sulphate and the organic phases were combined. The sodium sulphate was washed 2 times with 10 ml of ethyl acetate and the ethyl acetate was collected in the round-bottomed flask. Then the extract was evaporated under reduced pressure at  $45^{\circ}\text{C}$  until a volume of about 3 ml. Further evaporation to dryness was carried out under a gentle stream of nitrogen at room temperature. The residue was immediately dissolved in 500  $\mu$ l of water at pH 4.0. The samples were filtered before injection using a Millex-HV syringe Driven Filter Unit (pore size 0.45  $\mu\text{m}$ ) (Millipore, USA).

### **2.1.4. HPLC-UV analysis of patulin**

The HPLC was a Varian Vista 5500 (USA). It was equipped with a Rheodyne Model 7125 six-way injector with a 20  $\mu$ l loop. An Inertsil 5 ODS-2 Stainless Steel column (5  $\mu\text{m}$ , 250

mm x 4.6 mm), protected by a guard column, was used (Varian, USA). As mobile phase acetonitrile/perchloric acid in water (0.0175% (v/v)) (7/93, v/v) was used at 1 ml/min. After 10 minutes a change was made to acetonitrile at 2 ml/min to elute polluting compounds from the extract. After 10 minutes there was again a shift to acetonitrile/perchloric acid in water (0.0175% (v/v)) (7/93, v/v) at 1 ml/min. The column and guard column were maintained at 36°C during the HPLC analysis. Detection was carried out using a Gilson 119 UV/VIS detector set at 276 nm. Data collection and subsequent processing were performed using Gilson Unipoint software (USA).

## 2.2. Validation

The complete validation procedure, except the determination of the linearity, was carried out separately for clear and cloudy apple juice.

**Linearity** was tested using working standard solutions of patulin in the concentration range of 0.1 to 2 µg/ml. The analytical procedure was performed in duplicate and 11 data points were used.

**Recovery** was determined using spiked apple juice in the concentration range of 5 to 100 µg/kg. The analytical procedure was performed in duplicate and 11 data points were used.

For the **limit of detection** (LOD) and **limit of quantification** (LOQ) 10 samples of apple juice, without patulin, were analyzed. To be sure that no patulin was present in the apple juice, fresh apple juice was made from fresh apples that were washed and inspected for damages. The LOD and LOQ were calculated using equation 2.1 and 2.2 (Liteanu & Rîcă, 1980).

$$LOD = \bar{x}_{bl} + 3 \times SD_{bl} \quad (2.1)$$

$$LOQ = \bar{x}_{bl} + 6 \times SD_{bl} \quad (2.2)$$

with  $\bar{x}_{bl}$  = mean of the 10 samples

$SD_{bl}$  = standard deviation of the 10 samples

The **reproducibility** was determined using spiked apple juice with 11 different concentrations in the range of 5 to 100 µg/kg. The analytical procedure was performed in duplicate. The ratio

between the difference of the two measurements for the highest concentration and the difference for the lowest concentration has to be lower than 25 (equation 2.3);

$$\frac{|c_{11,1} - c_{11,2}|}{|c_{1,1} - c_{1,2}|} < 25 \quad (2.3)$$

with  $c_{11,1}$  and  $c_{11,2}$  = results of the duplicates for the highest concentration tested

$c_{1,1}$  and  $c_{1,2}$  = results of the duplicates for the lowest concentration tested

When the ratio is higher than 25, the reproducibility of the method decreases with increasing concentration. In this case, the range has to be split in two ranges and for every range the reproducibility is determined. The standard deviation and the inter-laboratory reproducibility were calculated using equation 2.4 and 2.5 (ISO, 1989).

$$SD = \sqrt{\frac{\sum_{i=1}^k (c_{i,1} - c_{i,2})^2}{(2 \times k)}} \quad (2.4)$$

$$r = 2.8 \times SD \quad (2.5)$$

with  $SD$  = inter-laboratory standard deviation

$c_{i,1}$  and  $c_{i,2}$  = concentrations of the duplicates

$k$  = number of independent samples

$r$  = inter-laboratory reproducibility

Due to a lack of Certified Reference Material, an apple juice containing patulin was purchased at Fapas. This apple juice had been tested in the past during a Fapas proficiency test and was delivered together with an assigned value. This apple juice was analysed in the lab to assess the **accuracy** of the method and the result of the analysis was used to determine the z-score (equation 2.6) (Fapas, 2002).

$$z = \frac{(x - X)}{\sigma_p} \quad (2.6)$$

with  $x$  = the participant's reported result

$X$  = the assigned value

$\sigma_p$  = the target value for standard deviation

### **2.3. Separation of cloudy apple juice to a liquid and solid phase**

Commercial cloudy apple juice, without detectable patulin was used. In order to fractionate cloudy apple juice, pectinase (10 ml) was added to cloudy apple juice (500 ml) and incubated during 2 hours at 37°C. The apple juice was centrifuged at 2560 g for 5 minutes.

### **2.4. Spiking and storage experiments**

The appropriate matrix (apple juice or solid phase or cloudy apple juice) was spiked by evaporating the appropriate amount of the patulin stock standard solution and dissolving the residue immediately in the matrix. The apple juice was stored at 7°C in the dark until analysis.

### **2.5. Total amino acid content**

Amino acid analysis was carried out using the method described by Ooghe (1983). Lithium acetate dihydrate, caprylic acid, trifluoroacetic acid and formic acid were supplied by Fluka Chemie GmbH Buchs, Germany. Methanol (HPLC-grade) was delivered by Labscan Ltd, Ireland. The total amino acid profile was determined for clear apple juice, the liquid phase of cloudy apple juice (without the solid particles) and cloudy apple juice. Based on the difference in total amino acid profile between cloudy apple juice and the liquid phase of cloudy apple juice and the amount of solid particles in cloudy apple juice, the amino acid profile of the solid particles of cloudy apple juice was determined.

## **3. Results**

### **3.1. Validation**

Firstly the above described method for patulin analysis was completely validated. The standard curve of patulin was linear in the range of 2 to 40 ng. The slope of the linear regression was found to be  $174.25 \pm 1.81$  and the intercept  $-3.93 \pm 2.17$  ( $R^2 = 0.9932$ ).

The recovery rates were determined using spiked apple juice with 11 different concentrations in the range of 5 to 100 µg/kg. The analytical procedure was performed in duplicate. For clear apple juice a recovery rate of 95% was determined, however for cloudy apple juice a time dependent recovery of patulin was observed (Table 2.1). When spiked cloudy apple juice was analysed on successive days, recoveries on day 3 were up to 20% lower than on day 1. This



observation was not reported before in literature. This reduction was further studied as described in section 3.2 of this chapter. For cloudy apple juice a recovery of 75% was obtained.

**Table 2.1: Recovery for cloudy apple juice at 3 concentrations for day 1, day 2 and day 3**

Spiked concentration on day 0 ( $\mu\text{g}/\text{kg}$ )	Recovery (%)		
	Day 1	Day 2	Day 3
15	92	76	74
50	90	74	73
100	91	81	79

The implemented analytical procedure for patulin determination, had a limit of detection (LOD) of  $5.2 \mu\text{g}/\text{kg}$  and a limit of quantification (LOQ) of  $8.1 \mu\text{g}/\text{kg}$  for clear apple juice. For cloudy apple juice, the LOD and LOQ were respectively  $8.6 \mu\text{g}/\text{kg}$  and  $16.0 \mu\text{g}/\text{kg}$ .

The reproducibility was determined for the standards and for spiked apple juice, to separate the effect of the HPLC-UV analysis and the sample preparation. The reproducibility was  $0.03 \mu\text{g}/\text{ml}$ , within the studied concentration range, for the patulin standards (injected concentration) or  $1.3 \mu\text{g}/\text{kg}$  in apple juice. This reproducibility can be ascribed to the HPLC-UV analysis. Statistical analysis revealed that the reproducibility for clear apple juice depended upon the concentration of patulin in the apple juice. Therefore, the range from 5 to  $100 \mu\text{g}/\text{kg}$  was divided into 2 regions : 5 to  $45 \mu\text{g}/\text{kg}$  and 50 to  $100 \mu\text{g}/\text{kg}$ . The reproducibility for clear apple juice was  $1.6$  and  $10.8 \mu\text{g}/\text{kg}$  for the range 5 to  $45 \mu\text{g}/\text{kg}$  and 50 to  $100 \mu\text{g}/\text{kg}$  respectively. These results indicate that the relative reproducibility decreases for the higher concentrations. The reproducibility of cloudy apple juice was  $7.9 \mu\text{g}/\text{kg}$ . A comparison of the reproducibility for the standards and for spiked apple juice samples showed that  $0.3 \mu\text{g}/\text{kg}$  (5 to  $45 \mu\text{g}/\text{kg}$  range) and  $9.5 \mu\text{g}/\text{kg}$  (50 to  $100 \mu\text{g}/\text{kg}$  range) of the reproducibility can be ascribed to sample preparation for clear apple juice and  $6.6 \mu\text{g}/\text{kg}$  for cloudy apple juice.

Because of the lack of certified reference material, the accuracy of the method was determined using samples of apple juice, which had been tested in a Fapas proficiency test. A z-score of 0.22 and  $-0.12$  was achieved for clear and cloudy apple juice respectively.

### 3.2. Interaction between patulin and solid particles

In a first series of confirmatory experiments, cloudy apple juice was separated in a clear liquid and a solid phase. These two fractions and commercial clear apple juice (included as a reference) were spiked with patulin at 70 µg/kg and analysed on their patulin content after 1, 2, 3 and 6 days of incubation in similar conditions as applied before (analysis after 6 days only for the solid particles of cloudy apple juice). Results are shown in Table 2.2. For clear apple juice, the recovery remained constant throughout the whole incubation period and for the liquid phase of cloudy apple juice there was a decrease from 95 % on day 1 to 86% on day 3. However, for the solid particles a major loss of the recovered patulin was observed (95% on day 1 while on day 6 only 49% was recovered).

**Table 2.2: Recovery for 3 spiked matrices (clear apple juice (AJ), liquid phase of cloudy apple juice and solid phase of cloudy apple juice) for day 1, day 2, day 3 and day 6**

Matrix	Recovery (%)			
	Day 1	Day 2	Day 3	Day 6
Clear AJ	95	94	94	
Liquid phase of cloudy AJ	95	91	86	
Solid phase of cloudy AJ	95	88	70	49

In a subsequent series of experiments, the mechanism of the observed phenomenon was elaborated. Therefore, the total amino acid profile, including both free and protein bound amino acids, was determined (Table 2.3). In clear apple juice and the liquid phase of cloudy apple juice, 0.073 g and 0.107 g of amino acids were present in 100 g of product, respectively. In the solid phase of the cloudy apple juice however, the total amino acid content amounted up to 0.792 g per 100 g of product. So up to 10 times more amino acids were found in the solid phase of the cloudy apple juice. Considering the relative concentration of each amino acid on total amino acids basis however, no significant difference could be observed between the various juices and fractions analyzed.

**Table 2.3: Total amino acid profile of clear apple juice (AJ), liquid phase of cloudy apple juice and solid phase of cloudy apple juice**

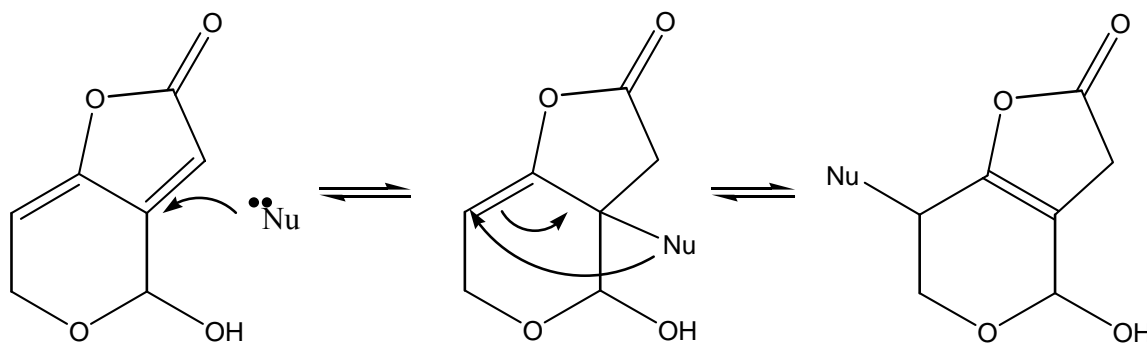
Amino acid	Level of amino acid (g/100 g)		
	Clear AJ	Liquid phase of cloudy AJ	Solid phase of cloudy AJ
Asp	0.056	0.059	0.566
Thr	0.001	0.004	<0.057
Ser	0.002	0.004	<0.057
Asn	<0.001	<0.001	<0.057
Glu	0.007	0.012	0.113
Gln	<0.001	<0.001	<0.057
Pro	0.001	0.002	<0.057
Gly	0.001	0.003	<0.057
Ala	0.002	0.004	0.057
Val	0.001	0.003	<0.057
Cys	<0.001	0.001	<0.057
Met	<0.001	0.001	<0.057
Ile	0.001	0.002	<0.057
Leu	<0.001	0.003	<0.057
Tyr	<0.001	0.002	<0.057
Phe	0.001	0.002	<0.057
His	<0.001	0.001	<0.057
Lys	<0.001	0.002	0.057
Arg	<0.001	0.002	<0.057
Total	0.073	0.107	0.79

## 4. Discussion

As shown in the first series of experiments, a decrease in patulin recovery was observed if cloudy apple juice was spiked with this mycotoxin. Remarkably as well, the recovery remained constant upon 2 days of incubation of the spiked cloudy apple juice as a function of storage time. Therefore, because of the acidic pH of apple juice and the selected storage conditions (maximum 7°C and dark), patulin degradation as such seems unlikely to be responsible for the observed decreased recovery. During a similar spiking and storage

experiment on the clarified and cloudy portion of a cloudy apple juice, it became obvious that the solid particles were responsible for the observed decrease in patulin recovery. This is in agreement with the significant lower recovery observed in spiked cloudy apple juice compared to clear juice. These results are moreover in agreement with the previously reported observation by Bissessur et al. (2001) that the patulin contamination of cloudy apple juice can be reduced upon clarification and that the solid residue becomes enriched with patulin. These authors however, did not elaborate further on the mechanisms involved in these observations.

During research on the toxicity of patulin, it has been reported that patulin is able to bind with thiol groups of small molecules like glutathione and amino acids by means of a covalent binding (Lieu & Bullerman, 1978; Fliege & Metzler, 2000). Moreover, patulin is able to crosslink proteins. The functional groups of proteins involved in patulin-mediated crosslink formation were characterized as Cys-SH, Lys- $\epsilon$ NH<sub>2</sub>, His-NH, and  $\alpha$ -NH<sub>2</sub>. The multitude of potentially reactive sites combined with the ability of patulin to form heterobifunctional crosslinks implies that theoretically, every protein is a possible target for patulin-induced crosslink processes (Fliege & Metzler, 1999b). Possibly the interactions between patulin and proteins become more important in cloudy apple juice compared to clear apple juice. The amino acid profile of these two juices could be different which could lead to an altered crosslinking behaviour. Secondly, the amount of proteins and amino acids could be different in the two types of juice. In order to confirm these statements, the total amino acid profile of the 3 investigated matrices (clear apple juice, the liquid phase of cloudy apple juice and the solid phase of cloudy apple juice) was determined. No significant differences could be observed in the amino acid profile of the different matrices. The overall level of amino acids was however, significant higher in the solid particles than in the juice. About 10 times more amino acids were found in the solid part than in clear apple juice and almost 8 times more than in the liquid phase of cloudy apple juice. This confirms that patulin is able to interact with the solid particles in apple juice and most likely the interactions with the proteins in the solid particles of cloudy apple juice are responsible for this observation. The interaction is based on a Michael-addition (Fliege & Metzler, 1999b), as shown in Figure 2.1. Therefore, it seems unlikely that this covalent binding can be broken during extraction. In agreement, the binding of mycotoxins to matrix components has been reported in other studies. Kim et al. (2003) and Seefelder et al. (2003) observed the binding of fumonisin B<sub>1</sub> to polysaccharides and proteins. However, the mechanism of interaction for fumonisin B<sub>1</sub> is different as for patulin, since patulin is electrophilic, while fumonisin B<sub>1</sub> will act as a nucleophilic compound.



**Figure 2.1: Proposed reaction mechanism between patulin and proteins containing a nucleophilic group (Nu: nucleophilic group of a protein)**

Until now little attention has been given to the influence of bound patulin on the analytical performance of a method. At the moment it is not known what happens with the bound patulin in the gastrointestinal tract. Since release of patulin can not be excluded, this bound fraction of patulin should be included in the reported contamination level, otherwise an underestimation may occur. The present study shows that up to 20% of the patulin in cloudy apple juice is bound, resulting in an underestimation of 20% since this fraction is not determined during HPLC-UV analysis. Moreover, this study gives indirect proof that patulin binds to proteins present in apple juice. However, HPLC-analysis of the total amino acid profile of apple juice spiked with patulin in order to detect changes in amino acid concentration compared to apple juice without patulin would not be suited to elaborate this further. Due to the excess of amino acids compared to the amino acids bound to patulin, no decrease in amino acid content compared to apple juice without patulin would be observable. Also the acidic hydrolysis performed during the amino acid analysis could have an influence on the bound between patulin to the amino acids and release patulin. Therefore, LC-MS should be used to monitor the presence of the patulin–protein adducts in apple juice.

## 5. Conclusion

The validation of the method showed that the method gives accurate results and will be used in Chapter 3 for the analysis of patulin apple juice. Moreover, it was observed that patulin is able to interact with the solid particles of cloudy apple juice and this is probably due to the binding of patulin with proteins.



### Occurrence of patulin in organic, conventional and handcrafted apple juices marketed in Belgium<sup>1</sup>

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<sup>1</sup> Based on: Baert, K., De Meulenaer, B., Kamala, A., Kasase, C., Devlieghere, F., 2006. Occurrence of patulin in organic, conventional, and handcrafted apple juices marketed in Belgium. *Journal of Food Protection* 69, 1371-1378.





## Chapter 3

# Occurrence of patulin in organic, conventional and handcrafted apple juices marketed in Belgium

### Summary

The aim of this chapter was to compare the occurrence of patulin in a high number of organic, conventional and handcrafted apple juices marketed in Belgium. The validated method, described in Chapter 2, was used to analyse 177 apple juices, of which 65 juices were organic, 90 conventional and 22 handcrafted. Patulin was detected in 22 apple juices (12%) and quantification was possible in 10 (6%) of them. The patulin content exceeded the European maximum limit of 50 µg/kg in two organic apple juices. Although, the incidence of patulin in organic apple juice (12%), conventional apple juice (13%) and handcrafted apple juice (10%) was not significantly different ( $p = 0.863$ ), the mean level of patulin in contaminated samples was significantly higher in organic apple juice (43.1 µg/kg) than in conventional apple juice (10.2 µg/kg) ( $p = 0.02$ ) and in handcrafted apple juice (10.5 µg/kg) ( $p = 0.037$ ). Interestingly, cheaper apple juices are not more contaminated with patulin in comparison to expensive apple juices. On the contrary, it was observed that the highest patulin concentrations were determined for the most expensive apple juices. This observation can be attributed to the higher cost price of organic apple juice.

## 1. Introduction

Organic agricultural and food processing practices are wide ranging and overall seek to foster the development of a food production system that is socially, ecologically and economically sustainable (Bourn & Prescott, 2002). In Europe, organic food products are produced in accordance with the European regulation 2092/91 on organic production of agricultural products. This regulation has been amended several times over the years, but it is still the basis for organic production. This law regulates the labelling and production of organic products and also the inspection system. During production, the use of for example plant-protection products, detergents, fertilisers and soil conditioners is restricted (European Council, 1991). In Belgium and other European countries, organic food products became more popular as a result of several food crises in the recent past. In 1998 0.8% of the area of land used for agriculture in Belgium was worked organically and this had risen to 1.7% in 2004 (National Institute of Statistics, 2007b). In addition, the sale of organic products has increased. In Belgium, the turnover of organic products was 62.5 million euro in 1997 and subsequently increased by almost 400% to 311 million euro by 2002. An increase has also been reported for other European countries. From 1997 until 2002 there was an increase of 47%, 175%, 43% and 321% in Germany, France, The Netherlands and The United Kingdom, respectively (National Institute of Statistics, 2007a). However, the influence of the reduced use of pesticides on the presence of mycotoxins like patulin, is not yet clear. Two possible mechanisms are postulated. On one hand the reduced use of fungicides can result in an increase of mould growth. On the other hand the reduced use of insecticides, can result in an increase of insect damage, which can lead to more fungal invasion (Scientific Committee on plants, 1999). Until now, several studies have compared the occurrence of different mycotoxins in organic and conventional foods, but only conflicting results have been reported. Ghidini and co-workers reported that the level of aflatoxin M<sub>1</sub> in milk was significantly higher in organic than in conventional milk (Ghidini et al., 2005), while other studies showed that the mycotoxin level is higher in conventional than in organic foods (Döll et al., 2002; Schollenberger et al., 2002; Cirillo et al., 2003; Tafuri et al., 2004; Schollenberger et al., 2005). Also some cases have been reported where no difference was observed between the two types of agricultural practices (Skaug, 1999; Beretta et al., 2002; Malmauret et al., 2002; Biffi et al., 2004). For patulin in particular, six studies have been published (Beretta et al., 2000; Malmauret et al., 2002; Ritieni, 2003; Tangni et al., 2003; Boonzaaijer et al., 2005; Piemontese et al., 2005). However, no unequivocal results are

available on the occurrence of patulin in organic and conventional apple juice in Europe. Also for the Belgian situation in particular no clear results are available. An important limitation of the published studies is the limited amount of samples that are analysed for a certain apple product. Within the framework of probabilistic risk assessment, a large dataset is required to conduct a reliable probabilistic risk assessment. Since the use of probabilistic risk assessment is becoming more important within the framework of evaluating contaminants, this is an important criterion to conduct studies on the occurrence of contaminants in foodstuffs. Therefore, the aim of the present study was to compare the patulin contamination of organic and conventional apple juices consumed by the Belgian population for a high number of apple juices using the analytical method described in Chapter 2, in order to collect data that can be used for a probabilistic risk assessment.

## **2. Materials and methods**

### **2.1. Patulin analysis**

Patulin analysis of the apple juice samples was performed using the method described in Chapter 2 – section 2.1.

### **2.2. Samples**

A total of 177 apple juices (65 organic, 90 conventional and 22 handcrafted) were purchased at random in supermarkets, bio-shops (shops where only products of organic origin are sold), health food shops, farmers, small shops and discounters, in the Northern part of Belgium (Flanders). The total number of samples was based on the formula to calculate the sampling size based on the expected prevalence (EFSA, 2006). Every brand that was available in the visited shops was included in the study and for each brand 3 or 4 batches were analysed (1 sample per batch). The characteristics of the analysed apple juices are summarised in Table 3.1. Since only one handcrafted brand was organic, no separation was made between handcrafted organic and handcrafted conventional. The handcrafted, organic apple juice was included in the organic apple juices. The samples were shaken thoroughly and 200 ml stored at  $-20^{\circ}\text{C}$  for maximum one month until analysis. Each sample was analysed once and when patulin was detected above the limit of detection (LOD), a second confirmatory analysis was conducted. The recovery rate (Chapter 2 – section 3.1) was taken into account for the calculation of the patulin concentration in apple juice samples.

**Table 3.1: Summary of apple juice characteristics**

Agricultural practice <sup>a</sup>	Clear /cloudy	Additional information	Shop	Number of samples
O	cloudy		bio-shop	5
O	cloudy		HFS <sup>c</sup>	3
O	cloudy		supermarket	5
O	cloudy		farmer	1
O	cloudy	100% AJ <sup>b</sup> from organically grown apples	bio-shop	1
C	cloudy	100% jonagold AJ, addition of ascorbic acid	supermarket	7
O	cloudy	apple and orange juice, from the age of 4 months	bio-shop	1
O	clear	concentrate	bio-shop	2
C	cloudy	addition of ascorbic acid	supermarket	9
O	cloudy	freshly squeezed	HFS	2
O	cloudy	freshly squeezed	bio-shop	10
C	cloudy	freshly squeezed, natural cloudy	HFS	3
C	cloudy	freshly squeezed, natural cloudy	farmer	1
C	cloudy	freshly squeezed, natural cloudy	supermarket	1
C	cloudy	freshly squeezed, natural cloudy	shop	2
H	cloudy	from environment-friendly produced apples, pasteurised	shop	2
H	cloudy	from environment-friendly produced apples, pasteurised	farmer	6
O	clear	from the age of 4 months (baby food), addition of ascorbic acid	supermarket	2
O	clear	from the age of 4 months (baby food), addition of ascorbic acid	bio-shop	1

**Table 3.1: Summary of apple juice characteristics (Continued)**

Agricultural practice <sup>a</sup>	Clear /cloudy	Additional information	Shop	Number of samples
C	clear	made from concentrated AJ	discounter	9
O	cloudy	made from concentrated unfiltered AJ, pasteurised	bio-shop	3
C	cloudy	made with apples from standard tree, natural cloudy	HFS	2
O	cloudy	made with apples from standard tree, natural cloudy	bio-shop	2
C	clear	made with concentrated AJ	supermarket	46
O	cloudy	mild pasteurisation, store at 6°C	supermarket	7
O	cloudy	natural cloudy	bio-shop	5
O	cloudy	natural cloudy	supermarket	1
C	cloudy	natural cloudy, addition of ascorbic acid	supermarket	5
H	cloudy	natural pure	farmer	3
H	cloudy	natural, environment-friendly production	farmer	1
O	cloudy	not from concentrate	bio-shop	6
H	clear	not out concentrate	shop	2
H	cloudy	not out concentrate	shop	1
H	clear	pasteurised AJ	supermarket	2
O	cloudy	pure apple juice, freshly squeezed	bio-shop	1
C	cloudy	pure cloudy, addition of ascorbic acid	supermarket	1
C	clear	sparkling AJ, made with concentrate, addition of ascorbic acid	supermarket	2
C	cloudy	store at 7°C, pasteurised, addition of ascorbic acid	supermarket	2

**Table 3.1: Summary of apple juice characteristics (Continued)**

Agricultural practice <sup>a</sup>	Clear /cloudy	Additional information	Shop	Number of samples
O	cloudy	unfiltered	bio-shop	3
O	cloudy	unfiltered	farmer	2
O	cloudy	unfiltered	HFS	1
H	cloudy	unfiltered	farmer	1
H	cloudy	with pear, from environment-friendly produced apples, pasteurised	farmer	1
O	cloudy	from environment-friendly produced apples, pasteurised	shop	1
H	cloudy	with strawberry, from environment-friendly produced apples, pasteurised	farmer	1
H	cloudy	without label	farmer	2

<sup>a</sup>C: conventional; O: organic; H: handcrafted; <sup>b</sup>AJ: apple juice; <sup>c</sup>HFS: health food shop

### 2.3. Quality control

To evaluate the performance of the method for patulin analysis, a control chart was constructed. Therefore, every day the method was executed, a secondary standard that had been tested against a test material of a Fapas proficiency test was analysed together with the samples. The test material of Fapas was an apple juice containing patulin that had been tested in the past during a Fapas proficiency test and was delivered together with an assigned value. The data of the first 20 analyses of secondary standards were used to determine the mean and standard deviation (SD). As a warning limit,  $\text{mean} \pm 2.SD$  was used, and  $\text{mean} \pm 3.SD$  was used as an alarm limit. During the complete study the analysed concentration of the secondary standards were within the warning limits.

### 2.4. Statistical analysis

Statistical analysis was performed by the statistical program SPSS 11.0 for windows (SPSS Inc, USA). For the comparison of means, the Mann-Whitney test was used. The  $\chi^2$  -test was used to compare frequencies of occurrence.

## 3. Results

The validated method was used to analyse 177 apple juice samples, of which 65 samples were organic, 90 conventional and 22 handcrafted. The more limited number of handcrafted apple juices included in this study, was due to the limited availability of these products on the Belgian market. The characteristics of the analysed apple juices are summarised in Table 3.1. Patulin was detected in 22 samples (12%) and in 10 (6%) of them quantification was possible (Table 3.2). Table 3.3 gives a complete overview of the samples with a patulin content above the LOD. The patulin content was higher than the maximum limit of 50  $\mu\text{g}/\text{kg}$  in 2 of the apple juices tested and both samples were organic. One of the samples was a concentrate that contained 328.7  $\mu\text{g}/\text{kg}$ , however the manufacturer indicated that the consumer has to dilute the product 5 to 7 times. Therefore, a concentration of 65.7  $\mu\text{g}/\text{kg}$  was used in the data analysis. The mean level of patulin in contaminated samples was 43.1  $\mu\text{g}/\text{kg}$  for organic apple juice, 10.2  $\mu\text{g}/\text{kg}$  for conventional and 10.5  $\mu\text{g}/\text{kg}$  for handcrafted apple juice (Table 3.2). Statistical analysis showed that the level of patulin contamination was significantly higher in contaminated organic apple juice than in conventional ( $p = 0.02$ , Mann-Whitney test) and in

handcrafted apple juice ( $p = 0.037$ , Mann-Whitney test). When the mean patulin concentration was calculated for all apple juices (for samples below LOD, LOD/2 was used) again the contamination for organic apple juice ( $8.8 \mu\text{g}/\text{kg}$ ) was higher compared to conventional ( $4.1 \mu\text{g}/\text{kg}$ ) and handcrafted apple juice ( $4.4 \mu\text{g}/\text{kg}$ ), but not significantly. However, the incidence of patulin in organic (12%), conventional (13%) and handcrafted apple juice (10%) was not significantly different ( $p = 0.863$ ,  $\chi^2$  -test).

Besides a comparison between conventional, organic and handcrafted apple juice, the apple juices were also compared based on their cloudiness. A significant higher contamination was observed for cloudy apple juice ( $6.7 \mu\text{g}/\text{kg}$ ) compared to clear ( $4.5 \mu\text{g}/\text{kg}$ ) ( $p = 0$ , Mann-Whitney test). Since, it was observed that organic apple juice was in most cases cloudy, a comparison of the 3 types of apple juice was made only for the cloudy apple juices in order to determine whether the higher concentrations can be ascribed to the fact that the apple juices are organic or cloudy (Table 3.4). Although, the mean patulin level was higher for contaminated cloudy organic apple juice ( $43.1 \mu\text{g}/\text{kg}$ ) compared to both cloudy conventional ( $14.0 \mu\text{g}/\text{kg}$ ) and cloudy handcrafted apple juice ( $10.2 \mu\text{g}/\text{kg}$ ), statistical analysis could not demonstrate significant differences (respectively  $p = 0.4$  and  $p = 0.222$ , Mann – Whitney test). Also when the mean patulin concentration was calculated for all cloudy apple juices, a higher contamination was observed for cloudy organic apple juice ( $9.2 \mu\text{g}/\text{kg}$ ) than cloudy conventional ( $4.7 \mu\text{g}/\text{kg}$ ) and cloudy handcrafted apple juice ( $4.4 \mu\text{g}/\text{kg}$ ).

A division of the apple juice into different price ranges showed that the highest concentrations were correlated with the highest price ranges (Table 3.5). However, when only conventional apple juice was studied no relation between the patulin concentration and the prices was observed.



**Table 3.2: Summary of patulin levels in apple juice**

	Conventional	Organic	Handcrafted	Clear	Cloudy	Total
<b>Number of samples</b>						
Total	90	65	22	67	110	177
<LOD <sup>a</sup>	78	57	20	55	100	155
LOD-LOQ <sup>b</sup>	8	3	1	5	7	12
LOQ-25 µg/kg	4	0	1	5	0	5
25-50 µg/kg	0	3	0	0	3	3
>50 µg/kg	0	2	0	1	1	2
Overall incidence	12 (13%)	8 (12%)	2 (10%)	11 (16%)	11 (10%)	22 (12%)
<b>Level of patulin (µg/kg)</b>						
Mean ± standard deviation (µg/kg) <sup>c</sup>	10.2 ± 3.6	43.1 ± 36.9	10.5 ± 0.5	14.1 ± 17.3	30.3 ± 32.8	22.2 ± 26.9
	(n <sup>e</sup> = 12)	(n= 8)	(n= 2)	(n= 11)	(n= 11)	(n= 22)
Mean ± standard deviation (µg/kg) <sup>d</sup>	4.1 ± 2.8	8.8 ± 17.8	4.4 ± 2.0	4.5 ± 7.8	6.7 ± 12.7	5.8 ± 11.2
	(n= 90)	(n= 65)	(n= 22)	(n= 67)	(n= 110)	(n= 177)

<sup>a</sup>LOD: Limit of detection; <sup>b</sup>LOQ: Limit of quantification; <sup>c</sup>The mean patulin concentration of contaminated samples (samples with a patulin content >LOD); <sup>d</sup>The mean patulin concentration of all samples. For a sample with a concentration below the LOD, a concentration equal to half the LOD was used; <sup>e</sup>n= number of samples

**Table 3.3: Characteristics of samples contaminated with patulin at a level above the limit of detection**

Code	Agricultural			Shop	Patulin concentration ( $\mu\text{g}/\text{kg}$ ) ( $n^d = 2$ )
	practice <sup>a</sup>	Clear /cloudy	Additional information		
S4	C	clear	made with concentrated AJ <sup>b</sup>	supermarket	$10.7 \pm 1.6$
S15	O	cloudy	mild pasteurisation, store at 6°C	supermarket	<LOQ <sup>c</sup> ( $12.1 \pm 7.9$ )
S21	C	cloudy	freshly squeezed, natural cloudy	HFS <sup>e</sup>	<LOQ ( $12.5 \pm 7.9$ )
S31	O	cloudy		bio-shop	$34.1 \pm 7.9$
S49	C	clear	made from concentrated AJ	supermarket	$9.4 \pm 1.6$
S54	O	cloudy		bio-shop	<LOQ ( $13.9 \pm 7.9$ )
S58	C	cloudy	100% jonagold AJ, addition of ascorbic acid	supermarket	<LOQ ( $15.5 \pm 7.9$ )
S60	O	cloudy	natural cloudy	bio-shop	$42.0 \pm 7.9$
S61	C	clear	made with concentrated AJ	supermarket	$13.6 \pm 1.6$
S63	H	clear	pasteurised AJ	supermarket	$10.9 \pm 1.6$
S65	C	clear	made with concentrated AJ	supermarket	<LOQ ( $6.0 \pm 1.6$ )
S73	C	cloudy	100% jonagold AJ, addition of ascorbic acid	supermarket	<LOQ ( $15.6 \pm 7.9$ )
S95	C	clear	sparkling AJ, made with concentrate, addition of ascorbic acid	supermarket	<LOQ ( $7.0 \pm 1.6$ )
S96	O	cloudy	unfiltered	bio-shop	$39.9 \pm 7.9$
S106	O	cloudy		supermarket	$122.6 \pm 7.9$
S111	O	cloudy	not from concentrate	bio-shop	<LOQ ( $14.7 \pm 7.9$ )
S117	C	clear	with concentrated AJ	supermarket	<LOQ ( $6.2 \pm 1.6$ )
S121	C	clear	made with concentrated AJ	supermarket	<LOQ ( $6.2 \pm 1.6$ )

**Table 3.3: Characteristics of samples contaminated with patulin at a level above the limit of detection (Continued)**

Code	Agricultural			Shop	Patulin concentration ( $\mu\text{g}/\text{kg}$ ) ( $n^d = 2$ )
	practice <sup>a</sup>	Clear /cloudy	Additional information		
S126	O	clear	unsprayed fruit from standard trees, concentrate	bio-shop	$65.7 \pm 10.8$
S142	C	clear	made from concentrated AJ	supermarket	$11.6 \pm 1.6$
S169	C	clear	made with concentrated AJ	supermarket	<LOQ ( $8.5 \pm 1.6$ )
S173	H	cloudy	without label	farmer	<LOQ ( $10.2 \pm 7.9$ )

<sup>a</sup>C: conventional; O: organic; H: handcrafted; <sup>b</sup>AJ: apple juice; <sup>c</sup>LOQ: Limit of quantification; <sup>d</sup>n = number of samples; <sup>e</sup>HFS: Health food shop

**Table 3.4: Summary of patulin levels in cloudy apple juice**

	Conventional	Organic	Handcrafted
<b>Number of samples</b>			
Total	33	59	18
<LOD <sup>a</sup>	30	52	17
LOD-LOQ <sup>b</sup>	3	3	1
LOQ-25 µg/kg	0	0	0
25-50 µg/kg	0	3	0
>50 µg/kg	0	1	0
Overall incidence	3 (9%)	7 (12%)	1 (6%)
<b>Level of patulin (µg/kg)</b>			
Mean ± standard deviation (µg/kg) <sup>c</sup>	14.0 ± 2.1 (n <sup>e</sup> = 3)	43.1 ± 36.9 (n= 7)	10.2 (n= 1)
Mean ± standard deviation (µg/kg) <sup>d</sup>	4.7 ± 2.5 (n= 33)	9.2 ± 18.3 (n= 59)	4.4 ± 1.5 (n= 18)

<sup>a</sup>LOD: Limit of detection; <sup>b</sup>LOQ: Limit of quantification; <sup>c</sup>The mean patulin concentration of contaminated samples (samples with a patulin content >LOD); <sup>d</sup>The mean patulin concentration of all samples. For a sample with a concentration below the LOD, a concentration equal to half the LOD was used; <sup>e</sup>n= number of samples

## 4. Discussion

The present study shows that the patulin level is significantly higher in contaminated organic apple juice than in conventional and handcrafted apple juice, although the incidence of patulin in the 3 types of juice was not significantly different. This indicates that patulin does not occur more frequently in organic apple juice than in other apple juices, but when it occurs it can be present in higher concentrations. The observed significant difference between the patulin content of contaminated organic and conventional apple juice samples was in accordance with the results of Beretta et al. (2000), Malmauret et al. (2002) and Piemontese et al. (2005). Beretta et al. observed in Italy a significant difference between juice containing apples from conventional and organic agriculture, however only 21 samples were analysed (Beretta et al., 2000). In another Italian study 169 fruity foodstuffs were analysed, of which 57 were apple juice. Patulin was detected with a significant higher mean concentration in the

**Table 3.5: Patulin concentration of apple juice in function of price range**

Price range (€/litre)	All apple juices		Conventional apple juice	
	Mean concentration of contaminated samples (µg/kg)	Mean concentration of all samples (µg/kg)	Mean concentration of contaminated samples (µg/kg)	Mean concentration of all samples (µg/kg)
< 0.75	8.4 ± 2.3 (n <sup>a</sup> = 3)	3.4 ± 2.0 (n= 25)	8.4 ± 2.3 (n= 3)	3.4 ± 2.0 (n= 25)
0.75 ≤ < 1.5	10.6 ± 4.8 (n= 5)	4.3 ± 2.9 (n= 41)	10.6 ± 4.8 (n= 5)	4.3 ± 2.9 (n= 40)
1.5 ≤ < 3.5	27.6 ± 32.3 (n= 12)	6.7 ± 13.4 (n= 98)	11.2 ± 2.9 (n= 4)	4.4 ± 3.2 (n= 25)
3.5 ≤	39.8 ± 36.6 (n= 2)	9.1 ± 17.3 (n= 13)	-	4.1 (n= 1)

<sup>a</sup>n= number of samples

organic products compared to conventional, but for apple juice in particular, the higher mean concentration in organic apple juice was not significantly different from the conventional apple juice (Piemontese et al., 2005). In a French study 6 conventional and 6 organic samples of apple were analysed. Although the organic apples were more contaminated by patulin than the conventional ones, the difference was not significant (Malmauret et al., 2002). On the contrary, a Dutch study reported no difference between conventional and organic apple products. However, the detection limit was set at 25 µg/kg and only one sample (which was organic apple juice) of the 63 analysed samples contained patulin above the detection limit (Boonzaaijer et al., 2005). Tangni et al. (2003) analysed four organically produced apple juices and the patulin level was lower than for the conventional apple juice. However, the number of samples was too small to draw conclusions. Also, Ritieni (2003) did not observe a significant difference between apple products (including apple juice, baby food, apple vinegar and apple puree) produced by the 2 agricultural practices. The difference between the present study and the five cited studies is the higher number of apple juices included in this study, that allow to draw more firm conclusions and to use the data for a probabilistic risk assessment.

Deterministic risk assessment shows that for a child of 18 kg consuming 0.2 litre of apple juice a day (the volume of a small package of apple juice in Belgium), the mean patulin intake is 0.046; 0.098 and 0.049  $\mu\text{g}/\text{kg}$  bw/day ( $\mu\text{g}/\text{kg}$  body weight/day) for respectively conventional, organic and handcrafted apple juice. This corresponds to respectively 11, 24 and 12% of the TDI (tolerable daily intake) which is 0.4  $\mu\text{g}/\text{kg}$  bw/day (JECFA, 1995). However, when in the worst case a child consumes the most contaminated apple juice of this study, an intake of 1.36  $\mu\text{g}/\text{kg}$  bw/day is reached which corresponds to 340% of the TDI. In order to perform a more realistic assessment, a probabilistic risk assessment is necessary to determine how many children exceed the TDI.

Since it was observed that organic apple juice was mostly cloudy, a comparison was made for organic, conventional and handcrafted apple juice in which only the cloudy apple juices were included. This comparison showed that higher patulin concentrations occur in organic, cloudy apple juice than in the other two, indicating that the higher patulin levels are a consequence of the fact that the juices are produced in accordance with the organic agricultural practices and not the fact that they are cloudy.

In order to determine whether there is a correlation between the price and the safety, more precisely the patulin content of apple juice, the apple juices were divided in different price ranges. Data analysis showed that the highest patulin concentrations were observed for the apple juices in the highest price ranges. However when only conventional apple juices were compared, no relation between the price and the patulin concentration was observed. Since organic apple juices are more expensive than conventional apple juices, the higher patulin concentrations of more expensive apple juices can be ascribed to the organic production.

## 5. Conclusion

The results of this study demonstrate that although the incidence of patulin contamination in organic, conventional and handcrafted made apple juice is not statistically significant, the level of contamination is higher for organic apple juice than for conventional and handcrafted apple juice. Further data analysis showed that the higher concentrations are a consequence of the fact that the juices are organic and not that they are cloudy. From the presented study it can also be concluded that people that buy cheaper apple juice are not more exposed to patulin. A deterministic risk assessment showed that the observed patulin concentration in

apple juice can result in exceeding the TDI. Therefore, a probabilistic risk assessment will be performed in Chapter 4 to estimate the risk of children consuming apple juice.





### Variability and uncertainty assessment of patulin exposure for preschool children in Flanders<sup>1</sup>

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<sup>1</sup> Based on: Baert, K., De Meulenaer, B., Verdonck, F., Huybrechts, I., De Henauw, S., Vanrolleghem, P.A., Debevere, J., Devlieghere, F. Variability and uncertainty assessment of patulin exposure for preschool children in Flanders. *Food and Chemical Toxicology* (in press: <http://dx.doi.org/10.1016/j.fct.2007.03.008>).



## Chapter 4

# Variability and uncertainty assessment of patulin exposure for preschool children in Flanders

### Summary

The objective of the present study was to evaluate the patulin exposure of children consuming organic, handcrafted or conventional apple juice through a probabilistic approach and to evaluate the effectiveness of several risk management options aiming to reduce the patulin exposure. A large part of the data on patulin contamination of apple juice fell under the limit of detection (LOD). Different methods were tested to deal with these so-called left censored data and a uniform distribution with uncertain bounds was selected to handle this censorship. Variability and uncertainty assessment of patulin exposure showed that 0.9% [90% confidence interval (CI): 0.3-1.8%] of the children consuming organic apple juice, exceeded the tolerable daily intake (TDI) for patulin. For consumers of conventional and handcrafted apple juice, this was respectively 0.1% [90% CI: 0-0.3%] and 0% [90% CI: 0-0.2%]. Rejection of apples juices containing more patulin than 25 µg/kg reduced the percentage of the children whose patulin intake exceeds the TDI to 0% [90%CI: 0-0.2%]. Reduction of the apple juice consumption was less effective than the application of a more stringent maximum level than the current one (25 µg/kg instead of 50) and was only useful when the patulin concentration of apple juice was below 25 µg/kg.

## 1. Introduction

The aim of this chapter was to evaluate the exposure to patulin and to determine whether the patulin concentration observed in apple juice (Chapter 3) can lead to an intake that causes adverse health effects. To our knowledge, five different studies have been conducted in the past to evaluate the exposure to patulin. A Scientific Cooperation (SCOOP) study conducted by the Directorate – General Health and Consumer Protection of the European Union (EU) on the assessment of the dietary intake of patulin by the population of EU member states showed that in most countries, apple juice and apple nectar are the main sources of patulin intake, particularly for young children (Directorate – General Health and Consumer Protection, 2002). An important limitation of the latter study and four other exposure studies (Thuvander et al., 2001; Tangni et al., 2003; Leblanc et al., 2005; Piemontese et al., 2005) is the use of point estimates or simple distributions to assess the exposure. As described in Chapter 1 – section 1.6, point estimates or simple distributions have the disadvantage that only a limited risk characterisation can be conducted.

Therefore, a large number of apple juice samples (177) were tested on their patulin content as described in Chapter 3. The aim of the present study was to estimate the consumers' exposure to patulin due to the consumption of apple juice, based on a probabilistic method. Since the results presented in Chapter 3 indicated that patulin concentrations are higher in organic apple juices than in conventional and handcrafted apple juices, the exposure was estimated separately for the three types of apple juice. The simulated exposures were then compared with the TDI aiming to help risk managers in the regulatory decision making process. Since the SCOOP task has shown that small children have a higher patulin intake compared to other population groups and apple juice and apple nectar are the main sources of patulin intake in most countries (Directorate – General Health and Consumer Protection, 2002), the focus of this probabilistic exposure assessment was on this particular population group and these specific matrices. Secondly, the study aimed to evaluate the effectiveness of alternative risk management options to reduce the patulin exposure in children. A first risk management option tested in the model was the proposal of the EU commission to lower the regulatory limit for patulin contamination in apple juice (European Commission, 2003b). A second scenario evaluated in the model was a decrease in apple juice consumption by young children as advised by paediatrics (American Academy of Pediatrics, 2001).

## 2. Materials and methods

### 2.1. Consumption data

Data on apple juice consumption were obtained from a large-scale epidemiological study investigating nutrition habits of preschool children in Flanders (2.5-6.5 years old) (Huybrechts et al., 2006). This study was conducted by the Department of Public Health, Ghent University. A total of 2095 children were asked to complete a food-frequency questionnaire (FFQ), a 3-day estimated diet record (EDR) and a general questionnaire. A total of 1579 FFQ's and 1052 EDR's were collected by the end of the fieldwork. The semi-quantitative FFQ included questions on the average consumption of 47 food items (including fruit juice), during the past year. The parents were also asked to collect a structured EDR over three consecutive days. Detailed information on the type (including brand name) and portion size of the foods consumed was collected in the EDR, using an open entry format. On a separate sheet, recipes could be described in more detail. To ensure that all days of the week were registered equally, the research team determined beforehand the days that the parents had to register. In a general questionnaire, the parents were asked to report the weight and length of their child. Since apple juice was not included as a separate food category in the FFQ and because the EDR only include three days of a child's life, it was not possible to divide the population in "apple juice consumers" and "non consumers". However, the apple juice consumption from the EDR could be used in the exposure assessments. The fieldwork of the study was carried out from November 2002 until February 2003. A brief description of the apple juice consumption data is given in Table 4.1.

**Table 4.1: Descriptive statistics of the consumption of apple juice (g/kg body weight/day) by Flemish preschool children (Huybrechts et al., 2006)**

P0 <sup>a</sup>	P85	P90	P95	P97.5	P99	P99.5	P99.9	P100	Mean
0	0	10.5	14.3	22.2	31.0	38.3	56.7	67.3	2.2

<sup>a</sup>P0: 0<sup>th</sup> percentile

## 2.2. Contamination data of organic, handcrafted and conventional apple juice

The contamination data used in the present study were derived from the previous study described in Chapter 3 in which 177 apple juice samples were analysed.

## 2.3. Alternatives for values below LOD

The contamination data consisted for a large part of left censored data (Table 3.2). The censored data were treated in several ways (Figure 4.1):

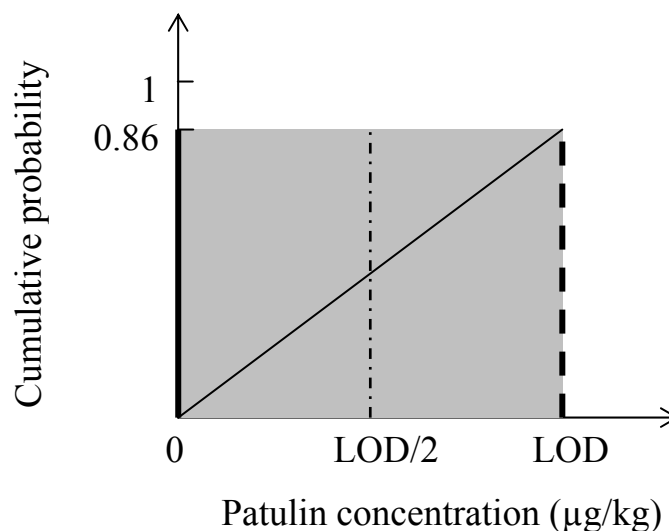
H1: The censored data were replaced by the corresponding LOD (e.g. Tressou et al., 2004; Govaerts et al., 2005)

H2: The censored data were replaced by the corresponding LOD divided by 2 (e.g. Tressou et al., 2004; Govaerts et al., 2005)

H3: The censored data were replaced by zero (e.g. Tressou et al., 2004; Govaerts et al., 2005)

H4: The censored data were replaced by random samples from a uniform distribution with zero as minimum and LOD as maximum (RiskUniform (0; LOD)) (Govaerts et al., 2005)

H5: The censored data were replaced by random samples from a uniform distribution with  $\alpha$  as a minimum ( $\alpha$  = uniform distribution between zero and the LOD) and  $\beta$  as a maximum ( $\beta$  = a uniform distribution between zero and the LOD), with the restriction that  $\alpha$  is smaller than  $\beta$  (RiskUniform (RiskUniform (0; LOD); RiskUniform (0; LOD))).



**Figure 4.1: Graphical representation of the different treatments for censored data**  
(H1: - - - , H2: - . - . - , H3: — , H4: — , H5: ■ )

## 2.4. Exposure assessment

The patulin exposure was modelled by multiplying consumption data with contamination data of organic, conventional and handcrafted apple juice.

$$\begin{aligned} \text{patulin intake} \left[ \frac{\mu\text{g}}{\text{kg bw} \cdot \text{day}} \right] &= \text{concentration of patulin in apple juice} \left[ \frac{\mu\text{g}}{\text{kg}} \right] \\ &\times \text{apple juice consumption} \left[ \frac{\text{g}}{\text{kg bw} \cdot \text{day}} \right] \times 0.001 \left[ \frac{\text{kg}}{\text{g}} \right] \end{aligned} \quad (4.1)$$

In this exposure assessment it was assumed that a consumer uses only one of the three commodities (e.g. a consumer of organic apple juice will only consume organic apple juice). It was also assumed that the consumption pattern of the three groups of consumers (organic, handcrafted and conventional) is the same.

In order to characterize the variability, both the parametric and non-parametric approach were used. In the parametric approach, a theoretical distribution function is used to describe the data instead of the collected data itself. In order to have a good representation of the dataset, the parameters (e.g. mean, variance) of the probability distribution are to be estimated (Cullen & Frey, 1999). Fitting of the probability distributions to the data was performed using BestFit (Palisade, UK). The censored data were replaced by some specific values or random samples from distributions according to the treatments mentioned in section 2.3. In the non-parametric approach, the collected data points themselves are considered to form the distribution function (no fitting to a pre-defined model). In this study a discrete uniform distribution (RiskDuniform) was used for both the observed consumption and contamination values (subsequent sampling was done by replacement). A discrete uniform distribution is a special case of the Discrete distribution where all possible values have the same probability of occurrence (Vose, 2000). The censored data were replaced by some specific values or random samples from distributions according to the treatments mentioned in section 2.3. In conclusion, the variability of the consumption and the contamination parameters was characterized by a non-parametric, discrete, uniform distribution.

Uncertainty characterization was only performed in case of the non-parametric approach using non-parametric bootstrap. The bootstrap theory assumes that the distribution  $F$  (of e.g. patulin concentration in apple juice) can be reasonably approximated by the distribution  $F'$  of

n observed values. This is of course a more reasonable assumption when more data are collected. For a sufficiently large number of times, n random samples are taken with replacement from the distribution F' and each time, the statistical parameter of interest is calculated from that sample (Vose, 2000).

Propagation of variability and uncertainty was performed using second order Monte Carlo simulation. A second order or two-dimensional Monte Carlo simulation consists of two Monte Carlo loops nested one inside the other. The inner one deals with the variability of the input variables, while the outer one deals with uncertainty. The simulation was executed with the risk analysis software @RISK (@RISK 4.5 professional edition, Palisade, UK). Latin Hypercube sampling was used to randomly sample the probability distribution functions of input parameters (consumption and contamination data) and the samples were used to calculate the intake by the described model. One thousand simulations were carried out to describe the variability in the consumption of the population and in the contamination, and one thousand bootstrap iterations were carried out to estimate the confidence interval. This results in a total of 1000 x 1000 simulations.

## **2.5. Evaluating different risk management scenarios to reduce the exposure**

Six scenarios to reduce the exposure to values below the TDI were evaluated.

S1: Reduction of the patulin contamination to concentrations below 50 µg/kg (the samples above the legal limit (50 µg/kg) were removed from the dataset).

S2: Reduction of the patulin contamination to concentrations below 25 µg/kg (the samples above 25 µg/kg were removed from the dataset).

S3: Reduction of the fruit juice consumption to values below 200 ml/day (For this, the fraction of apple juice on the total fruit juice consumption was calculated. When the total fruit juice consumption was above 200 ml, the value was replaced by 200 ml. The allowed fruit juice consumption (200 ml) was then multiplied with the fraction of apple juice in the fruit juice consumption to calculate the allowed apple juice consumption. When the total fruit juice consumption was below 200 ml, the reported apple juice consumption was used.).

S4: Reduction of the fruit juice consumption to values below 170 ml/day (as described for S3).



S5: Reduction of the apple juice consumption to values below 200 ml/day (the values of apple juice consumptions above 200 ml were replaced by 200 ml).

S6: Reduction of the apple juice consumption to values below 170 ml/day (as described for S5).

Since concentrations above 25 µg/kg were only found for organic apple juice, S1 and S2 were solely tested for organic apple juice.

## **3. Results and discussion**

### **3.1. Evaluation of the proposed methods**

In order to select a good representation of the variability in the consumption as well as in the contamination, the parametric and non-parametric approaches were both evaluated. In case of the parametric approach, several parametric distributions (for example, a log-normal distribution, a gamma distribution, ...) were fitted to the consumption data. However, none of the distributions fitted the consumption and contamination data well (graphical representation showed systematic deviations, especially in the tails of the distribution, results not shown) and accurately represented their variability. This was due to the large number of non-consumptions (0 g/kg body weight/day) (Table 4.1) and the large number of contamination data below the LOD (Table 3.2). Since the sample size was large, the data were considered to be more reliable than assuming a certain distribution. For this reason, the non-parametric approach was selected.

As can be observed from Table 3.2, the contamination distributions contain a large part of left censored data. Indeed, between 87 and 91% of the data were under the LOD. This induces a bias that can be dealt with, by considering several treatments of the censorship. In literature (e.g. Tressou et al., 2004; Govaerts et al., 2005), the censored data are often replaced by the corresponding LOD (H1), LOD divided by 2 (H2) or by 0 (H3). These three methodologies were compared for one apple juice commodity, namely organic apple juice, in order to determine the influence of the methodology on the simulated exposure (Table 4.2). Although high patulin concentrations (up to 122 µg/kg of apple juice) were found compared to the LOD (5.2 µg/kg for clear apple juice and 8.1 µg/kg for cloudy apple juice), the choice of a substitute for the censored data had an influence on the high percentiles of the exposure. The

**Table 4.2: Comparison of the statistics on exposure (best estimation [90% confidence interval];  $\mu\text{g}/\text{kg bw}/\text{day}$ ) for organic apple juice calculated by different methodologies to replace values below the limit of detection (LOD) (H1: left censored data replaced by LOD; H2: left censored data replaced by LOD/2; H3: left censored data replaced by zero; H4: left censored data replaced by Riskuniform(0; LOD); H5: left censored data replaced by Riskuniform( $\alpha$ ;  $\beta$ ) with  $\alpha$  and  $\beta$  = Riskuniform(0; LOD))**

	H1	H2	H3	H4	H5
P0 <sup>a</sup>	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-0]
P50	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-0]
P85	0.001 [0-0.049]	0.0003 [0-0.025]	0 [0-0]	0.0001 [0-0.013]	0.0001 [0-0.024]
P90	0.085 [0.077-0.094]	0.043 [0.039-0.047]	0 [0-0]	0.036 [0.026-0.049]	0.039 [0.014-0.069]
P95	0.132 [0.116-0.166]	0.068 [0.058-0.090]	0 [0-0]	0.081 [0.065-0.102]	0.072 [0.027-0.117]
P97.5	0.216 [0.181-0.312]	0.125 [0.096-0.197]	0 [0-0.183]	0.148 [0.103-0.237]	0.135 [0.053-0.229]
P99	0.408 [0.261-0.815]	0.341 [0.155-0.782]	0.316 [0-0.775]	0.373 [0.184-0.774]	0.350 [0.143-0.822]
P99.5	0.601 [0.351-1.442]	0.617 [0.213-1.442]	0.627 [0.161-1.442]	0.634 [0.266-1.363]	0.615 [0.249-1.472]
P99.9	1.449 [0.533-3.068]	1.443 [0.506-3.246]	1.445 [0.522-3.245]	1.442 [0.499-3.268]	1.471 [0.526-3.066]
Mean	0.026 [0.019-0.039]	0.019 [0.012-0.031]	0.011 [0.003-0.024]	0.019 [0.012-0.032]	0.019 [0.010-0.032]

<sup>a</sup>P0: 0<sup>th</sup> percentile

mean exposure was the highest for H1 (0.026 [90% confidence interval (CI): 0.019-0.039]  $\mu\text{g}/\text{kg}$  body weight/day ( $\mu\text{g}/\text{kg}$  bw/day)), which is likely to be an overestimation due to the assumption that all censored data are equal to the LOD. For H3, the mean exposure (0.011 [90% CI: 0.003-0.024]  $\mu\text{g}/\text{kg}$  bw/day) was the lowest and this will most probably be an underestimation due to the assumption that all censored data are equal to zero. The mean exposure calculated by H2 was equal to 0.019 [CI: 0.012-0.031]  $\mu\text{g}/\text{kg}$  bw/day. Using H2 (LOD/2) is probably a good compromise which is commonly used but not without criticism (El-Shaarawi & Esterby, 1992). It was apparent that even the 99<sup>th</sup> percentile, with an exposure close to the tolerable daily intake (0.4  $\mu\text{g}/\text{kg}$  bw/day), was influenced by the treatment of the censored data. It was rather unexpected that the left censorship of the contamination data strongly influenced the right tail of the exposures to patulin. A further in-depth analysis of the data explained that high apple juice consumption (up to 67.3 g/kg bw/day) with a concentration below the LOD (e.g. 8  $\mu\text{g}/\text{kg}$ ) gave rise to a patulin intake of 0.5  $\mu\text{g}/\text{kg}$  bw/day, which is higher than the TDI. A proper treatment of censored concentration data is consequently not to be underestimated. Therefore, two other censored data treatments were tested. Those do not use a fixed value for the censored data but instead consider the variability (and uncertainty) by considering random samples. Firstly, the values below the LOD were replaced by random samples from a uniform distribution with zero as a minimum and the LOD as a maximum (H4). This treatment was used since it can be expected that a natural variability is present in the concentrations below the LOD. Secondly, the observed variability of the patulin concentrations of the apple juice above the LOD is also expected to occur for patulin concentrations below the LOD. This variability can be characterised by a uniform distribution. In addition, the boundaries of the uniform distribution were considered to be uncertain. Therefore, the censored data were replaced by random samples from a uniform distribution with  $\alpha$  as a minimum ( $\alpha$  = uniform distribution between zero and the LOD) and  $\beta$  as a maximum ( $\beta$  = a uniform distribution between zero and the LOD) (H5). Comparison of the simulated exposures for method H4 and H5 with method H2 (Table 4.2), showed that the simulated exposures for the three treatments are not significantly different. The mean exposure is similar for the three treatments with a larger 90% CI for H5. Since H5 is the most realistic representation of the censored data (Figure 4.1), this methodology was used further to assess the patulin exposure of Flemish preschool children via conventional, organic and handcrafted apple juice consumption.

### 3.2. Assessment of patulin exposure

As described in Chapter 3, higher patulin concentrations were found in organic apple juice (8.8 µg/kg) compared to conventional (4.1 µg/kg) and handcrafted apple juice (4.4 µg/kg). It was one of the aims of the present study to evaluate the implications of these higher concentrations with regard to public health. The estimated exposures to patulin together with the 90% CI are summarised in Table 4.3. For the three types of apple juice tested, 83% of the children have no intake of patulin via apple juice. For organic apple juice, the best estimate for the 99.5<sup>th</sup> percentile is higher than the TDI (0.43 µg/kg bw/day). Based on the best estimate, it can be observed that the exposure can be more than 3 times higher than the TDI for children consuming organic apple juice in 0.1% of the cases. The best estimate of the exposure through handcrafted and conventional apple juice was below the TDI. Although higher exposures are observed for organic apple juice compared to the other two types of apple juice, their CI overlap, which indicates that the average exposure to patulin of children will be roughly the same for the three kinds of apple juice tested.

**Table 4.3: Statistics on exposure (best estimation [90% confidence interval]; µg/kg bw/day) and probability to exceed the tolerable daily intake (TDI) for different apple juices (AJ)**

	Organic AJ	Conventional AJ	Handcrafted AJ
P0 <sup>a</sup>	0 [0-0]	0 [0-0]	0 [0-0]
P83	0 [0-0]	0 [0-0]	0 [0-0]
P90	0.039 [0.014-0.069]	0.030 [0.011-0.049]	0.037[0.013-0.066]
P95	0.072 [0.027-0.117]	0.059 [0.031-0.085]	0.065 [0.027-0.102]
P97.5	0.135 [0.053-0.229]	0.095 [0.057-0.133]	0.102 [0.047-0.151]
P99	0.350 [0.143-0.822]	0.156 [0.106-0.206]	0.150 [0.084-0.229]
P99.5	0.615 [0.249-1.472]	0.202 [0.141-0.287]	0.195 [0.109-0.290]
P99.9	1.471 [0.526-3.066]	0.328 [0.210-0.548]	0.298 [0.156-0.460]
Mean	0.019 [0.010-0.032]	0.009 [0.006-0.013]	0.010 [0.005-0.015]
Probability to exceed TDI	0.009 [0.003-0.018]	0.001 [0-0.003]	0 [0-0.002]

<sup>a</sup>P0: 0<sup>th</sup> percentile

The simulated exposures were similar to the results found in other studies (Directorate – General Health and Consumer Protection, 2002; Tangni et al., 2003; Leblanc et al., 2005; Piemontese et al., 2005) reporting mean exposures for children between 0.003 and 0.18 µg/kg bw/day (Table 4.4). However, a tendency exists to overestimate mean exposures when a deterministic approach is used.

A comparison of the percentage of children exceeding the TDI for the three types of apple juice (Table 4.3) shows that the probability of exceeding the TDI is higher for organic apple juice than for conventional and handcrafted apple juice. With 90% certainty, it can be stated that between 0.3 and 1.8% of the children consuming organic apple juice exceed the TDI while for conventional and handcrafted apple juice this is between 0 and 0.3% and between 0 and 0.2% respectively.

### **3.3. Evaluation of risk management measures to reduce patulin exposure**

Since the simulation of the exposure showed that the TDI for patulin is sometimes exceeded for Belgian children, different scenarios to reduce the patulin intake were evaluated by simulation.

In 2003 the European Commission has extended the regulation on contaminants in foodstuffs (466/2001) by setting maximum limits for patulin in different products by implementation of regulation 1425/2003 (European Commission, 2003b). For apple juice the maximum limit (ML) was set at 50 µg/kg. However, during the study on the occurrence of patulin in apple juice in Flanders, two organic apple juices were found with a patulin content above 50 µg/kg (Chapter 3). Therefore, the influence of a strict implementation of the current legislation on the patulin exposure in young children was tested by removing these two samples from the dataset (S1). In the regulation 1425/2003 it was also foreseen that the commission would review the maximum levels for patulin by 30<sup>th</sup> June 2005 at the latest in order to reduce these levels by taking into account the progress in scientific and technological knowledge and the implementation of the “Code of practice for the prevention and reduction of patulin contamination in apple juice and apple juice ingredients in other beverages” (European Commission, 2003a; European Commission, 2003b). However, until now no new legislation has been published. In order to test the effect of lowering the ML, the limit was reduced by

**Table 4.4: Overview of different studies on patulin exposure**

Country	Patulin intake ( $\mu\text{g}/\text{kg}$ bw/day)		Method	Commodity	Population	Reference
	Mean [CI] <sup>a</sup>	P95 <sup>b</sup> [CI] <sup>a</sup>				
France	0.005	0.032	Deterministic	AJ <sup>c</sup>	3-5 years	Directorate – General Health and Consumer Protection, 2002
Germany	0.043	0.129	Deterministic	AJ	4-6 years (girls)	Directorate – General Health and Consumer Protection, 2002
Austria	0.022	0.072	Deterministic	AJ	3-6 years	Directorate – General Health and Consumer Protection, 2002
Belgium	0.18	/ <sup>e</sup>	Deterministic	AJ	Children	Tangni et al., 2003
Italy	0.014	/	Deterministic	Organic FP <sup>d</sup>	1-10 years	Piemontese et al., 2005
Italy	0.003	/	Deterministic	Conventional FP	1-10 years	Piemontese et al., 2005
France	0.03	0.106	Simple distribution	FP	3-14 years	Leblanc et al., 2005
Belgium	0.019 [0.010-0.032]	0.072 [0.027-0.117]	Probabilistic	Organic AJ	2.5-6.5 years	Current study
Belgium	0.009 [0.006-0.013]	0.059 [0.031-0.085]	Probabilistic	Conventional AJ	2.5-6.5 years	Current study

<sup>a</sup>[CI]: [90% confidence interval]; <sup>b</sup>P95: 95<sup>th</sup> percentile; <sup>c</sup>AJ: apple juice; <sup>d</sup>FP: Fruit-based products; <sup>e</sup>/: value was not available

50%, resulting in a new limit of 25 µg/kg, which is also the current ML for solid apple products. For this test, all samples above 25 µg/kg, were removed from the dataset (S2). Since concentrations above 25 µg/kg were only found for organic apple juice, both techniques (S1 and S2) were tested for organic apple juice only. Table 4.5 shows the simulated exposures for both scenarios compared to the current situation (as assessed in section 3.2). It can be observed that the exposure is reduced when the contamination is below 50 µg/kg and will further reduce when the contamination decreases to 25 µg/kg. However, the obtained reduction is not significantly different. For the 99.9<sup>th</sup> percentile (best estimate), a reduction of 54% was achieved when the limit of 50 µg/kg is used and a reduction of 79% when a limit of 25 µg/kg is implemented. For the 95<sup>th</sup> percentile on the other hand, only a reduction of 4% was achieved when the limit of 50 µg/kg is followed, and a reduction of 11% when using a limit of 25 µg/kg. Also for the mean exposure the reduction was limited (26 and 47%, respectively). Based on these results, it can be stated that a reduction of the ML mainly affects the high intakes, which are the children at risk and to a lesser extent the mean and lower intakes. Comparing the percentage of the population exceeding the TDI (Table 4.5), it is showed that a decrease of the contamination below 50 µg/kg reduces the percentage of children exceeding the TDI (between 0.1% and 1.2%). A restriction to 25 µg/kg reduced the exposure higher than the TDI further towards 0 to 0.3% of the population under study. Therefore, it can be concluded that in order to reduce the exposure to patulin, a reduction of the legal limit to 25 µg/kg is necessary and causes a six fold reduction of the probability to exceed the TDI (based on the upper limit of the 90% CI). However it needs to be stressed that the implementation of a ML is only effective when this limit is followed and as a consequence it is necessary to provide apple juice producers with guidelines to achieve this limit. It was not useful to test a further reduction of the ML, since the maximum concentration observed in conventional and handcrafted apple juice was 15.6 and 10.9 µg/kg respectively (Table 3.2), and still there was a small probability to exceed the TDI. This means that even with a reduction of the limit to about 11 µg/kg there is still a small probability to exceed the TDI. This is caused by the high apple juice consumption by some children (up to 67.3 g/kg bw/day), which leads to a patulin intake higher than the TDI for concentrations below the LOD (e.g. 8 µg/kg).

**Table 4.5: Statistics on exposure (best estimation [90% confidence interval]; µg/kg bw/day) and probability to exceed the tolerable daily intake (TDI) through the consumption of organic apple juice for the current situation and two alternatives to reduce the exposure (S1: patulin contamination below 50 µg/kg; S2: patulin contamination below 25 µg/kg)**

	Current situation	S1	S2
P0 <sup>a</sup>	0 [0-0]	0 [0-0]	0 [0-0]
P50	0 [0-0]	0 [0-0]	0 [0-0]
P85	0.0001 [0-0.024]	<0.0001 [0-0.025]	0.0001 [0-0.025]
P90	0.039 [0.014-0.069]	0.040 [0.013-0.072]	0.038 [0.011-0.068]
P95	0.072 [0.027-0.117]	0.069 [0.025-0.112]	0.064 [0.022-0.102]
P97.5	0.135 [0.053-0.229]	0.118 [0.045-0.186]	0.099 [0.036-0.157]
P99	0.350 [0.143-0.822]	0.226 [0.107-0.443]	0.154 [0.066-0.222]
P99.5	0.615 [0.249-1.472]	0.379 [0.173-0.647]	0.196 [0.100-0.303]
P99.9	1.471 [0.526-3.066]	0.682 [0.320-1.402]	0.309 [0.171-0.491]
Mean	0.019 [0.010-0.032]	0.014 [0.007-0.021]	0.010 [0.004-0.015]
Probability to exceed TDI	0.009 [0.003-0.018]	0.005 [0.001-0.012]	0 [0-0.003]

<sup>a</sup>P0: 0<sup>th</sup> percentile

A second scenario that was tested, consisted of the reduction of apple juice consumption. From a nutritional point of view, it is advised to limit the consumption of fruit juices. Fruit juices contain fewer fibres than fresh fruit and more sugar in comparison to mineral water. In Belgium, young children are advised to limit their fruit juice intake to approximately 200 ml a day (Vlaams Instituut voor Gezondheidspromotie, 2004). The American Academy of Pediatrics recommends giving not more than 6 ounces (about 170 ml) a day, to children between one and six years old and preferably as part of a meal or a snack (American Academy of Pediatrics, 2001). However, from the consumption data (Table 4.1) it can be determined that these recommendations are exceeded. Therefore, the exposure to patulin was simulated assuming that the consumption of fruit juice did not exceed 200 ml (S3). Based on the proportion of apple juice consumption in the total fruit juice consumption and the allowed fruit juice consumption of 200 ml, the allowed apple juice consumption was calculated. Table 4.6 shows the obtained results for the 3 types of apple juice. Comparing the exposures for the reduced consumption with the ones for normal consumption (Table 4.3) it can be observed that for the 99.9<sup>th</sup> percentile, the exposure was reduced by 43% for organic apple juice, while



for conventional and handcrafted apple juice this was 49% and 54% respectively. These higher reductions for handcrafted and conventional apple juice can be linked to the lower patulin concentration observed in both commodities, showing that a reduction of the consumption has more effect when the patulin contamination is lower. The reduction of the apple juice consumption shows that for conventional and handcrafted apple juice it is 90% certain that none of the children will exceed the TDI. For organic apple juice there was a small reduction of the probability to exceed the TDI, but it was less effective than reducing the apple juice contamination. Moreover, it is note-worthy that the ML-values are supposed to be implemented since it concerns a law, while the Food Based Dietary Guidelines are only recommendations which are often ignored or unknown by our population. When the fruit juice consumption was restricted to 170 ml (S4), similar results were obtained (results not shown). However, when only patulin intake is concerned, a restriction of the apple juice consumption to 200 ml (S5) will also lower the patulin intake and reduce the probability to exceed the TDI to 0 (with 90% certainty) for conventional and handcrafted apple juice (Table 4.7). Similar results were obtained for a restriction to 170 ml (S6) (results not shown).

**Table 4.6: Statistics on exposure (best estimation [90% confidence interval];  $\mu\text{g}/\text{kg}$  bw/day) and probability to exceed the tolerable daily intake (TDI) for different apple juices (AJ) when fruit juice consumption is below or equal to 200 ml/day (S3)**

	Organic AJ	Conventional AJ	Handcrafted AJ
P0 <sup>a</sup>	0 [0-0]	0 [0-0]	0 [0-0]
P50	0 [0-0]	0 [0-0]	0 [0-0]
P85	0 [0-0.014]	0 [0-0.010]	0 [0-0.013]
P90	0.026 [0.009-0.047]	0.019 [0.007-0.033]	0.024 [0.008-0.041]
P95	0.048 [0.018-0.077]	0.038 [0.020-0.056]	0.044 [0.018-0.073]
P97.5	0.069 [0.028-0.121]	0.056 [0.033-0.078]	0.059 [0.027-0.090]
P99	0.183 [0.067-0.519]	0.085 [0.058-0.118]	0.085 [0.039-0.120]
P99.5	0.421 [0.132-0.939]	0.113 [0.077-0.157]	0.107 [0.050-0.136]
P99.9	0.844 [0.371-1.634]	0.167 [0.116-0.217]	0.136 [0.066-0.167]
Mean	0.006 [0.011-0.018]	0.005 [0.004-0.007]	0.006 [0.003-0.009]
Probability to exceed TDI	0.006 [0.001-0.014]	0 [0-0]	0 [0-0]

<sup>a</sup>P0: 0<sup>th</sup> percentile

**Table 4.7: Statistics on exposure (best estimation [90% confidence interval]; µg/kg bw/day) and probability to exceed the tolerable daily intake (TDI) for different apple juices (AJ) when apple juice consumption is below or equal to 200 ml/day (S5)**

	Organic AJ	Conventional AJ	Handcrafted AJ
P0 <sup>a</sup>	0 [0-0]	0 [0-0]	0 [0-0]
P50	0 [0-0]	0 [0-0]	0 [0-0]
P85	0 [0-0.023]	0 [0-0.016]	0 [0-0.021]
P90	0.035 [0.012-0.065]	0.026 [0.009-0.043]	0.033 [0.011-0.061]
P95	0.055 [0.020-0.085]	0.046 [0.024-0.067]	0.051 [0.021-0.083]
P97.5	0.077 [0.031-0.150]	0.064 [0.038-0.089]	0.068 [0.031-0.101]
P99	0.226 [0.074-0.626]	0.100 [0.066-0.139]	0.099 [0.043-0.128]
P99.5	0.495 [0.151-1.168]	0.136 [0.088-0.174]	0.120 [0.055-0.145]
P99.9	1.011 [0.426-1.751]	0.182 [0.132-0.224]	0.145 [0.068-0.170]
Mean	0.014 [0.007-0.022]	0.007 [0.004-0.009]	0.007 [0.004-0.011]
Probability to exceed TDI	0.008 [0.002-0.017]	0 [0-0]	0 [0-0]

<sup>a</sup>P0: 0<sup>th</sup> percentile

## 4. Conclusion

It can be concluded that a proper treatment of the censored data may not be underestimated and that it is important to know the influence of assumptions that are made concerning censored data. It was demonstrated that a uniform distribution with uncertain bounds can be used to handle this censorship. Children consuming organic apple juice have a higher probability to exceed the TDI in comparison to children consuming conventional and handcrafted apple juice (0.009 versus 0.001 and 0). The presented research showed that in order to reduce the probability to exceed the TDI it is in the first place necessary to lower the contamination of patulin in apple juice to concentrations below 25 µg/kg. However, the implementation of a new ML will only be effective, when the apple juice producers are provided with tools to reduce the patulin contamination of apple juice. In this respect it is necessary to evaluate different risk mitigation strategies using a farm to fork risk assessment. However, for such an approach information concerning the growth and patulin production by the mould is necessary. This will be further elaborated in Chapters 5 to 8.

### Influence of storage conditions of apples on growth and patulin production by *Penicillium expansum*<sup>1</sup>

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<sup>1</sup> Based on: Baert, K., Devlieghere, F., Flyps, H., Oosterlinck, M., Ahmed, M., Verlinden, B., Nicolai, B., Debevere, J., De Meulenaer, B. Influence of storage conditions of apples on growth and patulin production by *Penicillium expansum*. Submitted for publication in International Journal of Food Microbiology.



## Chapter 5

# Influence of storage conditions of apples on growth and patulin production by *Penicillium expansum*

### Summary

*Penicillium expansum* causes blue mould rot, a serious post-harvest disease of apples, and is the main producer of the mycotoxin patulin. The present study aimed to determine the influence of storage conditions (i.e. temperature and O<sub>2</sub> level) on growth and patulin production by different *P. expansum* strains on a simulation medium and on apples. Growth was strongly influenced by the temperature, while the used atmosphere (20, 3, and 1% O<sub>2</sub>; <1% CO<sub>2</sub>) had no effect. Optimal growth was observed at 25°C for every strain tested. Patulin production was stimulated when the temperature decreased (from 20 to 10 or 4°C), while a further decrease of the temperature to 1°C caused a reduction in patulin production. The temperature at which the stimulation changed into suppression was strain dependent. Similar results were observed for the O<sub>2</sub> level. A reduction of the O<sub>2</sub> level from 20 to 3% O<sub>2</sub> could stimulate or suppress patulin production depending on the strain, while a clear decrease of the patulin production was observed when the O<sub>2</sub> level was reduced from 3 to 1%. These results show that the induction of limited stress to the fungus, such as lowering the temperature or lowering the O<sub>2</sub> levels, stimulates patulin production. However, the combination of different stress conditions (e.g. low temperature and low O<sub>2</sub>) will result in a reduced formation of the toxin. The combination of stress conditions, at which the transition from stimulation to suppression is observed, is strain dependent. Moreover, patulin production is characterized by a high natural variability. The presented results show that in order to suppress patulin production and to guarantee food safety, the temperature and O<sub>2</sub> level during the storage of apples has to be as low as possible without causing deterioration of the quality.

## 1. Introduction

*Penicillium expansum* is a filamentous fungus that is widespread in the environment. The preferred hosts of *P. expansum* are pomiferous fruits and this species is also responsible for blue mould rot, a major post-harvest disease of apples worldwide (Xu & Berrie, 2005). This fungus grows at low temperatures, so cold storage does not prevent spoilage but only retards it. Damaged and mature fruits show the greatest susceptibility to this mould (ICMSF, 1998). In Europe, particularly Northern Europe, losses in cold storage and in packhouses due to blue mould vary from year to year but can reach 5-15% (Bernard et al., 1993). Furthermore, *P. expansum* is known as the main producer of the mycotoxin patulin as described in Chapter 1.

The growth rate and patulin production of *P. expansum* are influenced to a large extent by environmental and endogenous factors typical for the substrate (Drusch & Ragab, 2003). The most important ones are water activity and temperature, although atmosphere and pH have also a large influence (Northolt et al., 1978; McCallum et al., 2002). However, since blue mould rot is a problem principally occurring during storage, temperature and atmosphere composition are the most important factors determining mould growth and mycotoxin production during storage of apples. Apples are usually stored under controlled atmosphere conditions (low temperature (0.5-3.5°C) in combination with reduced O<sub>2</sub> (1-3% O<sub>2</sub>) and elevated CO<sub>2</sub> (0.8-3% CO<sub>2</sub>)). Until now, research to reduce patulin production during storage of apples has focused on the use of high CO<sub>2</sub> levels, up to 90% (Orth, 1976; Moodley et al., 2002). However, high CO<sub>2</sub> levels are not used in practice since they result in quality defects of apples like off-flavours and scalding (Sitton & Patterson, 1992). Low O<sub>2</sub> levels have also been evaluated at 25°C (Paster et al., 1995), but the combination of a low O<sub>2</sub> level at higher temperature can also not be used in practice as it causes physiological disorders. The aim of the present study was to determine the influence of the extrinsic factors, temperature and atmosphere composition, on growth and patulin production of different *P. expansum* strains in order to develop a farm to fork risk assessment for patulin in apple juice (Chapter 8). The emphasis of this work was on low temperature, low O<sub>2</sub> and low CO<sub>2</sub> conditions as applied in practice during long term storage of apples. This research was performed in two stages. Firstly, a simulation medium based on apple puree was used to evaluate patulin production as a function of growth for a large number of conditions. Secondly, the observed results were validated on apples as a growth medium.

## **2. Materials and methods**

### **2.1. Fungal strains**

Strains MUM 00.01, MUM 99.23, MUM 99.19, MUM 99.20, MUM 99.22 and MUM 99.24 of *Penicillium expansum* were obtained from the Centro de Engenharia Biológica da Universidade do Minho in Braga (Portugal). Strains MUCL 29381 and MUCL 20543 were obtained from the Belgian Co-ordinated Collections of Micro-organisms, collection of agro-industrial fungi-yeasts (BCCM/MUCL, Louvain-la-Neuve, Belgium).

### **2.2. Inoculum preparation**

For inoculum preparation, firstly the fungus was inoculated on malt extract agar (MEA) and incubated at 22°C until sporulation. MEA was prepared according to the Blakeslee's formulation (Santos et al., 2002) (20 g bacteriological agar (Oxoid, England) was mixed with 20 g malt extract (Oxoid, England), 20 g glucose (Sigma, Germany), 1 g mycopepton (Oxoid, England) and 1 litre of demineralised water). The spore solution (or spore inoculum) was prepared according to the method proposed by Marquenie et al. (2002). The concentration of the spore solution was determined by means of a Bürker haematocytometer (Marienfeld, Germany) and was adjusted using phosphate buffer, pH 7.4 (Sigma, Germany) until a final concentration of  $10^5$  spores per ml was obtained.

### **2.3. Apple Puree Agar Medium (APAM) preparation and inoculation**

To prepare the Apple Puree Agar Medium, apples (cultivar Boskoop) were carefully inspected for bruises and rotten spots. After washing and drying, the apples were cut into pieces and sound apple pieces were mixed until a fine puree was obtained. The puree was stored at -24°C until needed. To prepare APAM, 100 g of apple puree was weighed in a sterile Schott bottle and heated during 45 minutes in a hot water bath at 99°C. In this way a temperature of 90°C was reached during 3 minutes at the centre of the puree to kill vegetative cells. 50 ml sterile agar solution (4.2 g bacteriological agar (Oxoid, England) in 140 ml demineralised water) was then added to the puree to make the APAM. Under aseptic conditions, petri dishes (Novolab N.V., Belgium) were filled with 20 g APAM. Solidified APAM plates were inoculated aseptically by transferring 25 µl of the spore solution to the centre of each APAM plate.

## 2.4. Incubation of APAM plates at different temperatures in air

To incubate APAM plates at different temperatures in air, APAM plates were placed in recipients with a perforated, false bottom. Under this false bottom, a 1.5% NaCl solution (VWR, Belgium) was used to keep the water activity of the APAM plates at a constant level ( $a_w = 0.991$ ). Recipients were placed in the incubators and during incubation they were frequently opened to avoid the creation of anaerobic conditions. Growth of six strains of *P. expansum* (MUM 00.01, MUM 99.23, MUM 99.19, MUM 99.20, MUM 99.22 and MUM 99.24) was followed at 2, 4, 7, 10, 12, 16, 20, 25 and 30°C. Growth of 15 colonies was followed for every strain-temperature combination. Patulin production during growth was followed for *P. expansum* MUM 00.01 at 1, 4, 7, 10, 16, 20 and 30°C. The influence of strain variability on patulin production was determined by evaluating the patulin production of 8 different strains (MUM 00.01, MUM 99.23, MUM 99.19, MUM 99.20, MUM 99.22, MUM 99.24, MUCL 29381 and MUCL 20543) at 1, 4, 10 and 20°C when the colony surface reached 45 cm<sup>2</sup>.

## 2.5. Incubation of APAM plates at different controlled atmospheres

To investigate the effect of controlled atmospheres (CA) on growth and patulin production, inoculated plates were packaged separately in a plastic bag. The inoculated plate was fixed with tape on a piece of cardboard together with a plate containing soda lime (Fluka, Germany) and a plate with a 1.5% NaCl solution (VWR, Belgium). Soda lime was used to trap the CO<sub>2</sub> produced by *P. expansum*. The salt solution was necessary to maintain the water activity of the APAM plates. The plates on the cardboard were packaged in high O<sub>2</sub> gas barrier bags (NX90, EuralPack, Belgium) with an O<sub>2</sub> permeability of 5.2 ml/m<sup>2</sup>/24 h/atm at 85% R.H. and 23°C. The desired gas mixture was introduced into each bag and sealed by means of a gas packaging unit consisting of a gas mixer (Gasetechnik, Germany) and a gas packager (Multivac A300/42, Hagenmüller KG, Germany). The composition of the atmosphere was analysed by a Servomex 1450 Food Package Analyser (The Netherlands). O<sub>2</sub> and N<sub>2</sub> (fresh line brand) were obtained from Air Products (Belgium). APAM plates were packaged under atmospheres with 1% O<sub>2</sub> or 3% O<sub>2</sub>. For both conditions the CO<sub>2</sub> level was <1% and N<sub>2</sub> was used as a filler gas. Packages with 1% O<sub>2</sub> were repacked when the O<sub>2</sub> level was as low as 0.5%. For the bags containing 3% O<sub>2</sub>, the lowest allowed limit was 2% O<sub>2</sub>. A Teflon septum (Alltech, Belgium) was fixed on the plastic bags with silicone to allow measurement of the gas concentrations without causing leakages. Growth was followed at 3% O<sub>2</sub> (4, 10, 12 and



20°C) and 1% O<sub>2</sub> (4°C) for *P. expansum* MUM 00.01. Growth of 15 colonies was followed for every oxygen level. Patulin production during growth was followed for *P. expansum* MUM 00.01 at 3% O<sub>2</sub> (1, 4, 7, 10 and 20°C) and 1% O<sub>2</sub> (1 and 4°C). The influence of strain variability of patulin production was evaluated for 8 different strains (MUM 00.01, MUM 99.23, MUM 99.19, MUM 99.20, MUM 99.22, MUM 99.24, MUCL 29381 and MUCL 20543) at 3% O<sub>2</sub> (1 and 4°C) and 1% O<sub>2</sub> (1°C) when the colony surface reached 45 cm<sup>2</sup>.

## **2.6. Growth measurements and sampling method for patulin analysis on APAM**

After inoculation, the plates were checked every day to see if any visible growth started. As soon as visible growth started, perpendicular diameters of the colony were measured with a digital calliper at regular time intervals. The growth of 15 colonies was followed for every tested condition. To evaluate patulin production, samples were taken (n=3 or 5) when the colony diameter reached 1.5, 3, 4.5, 6 and 7.5 cm. The samples were weighed and stored in Falcon tubes (50 ml, Becton Dickinson, USA) at -24°C until further patulin analysis.

## **2.7. Inoculation of apples**

Before apples were inoculated, apples (cv. Boskoop) were washed with water and submerged in ethanol. After evaporation of the ethanol, 20 µl spore suspension (10<sup>5</sup> spores/ml) of *P. expansum* MUM 00.01 was injected with a sterile syringe (Micro-Fine 0.5 ml, Becton Dickinson, USA) in the apple at a depth of 1 cm. Apples were placed in sterile 1 litre containers (Gosselin, France).

## **2.8. Incubation of apples at different temperatures in air**

To incubate the apples at different temperatures in air, the plastic containers with apples were placed in incubators (1, 4 and 20°C). For every tested condition, 45 apples were inoculated. To prevent anaerobic conditions being generated due to the respiration of the apples and fungi, the containers were opened in a sterile environment on a regular basis (every day for apples stored at 20°C and every 5 days for apples stored at 1 and 4°C).

## **2.9. Incubation of apples at different controlled atmospheres**

Incubation of apples under controlled atmosphere was performed in hermetic incubators in which the temperature and the O<sub>2</sub> and CO<sub>2</sub> concentration were controlled. The temperature was set at 1 or 4°C. The composition of the atmosphere in the incubator was monitored using an O<sub>2</sub> and CO<sub>2</sub> analyser (Matec, Belgium) and adapted when necessary to maintain the CA. The plastic containers with the apples were not closed, in order to have the appropriate controlled atmosphere in the container. Patulin production was followed for *P. expansum* MUM 00.01 at 3% O<sub>2</sub> (1 and 4°C) and 1% O<sub>2</sub> (1°C). For every tested condition 45 apples were incubated.

## **2.10. Growth measurements and sampling method for patulin analysis in apples**

After inoculation, the apples were checked on a regular basis to see if visible growth had started. To follow up patulin production, samples were taken at certain colony diameters. The colony diameter was measured with a digital calliper in two perpendicular directions. Also the diameter of every apple was measured, in order to be able to calculate the diameter of the curved surface and the volume of the rotten spot (see section 2.12). The apple was cut in half, in order to measure the depth of the rotten spot. The complete apple was pureed and 20 g was weighed into a Falcon tube (50 ml, Becton Dickinson, USA). The samples were stored at -24°C until patulin analysis was performed.

## **2.11. Patulin analysis**

Patulin analysis was performed as described in Chapter 2 – section 2.1 with some modifications. Briefly, 20 drops of Rapidase Adex D (DSM, France) and 20 ml of water acidified with acetic acid (Chemlab Scientific Products, Belgium) to pH 4 were added to the samples (20 g). The samples were thoroughly shaken, wrapped in aluminium foil and placed overnight on a rotary mixer at room temperature. The samples were filtered by vacuum over a filter paper (Whatman 40, Ø 90 mm, England) in a Buchner funnel. The filtrate (10 ml) was extracted three times with ethyl acetate (20 ml) (Chemlab Scientific Products, Belgium) by shaking vigorously for 1 minute. The organic phases were combined and a clean up was performed with 1.5% sodium carbonate (VWR, Belgium). The organic phase was dried with anhydrous sodium sulphate (Chemlab Scientific Products, Belgium). The extract was

evaporated and the residue was immediately dissolved in 500 µl of water at pH 4.0. The samples were filtered using a Millex-HV syringe Driven Filter Unit (Millipore, USA) and analysed using HPLC-UV (Finnigan Autosampler Surveyor, Thermo, USA). This method for patulin determination in solid apple products has a limit of detection (LOD) of 4 µg/kg and a limit of quantification (LOQ) of 5 µg/kg.

## 2.12. Mathematical and statistical analysis

To calculate the volume of rot in an apple, it was assumed that the apples were perfect spheres and the rotten spot was divided into a segment of a sphere and a cone (Figure 5.1). The volume of rot was equal to the sum of the volumes of these two segments. Figure 5.1 shows the different variables of equation (5.1).

$$V_{rot} = V_{segment\ of\ a\ sphere} + V_{cone}$$

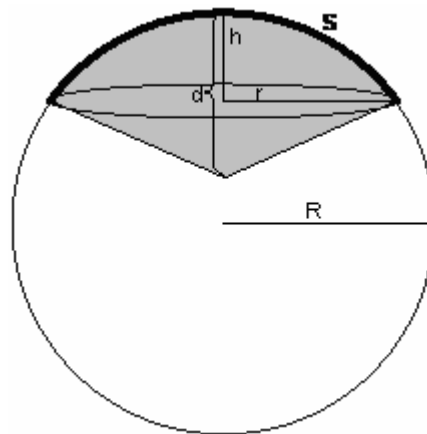
$$\Rightarrow V_{rot} = \frac{\pi \cdot h \cdot (3r^2 + h^2)}{6} + \frac{\pi \cdot r^2 \cdot (d - h)}{3} \quad (5.1)$$

with:  $h$  = height of the segment of a sphere =  $R - \sqrt{(R^2 - r^2)}$

$r$  = radius of the moulded spot (sum of the 2 measured perpendicular diameters of the rotten spot divided by 4) (cm)

$R$  = radius of the apple (sum of the 2 diameters of the apple divided by 4) (cm)

$d$  = depth of the rotten spot (cm)



**Figure 5.1: Schematic representation of an apple with the infected tissue in grey**

Statistical analysis was performed with SPSS 11.0 for Windows (SPSS Inc., USA). The dataset was checked with the Grubbs test for outliers, which were omitted from the results. Radial growth rates (mm/h) and the lag phase (h) at each temperature were derived from the

growth data using linear regression (Microsoft excel, Microsoft, USA) as described by Pardo et al. (2004). 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. Multiple regression of equation (5.2) was performed using the SAS/STAT software 8.2 (SAS Institute Inc., Cary NC. USA).

$$[\text{pat}] = a.T + b.S + c.O + d.T.S + e.T.O + f.S.O + g.T.S.O + h.T^2 + i.T^2.S + j.T^2.O + k.T^2.S.O \quad (5.2)$$

with [pat]: patulin concentration (mg/kg)

T: temperature (°C)

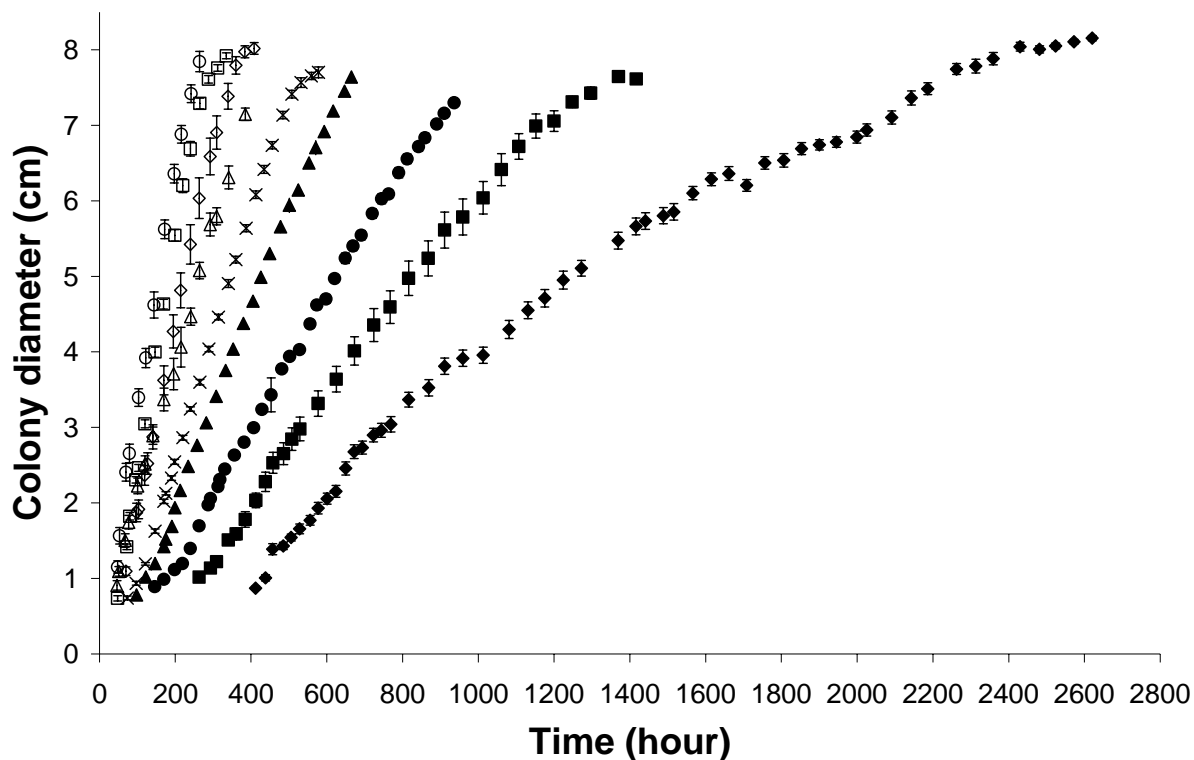
S: surface (cm<sup>2</sup>)

O: oxygen level (%)

### 3. Results

#### 3.1. Effect of temperature on growth of *P. expansum* in air (APAM)

For *P. expansum* MUM 00.01, the growth curves based on colony diameters were characterized by a lag phase, followed by linear growth for all temperatures studied (Figure 5.2). Growth stopped at a diameter of about 8 cm, due to the limited growth area on the petri dish (Ø 9 cm). The shortest lag phase and the highest growth rate (slope of the growth curve) were observed at 25°C. At lower temperatures, the influence of a small temperature difference had a more pronounced effect on the growth characteristics compared to the higher temperatures. At 2°C the lag time was 150 hours longer than at 4°C (400 and 250 hours, respectively), while the lag time for 25°C and 20°C differed only 24 hours (24 and 48 hours, respectively). A similar effect was observed for the growth rate. Moreover, at 30°C different morphologies of the colonies were observed compared to the other temperatures, such as colonies with extending white mycelium without spore formation or colonies with very dense white mycelium that stayed smaller than 5 to 6 cm.



**Figure 5.2:** Growth curves of *P. expansum* MUM 00.01 in air on APAM for different incubation temperatures (◆: 2°C, ■: 4°C, ●: 7°C, ▲: 10°C, ×: 12°C, ◇: 16°C, □: 20°C, ○: 25°C, △: 30°C) (error bars represent 95<sup>th</sup> confidence interval of the mean, n = 15)

### 3.2. Effect of oxygen level on growth (APAM)

At 4°C, two controlled atmospheres (CA) (3% O<sub>2</sub>; <1% CO<sub>2</sub> and 1% O<sub>2</sub>; <1% CO<sub>2</sub>) were tested together with air (20% O<sub>2</sub>; <1% CO<sub>2</sub>) for their influence on growth. For both CA conditions and air, strain MUM 00.01 grew at a similar rate (Table 5.1), which was not significantly different (p>0.05). Within the temperature range from 10 to 20°C also no effect of the oxygen level (3% O<sub>2</sub> and 20% O<sub>2</sub>) on the growth was observed (p>0.05).

### 3.3. Effect of temperature on patulin production (APAM)

In order to evaluate the influence of temperature on patulin production, APAM plates were in a first series of experiments inoculated with *P. expansum* MUM 00.01 and incubated at the appropriate temperature and 20% O<sub>2</sub>. At particular diameters, samples were analysed for their patulin content. For each temperature, except at 30°C, the same production profile was observed (Figure 5.3). Patulin concentrations increased as a function of time and consequently also as a function of the colony surface area. The lower the temperature at 20% O<sub>2</sub>, the longer

the incubation time to reach a certain surface area and the higher the patulin level (except for 1°C). The highest patulin concentrations at 20% O<sub>2</sub> for a given surface area were observed at 4°C and this was significantly higher than at 7 (p = 0.039) and 10°C (p = 0.047). A further increase of temperature to 16 and 20°C resulted in a decrease in patulin production. At 30°C the general accumulated patulin level remained low. At 1°C, the maximal patulin concentration was again lower than at 4°C. As can be observed from the error bars in Figure 5.3, patulin production was characterized by a large variability between replicates.

**Table 5.1: Growth rate of *P. expansum* MUM 00.01 on apples for different oxygen concentrations and different temperatures (n=15)**

Temperature (°C)	Growth rate (mm/h)		
	20% O <sub>2</sub>	3% O <sub>2</sub>	1% O <sub>2</sub>
4	0.0631 ± 0.0005 <sup>a</sup> <sub>b</sub>	0.0592 ± 0.0016 a	0.0600 ± 0.0011 a
10	0.1322 ± 0.0022 b	0.1354 ± 0.0015 b	n.d. <sup>c</sup>
12	0.1673 ± 0.0027 c	0.1623 ± 0.0007 c	n.d.
20	0.2985 ± 0.0059 d	0.3079 ± 0.0041 d	n.d.

<sup>a</sup>mean ± standard error; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different (p>0.05); <sup>c</sup>n.d.: not determined

At an oxygen level of 3% O<sub>2</sub> however, the highest patulin concentration was detected at 10°C which was significantly higher than 4°C (p = 0.033) and 1°C (p = 0.01) (Figure 5.3). In accordance to the results for 20% O<sub>2</sub>, reduced patulin production was observed at 1°C in comparison to 4°C. At 1% O<sub>2</sub> a significantly lower patulin production was observed at 1°C compared to 4°C (p = 0.024). Summarizing, the results showed that lowering the temperature to 4°C at 20% O<sub>2</sub> caused a stimulation of the patulin production, while for lower O<sub>2</sub> levels the optimum temperature for patulin production is higher than 4°C. Furthermore, a consistent decrease in patulin production at 1°C was observed for all O<sub>2</sub> levels tested.

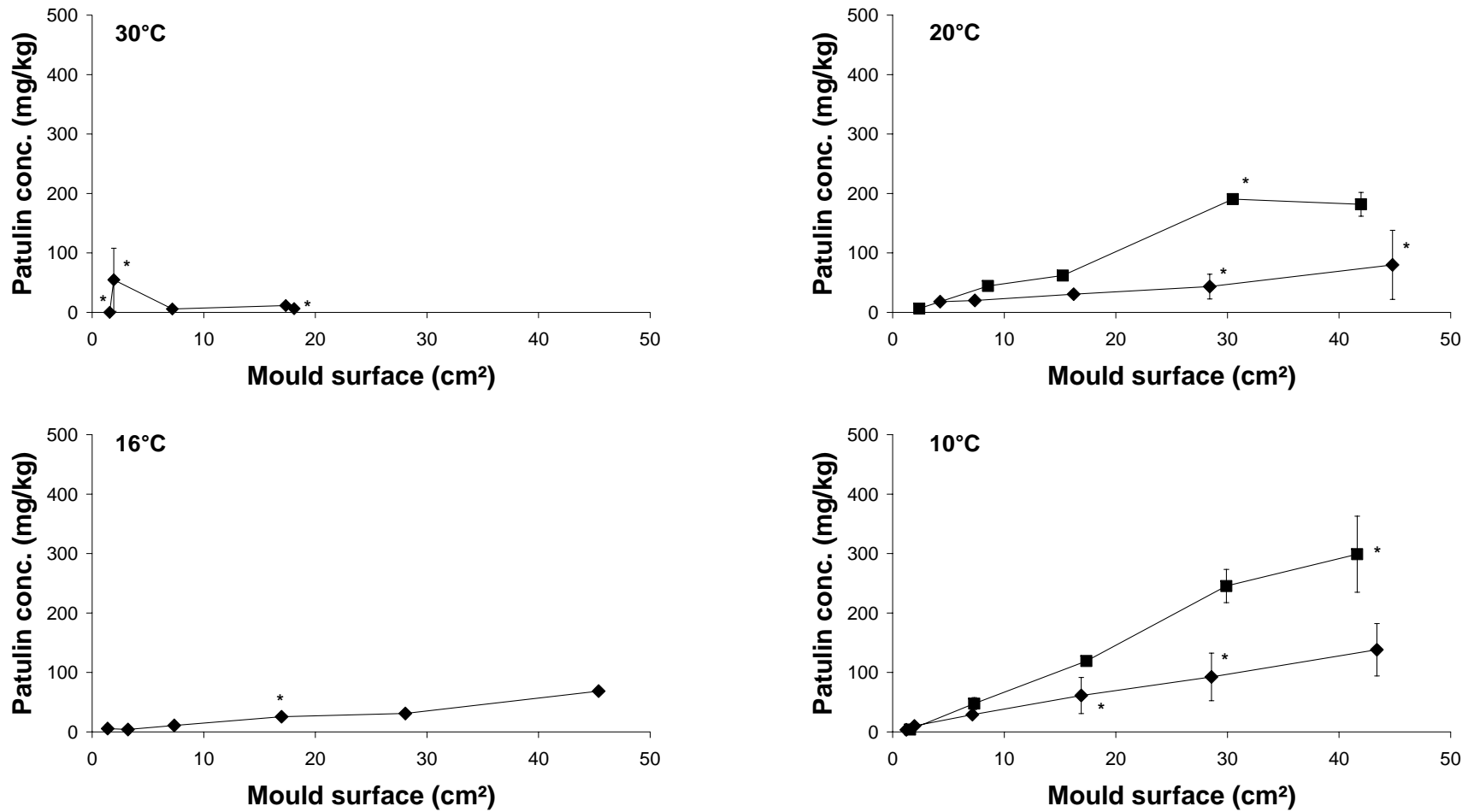


Figure 5.3: Patulin production (expressed as patulin concentration (mg/kg APAM)) by *P. expansum* MUM 00.01 in APAM at 30, 20, 16, 10, 7, 4 and 1°C and different oxygen levels (—◆—:20% O<sub>2</sub>, —■—: 3% O<sub>2</sub>, —▲—:1% O<sub>2</sub>) (error bars represent mean ± standard deviation; 30, 20, 16 and 10°C: n = 2, data points with \* n = 3; 7, 4 and 1°C: n = 5)

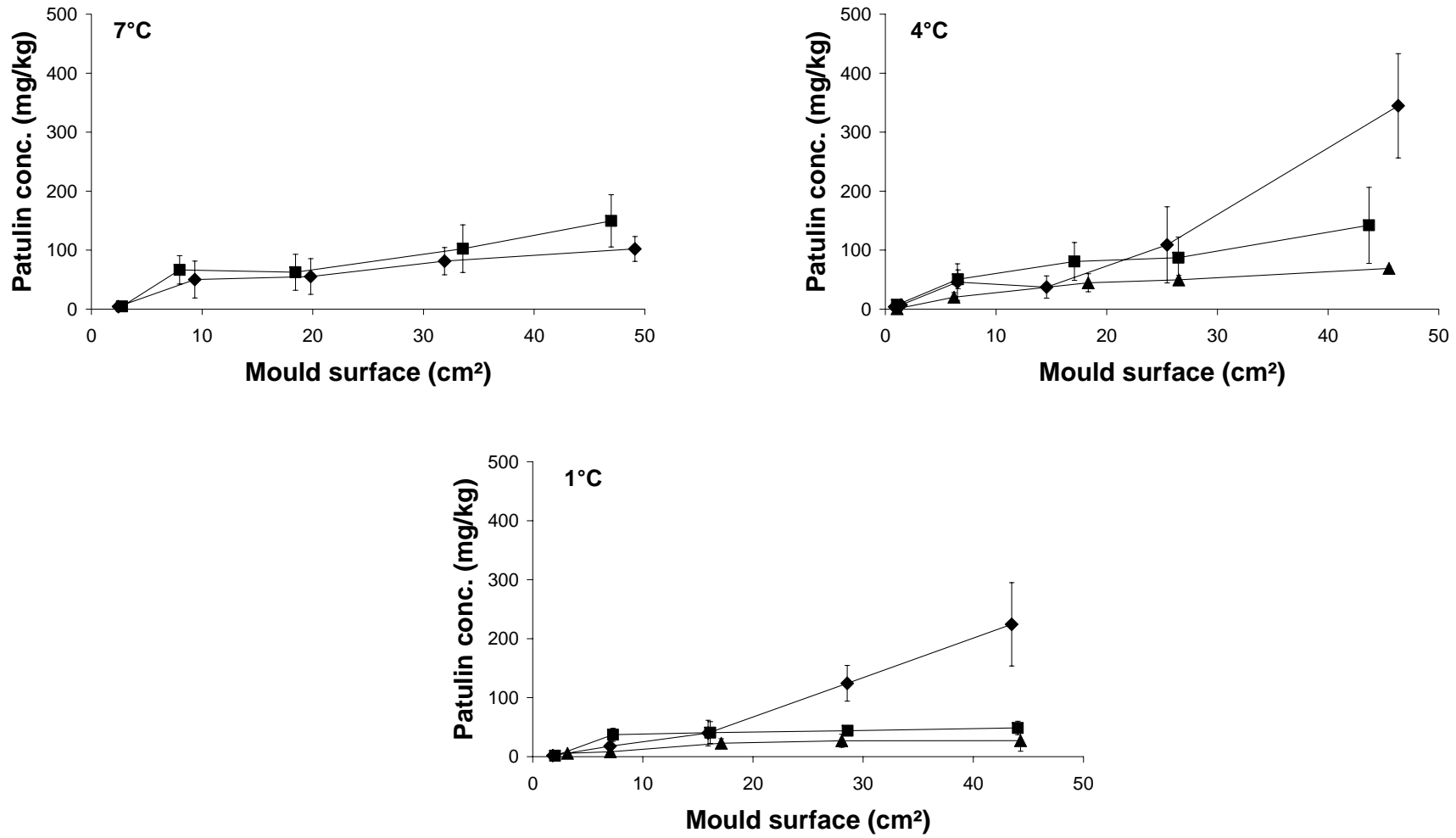


Figure 5.3: Patulin production (expressed as patulin concentration (mg/kg APAM)) by *P. expansum* MUM 00.01 in APAM at 30, 20, 16, 10, 7, 4 and 1°C and different oxygen levels (—◆—: 20% O<sub>2</sub>, —■—: 3% O<sub>2</sub>, —▲—: 1% O<sub>2</sub>) (error bars represent mean ± standard deviation; 30, 20, 16 and 10°C: n = 2, data points with \* n = 3; 7, 4 and 1°C: n = 5) (Continued)

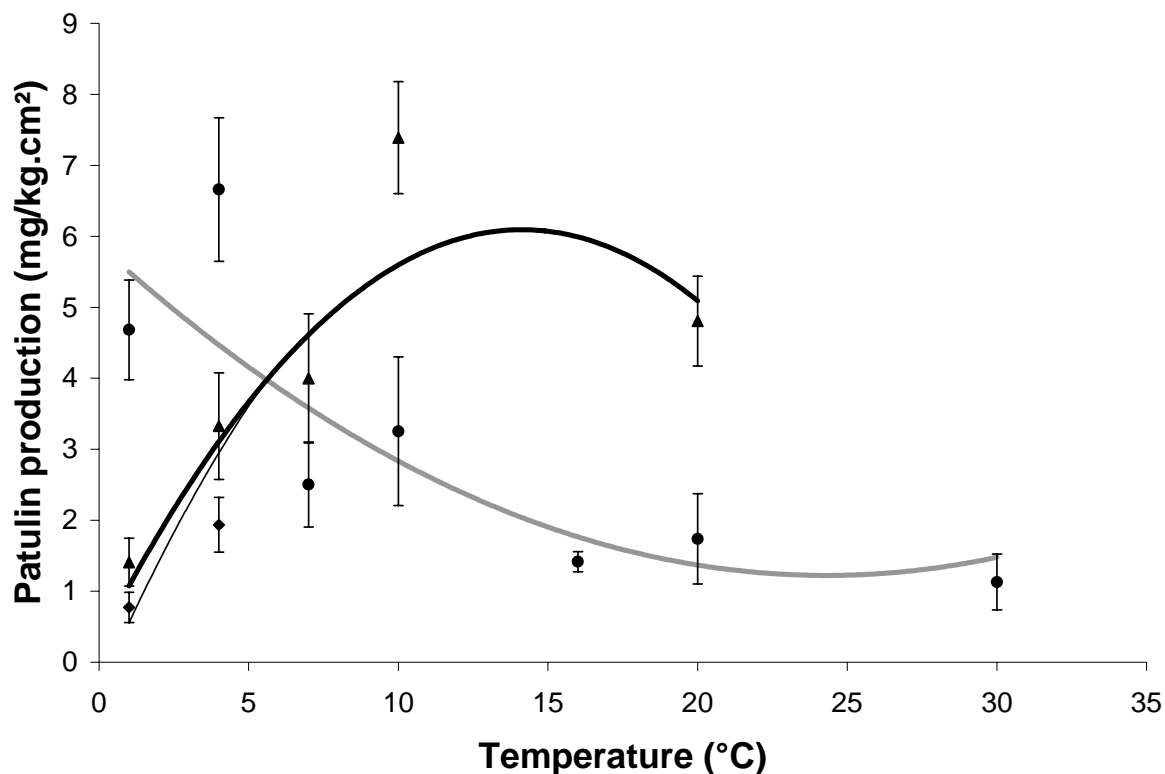


### 3.4. Effect of oxygen on patulin production (APAM)

The influence of O<sub>2</sub> on patulin production was highly influenced by the temperature. At 20 and 10°C higher patulin concentrations were achieved at 3% O<sub>2</sub> compared to 20% throughout the whole growth experiment (Figure 5.3). The maximum patulin concentrations achieved, doubled when the O<sub>2</sub> level dropped from 20 to 3%. Higher patulin concentrations were also observed at 7°C for 3% O<sub>2</sub>, however these differences were not statistically significant ( $p = 0.071$ ). Opposite results were obtained at 4 and 1°C (Figure 5.3). At 4°C, patulin production was comparable for the 3 atmospheres until about 25 cm<sup>2</sup>, but for larger diameters patulin production was considerably suppressed at 3% and 1% O<sub>2</sub> compared to 20% O<sub>2</sub>. At 1°C stagnation in patulin production was observed at a surface of about 20 cm<sup>2</sup> for 3 and 1% O<sub>2</sub> while at 20% O<sub>2</sub>, the increase in patulin concentration continued as a function of growth. The effect of O<sub>2</sub> on patulin production at low temperature was more pronounced at 1°C than at 4°C. At 4°C, a decrease in O<sub>2</sub> level from 20 to 3% reduced patulin biosynthesis with 60%, while further decrease to 1% O<sub>2</sub> resulted in a reduction of 80%. In contrast, at 1°C a decrease of the O<sub>2</sub> level from 20 to 3% reduced patulin formation by 78%, while a further decrease to 1% O<sub>2</sub> resulted in a mitigation of 88%.

### 3.5. Effect of temperature and oxygen on patulin production (APAM)

In order to study the trends observed in section 3.3 and 3.4 further, a quadratic model was fit by multiple regression to the obtained data. The quadratic model was selected since an optimum was observed for the patulin production. The main effects and interaction terms with surface as a variable were selected at a significant level of 0.05, indicating that the surface has a significant influence on the patulin concentration. Also temperature, O<sub>2</sub> and the interaction between temperature and O<sub>2</sub> were significant. Figure 5.4 shows the model for three fixed O<sub>2</sub> levels together with the observed data. The model shows the significant trends, which are in correspondence to the trends described in section 3.3 and 3.4, with the exception of the decrease in patulin production at 20% O<sub>2</sub> between 4 and 1°C.



**Figure 5.4:** Patulin production (lines represent model, symbols represent observed data) as a function of the temperature at different oxygen levels (● and ▲ :20% O<sub>2</sub>, ◆ and ■ :3% O<sub>2</sub>) (error bars represent 95<sup>th</sup> confidence interval of the mean)

### 3.6. Strain variability

In order to analyse whether the observed effect of temperature and oxygen on patulin production was general for all strains of *P. expansum*, patulin production was determined for eight *P. expansum* strains when the colony surface reached 45 cm<sup>2</sup>. Table 5.2 shows the time needed to reach a colony surface of 45 cm<sup>2</sup> for different temperatures. Strain MUM 00.01, which was used for the previous experiments, was the fastest growing strain. Strains MUM 99.24 and MUM 99.20 also grew faster than the other strains. *P. expansum* MUCL 20453 on the other hand was the slowest growing strain together with strains MUM 99.19 and MUCL 29381. The growth of the different strains is further studied in Chapter 7.

**Table 5.2: Incubation time (days) to reach a colony surface of 45 cm<sup>2</sup> for different temperatures and different strains of *P. expansum***

Strain	20°C	10°C	4°C	1°C
MUM 00.01	13	27	69	115
MUM 99.24	14	30	73	117
MUM 99.20	16	32	90	165
MUM 99.22	17	35	80	132
MUM 99.23	18	41	83	157
MUCL 29381	19	43	122	190
MUM 99.19	20	45	97	167
MUCL 20453	23	48	124	204

Table 5.3 shows the influence of temperature on patulin production for the various strains at 20% O<sub>2</sub>. For the fast growing strain MUM 99.24, an increase in patulin production was observed when the temperature was decreased to 10°C, but again a lower patulin concentration was observed at 1°C. In contrast, the slowly growing strain MUM 99.19, showed a continuous decreased patulin production upon a decreasing temperature. For strain MUCL 20453 no consistent trend could be observed, apart from a decrease in patulin production following a decrease of the temperature from 4 to 1°C. Also for strain MUM 99.23 no real trend could be observed. Strain MUM 99.20 and MUM 99.22 increased patulin production when the temperature was lowered from 20 to 10°C, but a further reduction to 1°C caused a mitigation in patulin production. Summarizing, a reduction of the temperature from 4 to 1°C reduces patulin production. However, for the higher temperatures the effect of temperature was strain dependent. This was also confirmed using analysis of variance, since the factor temperature was not significant ( $p = 0.556$ ) while the interaction between temperature and strain was significant ( $p < 0.001$ ).

At 3% O<sub>2</sub> a decrease in patulin production was observed when the temperature was lowered from 4 to 1°C, except for strain MUCL 20453 (Table 5.4). *P. expansum* MUCL 29381 had a patulin concentration below the LOD (0.004 mg/kg) for the two conditions tested. These results confirm the trend observed at 20% O<sub>2</sub>, that upon a further reduction of temperature to 1°C, patulin production is reduced.

**Table 5.3: Influence of temperature on patulin formation at 20% O<sub>2</sub> by different strains of *P. expansum* (colony surface is 45 cm<sup>2</sup>)**

Strain	Patulin concentration (mg/kg)			
	20°C	10°C	4°C	1°C
MUM 00.01	80 ± 34 <sup>a b</sup> n = 3	138 ± 31 ab n = 2	345 ± 45 b n = 5	224 ± 36 ab n = 5
MUM 99.24	84 ± 17 a n = 5	210 ± 38 a n = 5	n.d. <sup>c</sup>	135 ± 11 a n = 5
MUM 99.23	241 ± 23 a n = 4	286 ± 63 a n = 5	n.d.	341 ± 18 a n = 4
MUM 99.20	184 ± 64 a n = 5	219 ± 61 a n = 5	61 ± 20 a n = 5	67 ± 8 a n = 4
MUM 99.22	78 ± 10 a n = 4	236 ± 74 ab n = 5	94 ± 26 ab n = 5	126 ± 8 b n = 5
MUM 99.19	440 ± 36 a n = 4	349 ± 64 a n = 5	177 ± 31b n = 5	126 ± 15 b n = 5
MUCL 29381	0.07 ± 0.04 a n = 5	0.14 ± 0.18 a n = 5	0.04 ± 0.03 a n = 5	<0.004 n = 5
MUCL 20453	191 ± 41 ab n = 5	68 ± 4 ab n = 5	369 ± 85 ac n = 5	271 ± 17 c n = 4

<sup>a</sup>mean ± standard error; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different ( $p > 0.05$ ); <sup>c</sup>n.d.: not determined

The influence of O<sub>2</sub> level was evaluated at 1°C (Table 5.5). Strains MUM 99.24 en 99.23 produced less patulin when the oxygen level dropped from 20 to 1% O<sub>2</sub>, which corresponded to the results for strain MUM 00.01. The patulin concentration of MUCL 29381 was below the LOD and therefore no influence of the O<sub>2</sub> level on patulin production could be deduced for this strain. For the other slowly growing strains the patulin production increased when the oxygen level was lowered to 3% O<sub>2</sub>, but decreased again in the 1% O<sub>2</sub> condition. The observed results indicate that a reduction of the O<sub>2</sub> level to 1% at 1°C reduces patulin production, while for higher O<sub>2</sub> levels this effect is strain dependent. This was also confirmed using analysis of variance, since the interaction between oxygen level and strain was significant ( $p < 0.001$ ).

**Table 5.4: Influence of temperature on patulin formation at 3% O<sub>2</sub> by different strains of *P. expansum* (colony surface is 45 cm<sup>2</sup>)**

Strain	Patulin concentration (mg/kg)	
	4°C	1°C
MUM 00.01	142 ± 33 <sup>a</sup> b <sup>b</sup> n = 5	49 ± 6 b n = 5
MUM 99.24	147 ± 6 a n = 4	90 ± 8 b n = 4
MUM 99.23	147 ± 10 a n = 5	41 ± 3 b n = 5
MUM 99.20	165 ± 22 a n = 5	129 ± 8 a n = 5
MUM 99.22	300 ± 9 a n = 3	175 ± 21 b n = 5
MUM 99.19	509 ± 11 a n = 4	269 ± 66 a n = 3
MUCL 29381	<0.004 n = 5	<0.004 n = 5
MUCL 20453	583 ± 24 a n = 4	672 ± 27 b n = 5

<sup>a</sup>mean ± standard error; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different (p>0.05)

### 3.7. Effect of temperature and oxygen level on patulin production in apples

The previous experiments were performed on APAM plates. However, in practice *P. expansum* grows on apples. Therefore, the influence of temperature and oxygen on growth and patulin production in apples was also investigated and compared to the results obtained on APAM. For each condition 45 apples were inoculated. The number of apples on which fungal growth was observed decreased with decreasing temperature. For an oxygen level of 20% at 20, 4 and 1°C, growth was observed on 36 (80%), 12 (27%) and 5 apples (11%), respectively. This was in contradiction to the results on APAM plates, since growth was

observed on every inoculated APAM plate. This phenomenon was further studied in Chapter 6.

**Table 5.5: Influence of oxygen level on patulin formation at 1°C by different strains of *P. expansum* (colony surface is 45 cm<sup>2</sup>)**

Strain	Patulin concentration (mg/kg)		
	20% O <sub>2</sub>	3% O <sub>2</sub>	1% O <sub>2</sub>
MUM 00.01	224 ± 36 <sup>a</sup> <sup>b</sup>	49 ± 6 b	27 ± 9 b
	n = 5	n = 5	n = 5
MUM 99.24	135 ± 11 a	90 ± 8 b	54 ± 2 c
	n = 5	n = 4	n = 4
MUM 99.23	341 ± 18 a	41 ± 3 b	29 ± 3 b
	n = 4	n = 5	n = 4
MUM 99.20	67 ± 8 a	129 ± 8 b	114 ± 3 b
	n = 4	n = 5	n = 4
MUM 99.22	126 ± 8 a	175 ± 21 a	157 ± 7 a
	n = 5	n = 5	n = 4
MUM 99.19	126 ± 15 a	269 ± 66 a	158 ± 9 a
	n = 5	n = 3	n = 4
MUCL 29381	<0.004	<0.004	<0.004
	n = 5	n = 5	n = 5
MUCL 20453	271 ± 17 a	672 ± 27 b	423 ± 20 a
	n = 4	n = 5	n = 5

<sup>a</sup>mean ± standard error; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different (p>0.05)

Since the amount of patulin varied to a large extent between apples with the same volume of rot (Figures 5.5 and 5.6), it was necessary to check whether this variance was not caused by sampling or analytical analysis. Therefore, three apples were analysed in double by taking two sub samples of each apple that were analysed on their patulin content. The standard deviation of the doubles was not more than 10% indicating that the analytical method was well performed and the high standard deviations were due to natural variability.

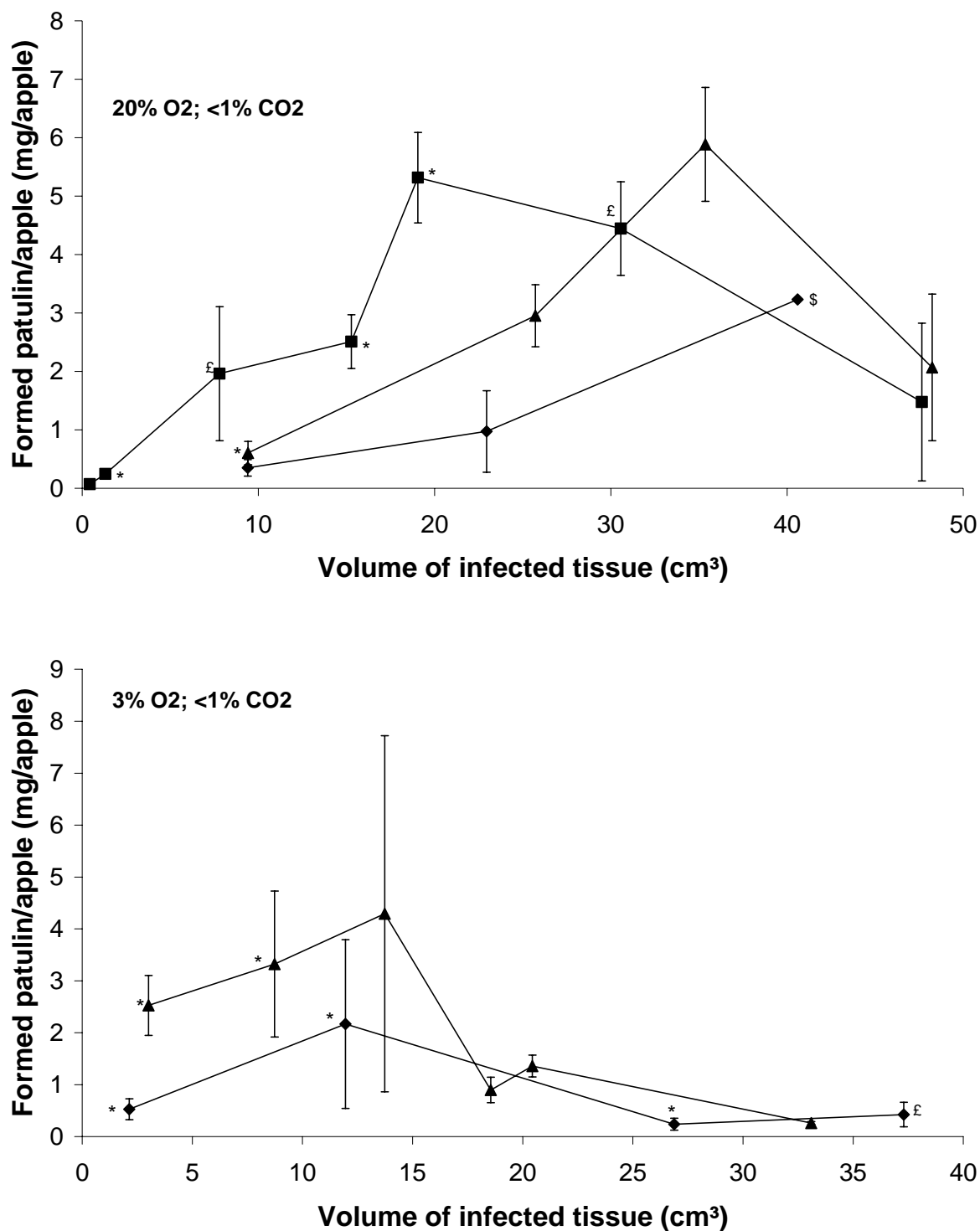
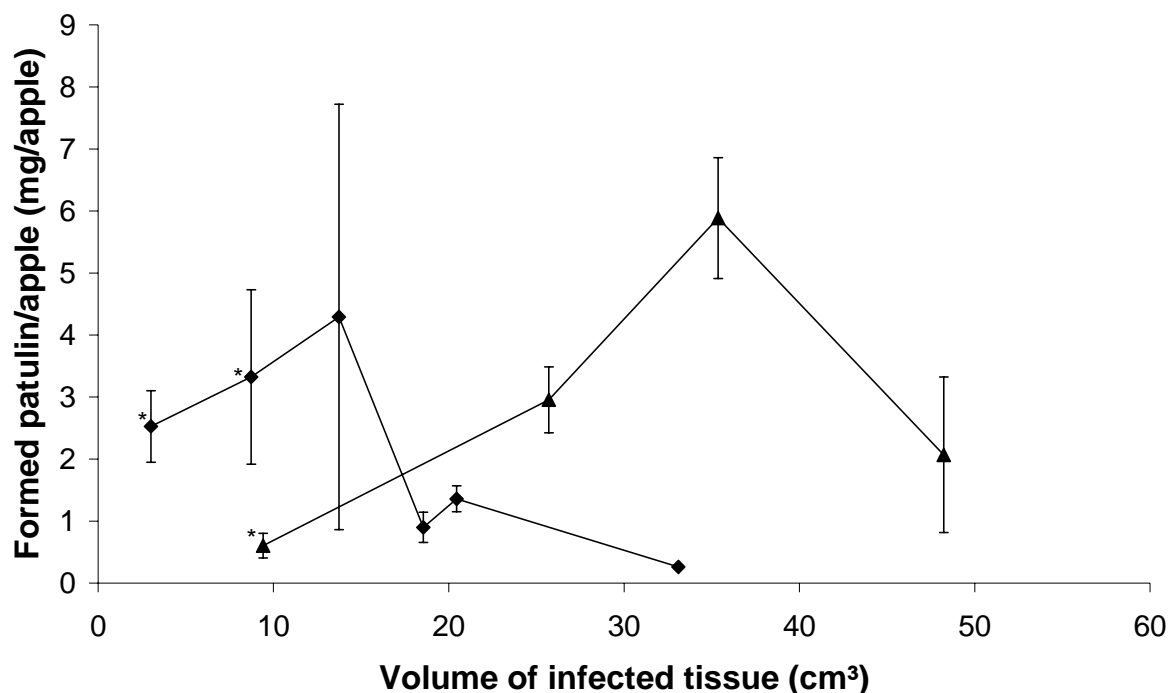


Figure 5.5: Patulin production by *P. expansum* MUM 00.01 in apples at 20% and 3% O<sub>2</sub> and at different temperatures (—◆—: 1°C, —▲—: 4°C, —■—: 20°C) (error bars represent mean ± standard deviation; 1 and 4°C: n = 2; 20°C: n = 5, data points with \$ n = 1, data points with \* n = 3, data points with £ n = 4)



**Figure 5.6:** Patulin production by *P. expansum* MUM 00.01 in apples at 4°C and at oxygen levels (—◆—:3% O<sub>2</sub>, —▲—:20% O<sub>2</sub>) (error bars represent mean ± standard deviation; n = 2, data points with \* n = 3)

At 20% O<sub>2</sub> (Figure 5.5), the amount of patulin increased, when the mould was growing and thus the volume of infected tissue enlarged. At 20 and 4°C, the amount of patulin decreased again from a certain volume onwards. This decrease was not observed for 1°C, but it should be noted that patulin formation was only followed until 40 cm<sup>3</sup> of infected tissue. In general, higher patulin levels were observed at 4°C than at 1°C. Similar curves were observed at 3% O<sub>2</sub> (Figure 5.5). Due to the variability of patulin production between replicates, it was difficult to determine consistent trends. In general however, the amount of patulin initially increased up to a particular volume of infected tissue, whereupon the amount of patulin in the apple decreased as function of infected volume. A decrease of temperature from 4 to 1°C at an O<sub>2</sub> level of 3 % caused a decrease in patulin biosynthesis as well.

Figure 5.6 shows the influence of oxygen level at 4°C. Taking the variability between replicates into consideration, consistent trends were again difficult to determine. For the same volume of infected tissue more patulin is produced initially at 3% than at 20% O<sub>2</sub>, whereas at larger volumes more patulin is produced at 20% O<sub>2</sub> than at 3%. In general however, more patulin was produced at 20% O<sub>2</sub> than at 3% O<sub>2</sub> which is in agreement with the results



observed on APAM. At 1°C it was not possible to draw conclusions concerning the influence of O<sub>2</sub> due the limited number of apples on which growth was observed (data not shown).

### 3.8. Correlation between patulin production in APAM and apple

In order to determine whether a correlation exists between patulin production in APAM and apple, the amount of patulin per volume of infected tissue (mg/cm<sup>3</sup>) was calculated for both APAM and apples (Figure 5.7). At 4 and 20°C no significant difference was observed between APAM and apple. However, at 1°C the amount of patulin per cm<sup>3</sup> was significantly higher for APAM than for apples and this was observed for the three tested atmospheres.

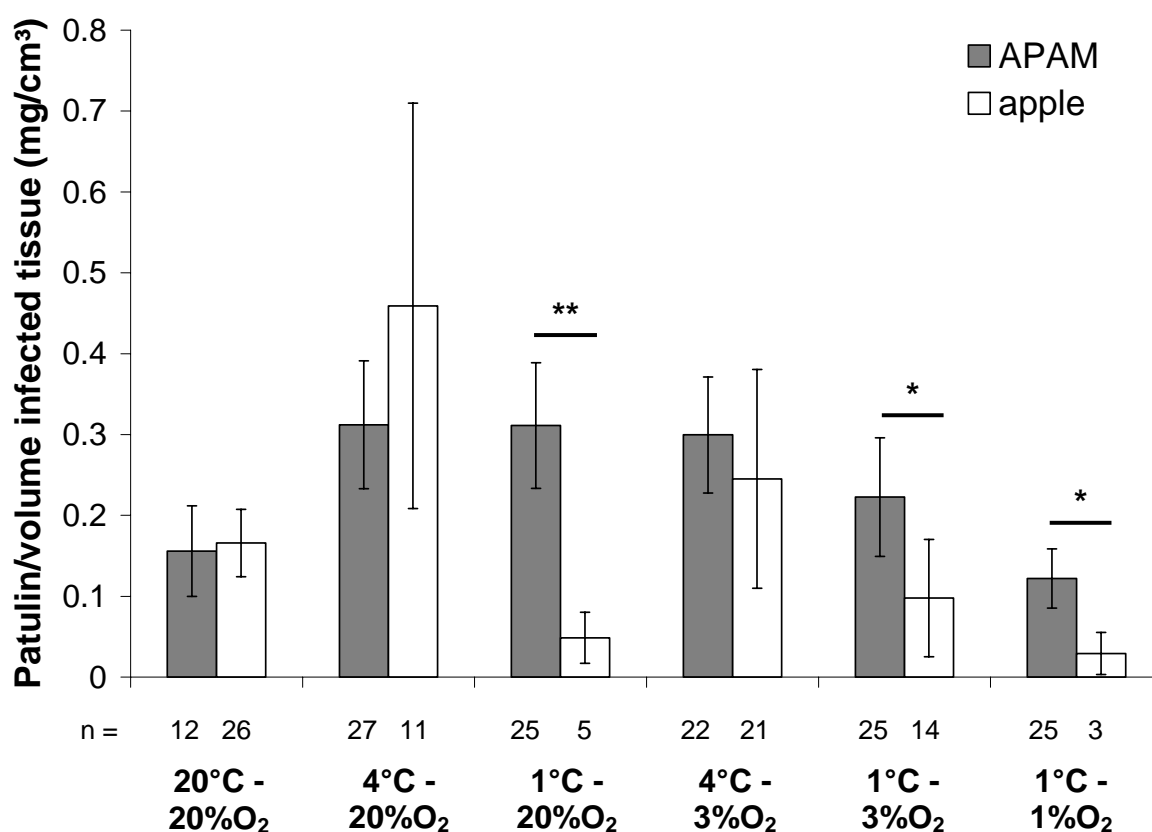


Figure 5.7: Comparison of patulin production by *P. expansum* MUM 00.01 in apples and APAM (error bars represent 95<sup>th</sup> confidence interval of the mean. \*, p<0.05; \*\*, p<0.001)

## 4. Discussion

Temperature had a profound effect on the growth characteristics of *P. expansum*. The effect of a small temperature deviation on fungal growth was less pronounced in the optimal temperature range than at low temperatures, indicating that a small deviation of storage temperature can have a large influence on the shelf life of pomiferous fruits. The deviating morphologies of the colonies at 30°C can be explained by the fact that 30°C is close to 35°C, the maximum temperature at which *P. expansum* is able to grow (Pitt & Hocking, 1997).

From the growth data observed under controlled atmospheres it can be concluded that the reduction of O<sub>2</sub> to 3% at higher temperature (20, 12 and 10°C) or to 1% at 4°C had no influence on the growth of *P. expansum*. This was in accordance to Orth (1976) who stated that low O<sub>2</sub> values in the atmosphere (2-0.5%) had very little influence on the growth of *P. expansum* in medium at 25°C. However, this was in contradiction to Sommer et al. (1981) who demonstrated that growth of *P. expansum* was reduced when the oxygen dropped below 2% at 23°C. Also Sitton and Patterson (1992) observed that lesion development caused by *P. expansum* was inhibited at O<sub>2</sub> concentrations below 2.3% at 0°C. However, reduced O<sub>2</sub> concentrations are not nearly as effective in preventing lesion development as elevated CO<sub>2</sub> levels (Orth, 1976; Sitton & Patterson, 1992). Unfortunately, high CO<sub>2</sub> regimes can produce serious side effects (off-flavours, scald, internal and external injury), which has limited their application (Sitton & Patterson, 1992).

Temperature had a strong influence on patulin production. The fact that lowering the temperature to 4°C causes higher patulin levels for some strains, gives cause for concern, since it indicates that refrigeration temperatures (4°C) do not prevent the production of patulin, but only delay (due to the slower growth) and enhance (higher patulin concentrations are detected) it. Only a further drop of the temperature to 1°C reduces the patulin production. However, this increase of patulin concentration with a temperature decrease from 20 to 4°C was not observed for all strains. Therefore probably it was also not in accordance to other studies. Strain MUM 99.19 (a slowly growing strain) produced less patulin when the temperature was lowered. McCallum et al. (2002) observed a decrease in patulin concentration when the temperature was decreased from 25 to 4°C. Paster et al. (1995) observed maximum patulin concentrations at 17°C and a decrease in patulin concentration

when the temperature increased to 25°C or lowered to 6°C and further to 3 and 0°C. Northolt et al. (1978) also observed an increase in patulin concentration when the temperature was lowered from 20 to 16°C, but a further decrease of the temperature to 4°C caused the opposite effect. Based on the presented results and other studies (Northolt et al., 1978; Paster et al., 1995; McCallum et al., 2002), it can be concluded that a reduction of the temperature to 1°C reduces patulin production in comparison to 4°C or a higher temperature. However, the statement formulated by Morales et al. (2006a) that patulin production is prevented during storage at 1°C could not be confirmed in the present study. For the higher temperatures (>1°C) lowering the temperature has no univocal effect on patulin production, since patulin production can be stimulated or suppressed depending on the strain. Therefore, it is recommended to perform cooled storage of apples at a temperature of 1°C in order to reduce the growth of the mould and to mitigate patulin formation in stored apples.

McCallum et al. (2002) divided the strains of *P. expansum* in aggressive (fast) and weak (slow) growers and stated that weak growers produced no detectable patulin, while aggressive growers produced higher levels of patulin. Also in this study one slowly growing strain produced in most cases no detectable levels of patulin. However, in contrast the other two slowly growing strains actually produced for some conditions more patulin than the fast growing strains, indicating that the link between slow growth and low patulin production is not general.

Despite the fact that the tested atmospheric conditions did not influence growth, patulin production was strongly influenced. At 20, 10 and 7°C a decrease in O<sub>2</sub> level corresponded to an increase in patulin production. These results were in contradiction to the study of Paster et al. (1995) where a decrease of O<sub>2</sub> from 20 to 2% at 25°C resulted in a decrease of the patulin production. However, this was tested at a CO<sub>2</sub> level of 3% while in the present study the CO<sub>2</sub> level was less than 1%. On the other hand, Orth (1976) reported that low O<sub>2</sub> values in the atmosphere (2-0.5%) hardly had an effect on the toxin production of *P. expansum* at 25°C. Only higher CO<sub>2</sub> and lower O<sub>2</sub> concentrations than those in air (e.g. 40% CO<sub>2</sub>, 6% O<sub>2</sub>) reduced patulin production. The presented study also showed that at 4 and 1°C the opposite effect was observed for *P. expansum* MUM 00.01 in comparison to the higher temperatures and a decrease in O<sub>2</sub> level corresponded to a decrease in patulin production. However this was not the case for every strain. Two other fast growing strains showed the same results at 1°C when the oxygen level was decreased while other strains showed deviating results at 3% O<sub>2</sub>.

Four strains (out of eight) produced more patulin at 3% O<sub>2</sub> compared to 20% O<sub>2</sub> at a temperature of 1°C. A further decrease of the O<sub>2</sub> level to 1% caused again a decrease in patulin production. From these results it can be concluded that a reduction of the O<sub>2</sub> level to 1% at 1°C is effective to reduce patulin production, but for higher temperatures and higher O<sub>2</sub> levels the effect on patulin production is strain dependent. However, a complete prevention of patulin production at 1°C and 2.5% O<sub>2</sub> or 1.5% O<sub>2</sub> as reported by Morales et al. (2006b) was not observed.

In general, it can be stated that an induction of limited stress to the fungus, such as lowering the temperature or lowering the oxygen levels, stimulates patulin production. However, the combination of different stress conditions (e.g. low temperature and low O<sub>2</sub>) will result in a reduced formation of the toxin. The combination of stress conditions, at which the transition from stimulation to suppression is observed, is strain dependent. Therefore, the effectiveness of CA storage is temperature related. The hypothesis that limited stress can stimulate patulin production was also put forward by McCallum et al. (2002). In the latter study, it was postulated that nutritional stress to the fungus, due to a shortage of carbohydrates or other nutrients, will increase patulin production.

Since *P. expansum* is mainly growing during the storage of apples, it is important to know the influence of different storage conditions on its ability to grow and produce patulin in apples. In contradiction to APAM, growth of *P. expansum* occurred only in a part of the inoculated apples and the number of apples in which growth occurred decreased when the temperature was lowered. This effect was not observed on APAM plates and indicates that although the apples are damaged and spores enter the wound, growth will occur only in a part of the apples, especially at a storage temperature of 1 or 4°C. Therefore, one has to be careful when synthetic or simulation growth media are used to study lag phases of moulds. The observed effect was further studied in Chapter 7.

Patulin production in apples was typically prone to a natural high variability. Also McCallum et al. (2002) observed high standard deviations (up to 116%) when patulin was analyzed in potato dextrose broth. In general, the amount of patulin increased when the volume of the rot became larger, after which a decrease in amount of patulin was observed. This decrease in the amount of patulin has been reported before and it has been postulated that it can be caused by intra- or extracellular enzymes, formed when *P. expansum* is stressed, that metabolize patulin

(Damoglou & Campbell, 1985). Due to the large confidence intervals it was difficult to draw conclusions concerning the influence of temperature and O<sub>2</sub> level on patulin production. In general, lower amounts of patulin were produced at 1°C than at 4°C, which was in accordance to the results on APAM. In order to study the influence of storage conditions on apples further, it is necessary to increase the number of repetitions for every data point and to extend the number of tested conditions to determine the trends in patulin production. However, such large-scale experiments are difficult to perform in practice. Therefore, it was tested whether the amounts of patulin produced in APAM are representative for the amounts in apple. At 20°C and 20% O<sub>2</sub> similar amounts of patulin per cm<sup>3</sup> were found for both apples and APAM, indicating that the results obtained on APAM can be extrapolated to apples. Also at 4°C and 20 % or 3% O<sub>2</sub>, similar results were obtained on APAM and apples. Only at 1°C a clear overestimation of patulin production per cm<sup>3</sup> was observed when APAM is used. Compared to the patulin production on APAM at 1°C, it seems that the apple causes a significant supplementary stress factor to the mould causing a reduction in patulin production. Such stress factors may include the intact tissue structure of the apple, which should be degraded in order to enable mould growth and which causes as well a reduced O<sub>2</sub> availability in the apple structure, compared to APAM. Based on these results together with the observations on APAM, it can be stated that the decrease in patulin production which was observed in APAM when the temperature drops to 1°C, will be even more pronounced in apples.

## 5. Conclusion

The simulation medium APAM can be used as a model system to evaluate growth and patulin production in apples, although an overestimation of patulin production at 1°C has to be taken into account. Storage conditions of apples have a profound effect on growth and patulin production of *P. expansum*. Growth is strongly influenced by the temperature, while the used atmosphere (20, 3 and 1% O<sub>2</sub>) has no effect. On the other hand, patulin production depends on the temperature and the O<sub>2</sub> level. However, whether the used condition stimulates or suppresses patulin production depends on the strain. In general it can be stated that intermediate stress levels stimulate patulin production while lowering the temperature to 1°C and the O<sub>2</sub> level to 1% O<sub>2</sub> reduced patulin production. This reduction at 1°C was even more pronounced in apples than in APAM indicating that apple as a food matrix is also an additional stress factor. Moreover, patulin production by *P. expansum* in apples is typically prone to a natural high variability. Based on the presented results it has to be stressed that the

temperature and O<sub>2</sub> level applied have to be as low as possible during the storage of apples in order to suppress patulin production and to guarantee food safety. The data describing the patulin production as a function of the growth will be used to develop a farm to fork risk assessment model as described in Chapter 8.

### Influence of inoculum size on the growth of *Penicillium expansum* in apples<sup>1</sup>

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<sup>1</sup> Based on: Baert, K., Devlieghere, F., Bo, L., Debevere, J., De Meulenaer, B. The effect of inoculum size on the growth of *Penicillium expansum* in apples. Submitted for publication in Food Microbiology.





## Chapter 6

# Influence of inoculum size on the growth of *Penicillium expansum* in apples

### Summary

The objective of this study was to evaluate the effect of the inoculum size on the germination and growth parameters of *Penicillium expansum* under different storage conditions in apples. Growth of *P. expansum* was observed in more than 90% of the inoculations, when the inoculum was equal or higher than  $2 \cdot 10^4$  spores. The use of a low inoculum level resulted in longer lag phases and a larger variability of the estimated lag phase, indicating that more replications are necessary to have a representative idea on the growth of the mould in the specified circumstances. At lower temperature more inoculum was necessary to reduce the variability of the estimated lag phase, showing that this effect is temperature dependent. Moreover, the effect of the inoculum level on the lag phase is even more pronounced for a slower growing strain. These results imply that the inoculum size influences the estimated growth parameters and should be considered in quantitative risk assessments and for the design of challenge tests and experiments to gather data for predictive growth models.

## 1. Introduction

Fungal growth involves two steps: spore germination and hyphal elongation to form a filamentous colony (Sautour et al., 2001). In the germination process, the dormant spore shifts from low to high metabolic activity. This process starts automatically if the spore is placed in suitable environmental conditions or after an activation process such as heat shock or chemical treatment (Paul et al., 1993). The germination process is characterized by three steps, namely activation, swelling and the formation of a germ tube (Dantigny et al., 2003). A spore is considered to be germinated when the length of the longest germ tube was equal to or greater than the greatest dimension of the swollen spore (Dantigny et al., 2006). Previous research has shown that the lag time for growth coincides with the completion of the germination (Dantigny et al., 2002). However, until now research has mainly focussed on preventing the growth of the mould while little is known on the germination of the mould. Moreover, studies on mould growth use mainly high inoculum levels, while in reality infection of a food product (e.g. apples) occurs with a low number of spores. A second limitation of growth experiments is the use of synthetic or simulation media that have different characteristics than the real food matrix.

Previous research has shown that under certain conditions (e.g. low temperature) no growth of *P. expansum* occurs when less than  $10^4$  spores are injected in an apple (Chapter 5). Therefore, the objective of this study was to evaluate the effect of the inoculum size on the germination and growth parameters of *P. expansum* under different storage conditions in apples.

## 2. Materials and methods

### 2.1. Fungal strains

Strains MUM 00.01 and MUM 99.19 of *P. expansum* were obtained from the Centro de Engenharia Biológica da Universidade do Minho in Braga (Portugal).

### 2.2. Inoculum preparation

The inoculum was prepared as described in Chapter 5 – section 2.2. The concentration of the spore solution was adjusted using phosphate buffer (Sigma, Germany) until a final concentration of about  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$  or  $10^8$  spores/ml.

### 2.3. Inoculation and incubation of apples

Apples (cultivar Boskoop) were inoculated as described in Chapter 5 – section 2.7 and incubated (4, 12 and 25°C). For each condition 7 apples were inoculated at one side, when the apples were incubated at 25°C, or at two opposite sides if the apples were incubated at 12 or 4°C. To maintain the air condition in the container while the apples respire, the containers were opened in a sterile environment on a regular basis (every day for apples stored at 25°C, every 2 days for apples stored at 12°C and every 5 days for apples stored at 4°C).

### 2.4. Growth measurements of *P. expansum* on apples

After inoculation, the apples were examined on a regular basis to see if any visible growth had started. As soon as visible growth started, the colony was measured with a digital calliper in two perpendicular directions at regular intervals to follow growth. Also the diameter of every apple was measured, in order to be able to calculate the diameter of the curved surface of the rotten spot (S) instead of using the measured diameter of the flat surface (2r). The diameter of the curved surface of a segment of a sphere (S) was calculated using the following equation (Figure 5.1):

$$S = R * 2 * \text{Arc sin}\left(\frac{r}{R}\right) \quad (6.1)$$

with R = radius of the apple (sum of the 2 perpendicular diameters of the apple divided by 4) (cm)

r = radius of the moulded spot (sum of the 2 measured perpendicular diameters of the rotten spot divided by 4) (cm)

Statistical analysis was performed with SPSS 11.0 for Windows (SPSS Inc., USA). The Kolmogorov-Smirnov test was used to test normality. Means were compared using analysis of variance (one-way 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. Radial growth rates ( $\text{mm h}^{-1}$ ) and the lag phase (h) at each temperature were determined by linear regression (Microsoft excel, Microsoft, USA) as described by Pardo et al. (2004).

### 3. Results

The effect of inoculum size on the germination of *P. expansum* MUM 00.01 was temperature dependent. Table 6.1 shows the percentage of inoculation spots where growth was observed after incubation. When  $\pm 2.10^1$  spores were injected in the apple no growth was observed. Inoculation with  $\pm 2.10^2$  spores resulted in growth of *P. expansum* at all temperatures except 4°C. For an inoculum level  $\geq 2.10^4$  spores, growth was observed in > 90% of the inoculation spots for every temperature studied.

**Table 6.1: Percentage of apples with growth of *P. expansum* MUM 00.01**

Temperature (°C)	Inoculum level (spores)					
	$2.10^6$	$2.10^5$	$2.10^4$	$2.10^3$	$2.10^2$	$2.10^1$
25	100	100	100	100	71	0
12	100	100	100	79	29	0
4	93	100	93	43	0	0

The growth rate and the duration of the lag phase of *P. expansum* MUM 00.01 was calculated from the growth curves (mould diameter as a function of time) using linear regression (Table 6.2). The linear model fitted the data properly ( $R^2 > 0.97$ ; MSE: 0.03-0.1). At lower inoculum levels ( $2.10^3$  and/or  $2.10^2$  spores) longer lag phases were observed compared to the higher inoculum levels, which were in some cases statistically significant. The standard deviation was also higher for lower inoculum levels, indicating that the variability between replicates is higher when less spores are inoculated. For the growth rate no clear effect of the inoculum size was observed (Table 6.3). The growth rates at lower inoculum levels were not different from higher inoculum levels except at 12°C ( $2.10^5$  and  $2.10^3$  spores;  $p=0.032$ ). A reduction of the inoculum size did not result systematically in an increase of the variability.

In order to confirm these results for *P. expansum* MUM 00.01, the growth parameters of another *P. expansum* strain (MUM 99.19) were determined at different inoculum levels and 25°C (Table 6.4). *P. expansum* MUM 99.19 was a slower growing strain in comparison to strain MUM 00.01. Longer lag phases and smaller growth rates were obtained at 25°C (Table 6.4) and at lower temperatures (Chapter 5) compared to strain MUM 00.01. The same effects of the inoculum level on the growth parameters and the variability were observed (Table 6.4).

**Table 6.2: Lag phase (h) of *P. expansum* MUM 00.01 on apples for different spore concentrations**

Temperature (°C)	Inoculum level (spores)					
	$2.10^6$	$2.10^5$	$2.10^4$	$2.10^3$	$2.10^2$	$2.10^1$
25	81 ± 22 <sup>a</sup> ab <sup>b</sup> n = 7	54 ± 6 a n = 7	82 ± 29 ab n = 7	115 ± 25 b n = 7	147 ± 59 <sup>c</sup> ab n = 5	/ <sup>d</sup>
12	192 ± 49 a n = 14	198 ± 61 a n = 14	220 ± 59 ab n = 14	301 ± 101 b n = 11	/	/
4	520 ± 102 a n = 13	522 ± 128 a n = 14	607 ± 213 a n = 13	/	/	/

<sup>a</sup> Values are mean ± standard deviation; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different ( $p > 0.05$ ); <sup>c</sup> Only apples with growth were used to calculate the average lag phase; <sup>d</sup> Growth in less than 50% of the apples.

Moreover, the results showed that the effect of the inoculum level on the lag phase is even more pronounced for this slower growing strain. Strain MUM 99.19 showed a significantly longer lag phase when  $2.10^4$  spores were used in comparison to  $2.10^6$  and  $2.10^5$  ( $p = 0.007$  and  $p = 0.01$ , respectively), while for strain MUM 00.01 the lag phase at  $2.10^5$  spores was significantly different from that with  $2.10^3$  spores ( $p = 0.005$ ).

## 4. Discussion

Growth of *P. expansum* on apples is influenced to a large extent by intrinsic and extrinsic factors (Drusch & Ragab, 2003), of which water activity and temperature are the most important ones (Northolt et al., 1978). In this study, only the effect of temperature was studied since apples were used as a growth medium and the intrinsic factors were consequently not variable. Besides the growth rate, the probability that a spore will germinate is also influenced by these intrinsic and extrinsic factors (Sautour et al., 2001). The number of spores that are present together with the probability to have germination will determine whether growth of *P. expansum* will occur. The present study shows that the growth of *P. expansum* in apples occurs in more than 90% of the inoculations when the inoculum is equal or higher than  $2.10^4$  spores. This is in contradiction with a previous growth experiment of *P. expansum* MUM 00.01. In the latter study growth occurred on a simulation medium in 100% of the cases

**Table 6.3: Growth rate (mm/h) of *P. expansum* MUM 00.01 on apples for different spore concentrations**

Temperature (°C)	Inoculum level (spores)					
	2.10 <sup>6</sup>	2.10 <sup>5</sup>	2.10 <sup>4</sup>	2.10 <sup>3</sup>	2.10 <sup>2</sup>	2.10 <sup>1</sup>
25	0.263 ± 0.021 <sup>a</sup> <sub>n = 7</sub>	0.236 ± 0.023 <sup>a</sup> <sub>n = 7</sub>	0.250 ± 0.029 <sup>a</sup> <sub>n = 7</sub>	0.226 ± 0.023 <sup>a</sup> <sub>n = 7</sub>	0.177 ± 0.050 <sup>c</sup> <sub>n = 5</sub>	/ <sup>d</sup>
12	0.163 ± 0.028 <sup>ab</sup> <sub>n = 14</sub>	0.186 ± 0.029 <sup>a</sup> <sub>n = 14</sub>	0.177 ± 0.017 <sup>ab</sup> <sub>n = 14</sub>	0.160 ± 0.011 <sup>b</sup> <sub>n = 11</sub>	/	/
4	0.061 ± 0.010 <sup>a</sup> <sub>n = 13</sub>	0.063 ± 0.008 <sup>a</sup> <sub>n = 14</sub>	0.056 ± 0.011 <sup>a</sup> <sub>n = 13</sub>	/	/	/

<sup>a</sup> Values are mean ± standard deviation; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different ( $p > 0.05$ ); <sup>c</sup> Only apples with growth were used to calculate the average growth rate; <sup>d</sup> Growth in less than 50% of the apples.

**Table 6.4: Growth rate (mm/h) and lag phase (h) of *P. expansum* MUM 99.19 on apples for different spore concentrations at 25°C**

	Inoculum level (spores)			
	2.10 <sup>6</sup>	2.10 <sup>5</sup>	2.10 <sup>4</sup>	2.10 <sup>3</sup>
Lag phase (h)	106 ± 14 <sup>a</sup> a <sup>b</sup> n = 7	109 ± 17 a n = 7	149 ± 30 b n = 7	173 ± 58 ab n = 7
Growth rate (mm/h)	0.177 ± 0.016 a n = 7	0.179 ± 0.013 a n = 7	0.160 ± 0.028 a n = 7	0.184 ± 0.023 a n = 7

<sup>a</sup> Values are mean ± standard deviation; <sup>b</sup>In each row, the same letter indicates that the data are not significantly different ( $p > 0.05$ )

(Chapter 5) when 2.10<sup>3</sup> spores were inoculated. Sautour et al. (2003) also reported growth at low inoculation levels. Growth of *P. chrysogenum* was observed on potato dextrose agar when 1 or 10 spores were inoculated, while in the present study no growth was observed when 2.10<sup>1</sup> spores were used for the inoculation. This indicates that the real food matrix reduces the probability of a spore to germinate in comparison to a synthetic or a simulation medium.

Using a low inoculum level will also result in a larger variability of the estimated lag phase, indicating that more replications are necessary to have a representative idea of the growth kinetics of the mould under the specified circumstances. This effect has also been observed for the lag time of bacteria (Robinson et al., 2001; Francois et al., 2006). Moreover, the present study demonstrated that this effect is more pronounced at low temperatures. Samapundo et al. (2007) studied the lag phase of single spores of *Aspergillus flavus* and *Fusarium verticillioides* under different growth conditions on yellow dent corn meal. In general, the spread of the lag phases of the single spores became wider when the conditions for growth became more limiting. These results indicate that more spores should be inoculated to reduce the variability between replicates when the growth conditions become limiting. This finding is in accordance with the presented results. The use of a small inoculum in the present study resulted in a longer lag phase in comparison to those observed when high inoculum sizes were used. This was also observed by Dantigny et al. (2002) for *Mucor racemosus* and by Sautour et al. (2003) for *P. chrysogenum*. However in the latter study, it was observed that *P. chrysogenum* grew significantly faster when 1 spore was inoculated

instead of 10 or more. This is in contrast with the present study where no significant difference was observed for the growth rate.

## 5. Conclusion

Based on these results it can be concluded that using large inoculum levels results in estimations of the lag phase that are smaller in comparison to low inoculum levels. Therefore, using high inoculum levels to estimate the growth parameters will result in estimating a worst case scenario, with the fastest growth and consequently the shortest shelf life. This implies that the inoculum size influences the estimated lag phase and should be considered for the design of challenge tests and experiments to gather data for predictive growth models (Chapter 7). Since predictive growth models are used in quantitative microbiological risk assessment, these findings are also important for the elaboration of quantitative microbiological risk assessment (Chapter 8).



### Modelling the effect of temperature on the growth rate and lag phase of *Penicillium expansum* in apples<sup>1</sup>

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<sup>1</sup> Based on: Baert, K., Valero, A., De Meulenaer, B., Samapundo, S., Ahmed, M., Bo, L., Debevere, J., Devlieghere, F. Modelling the effect of temperature on the growth rate and lag phase of *Penicillium expansum* in apples. Submitted for publication in International Journal of Food Microbiology.



## Chapter 7

# Modelling the effect of temperature on the growth rate and lag phase of *Penicillium expansum* in apples

### Summary

The objective of the present study was to develop validated models that describe the effect of storage temperature on the growth rate and lag phase of six *Penicillium expansum* strains. The growth of the selected strains was therefore studied on apple puree agar medium (APAM) at 30, 25, 16, 10, 4 and 2°C. Model validation was performed in two steps. Firstly, the developed models were validated on APAM for the six strains at three intermediate temperatures (20, 12 and 7°C) and secondly on apples at 25, 20, 12, 7, 4 and 2°C for three strains. Growth rates and lag phases were estimated using linear regression. Several secondary models were evaluated on their ability to describe the change in growth rate and lag phase as a function of temperature. For the growth rate, a modification of the extended Ratkowsky model was selected, as this model was flexible enough to adequately describe the evolution of the growth rate for the complete temperature range (2-30°C). Regarding the lag phase, the Arrhenius-Davey model provided the best adjustment to the observed data. The obtained Bias factors ( $B_f$ ) ranged from 0.91 to 1.14 and the accuracy factors ( $A_f$ ) were  $<1.2$  for the validation performed on APAM, indicating that the models were good predictors of the true mean colony growth rate and lag phase. Afterwards, an external validation was carried out in apples. For the growth rate,  $B_f$  ranged from 0.64 to 0.81 and  $A_f < 1.39$ , indicating conservative predictions. On the contrary for the lag phase, a clear deviation was observed between predictions and observed values on apples ( $0.35 < B_f < 0.7$  and  $A_f > 1.6$ ). This indicates that the food matrix has a large influence on the adaptation of the spores to the matrix, but once the spores have germinated, growth occurs at a similar rate on both APAM and apples. These results highlight that the use of simulation or synthetic media for the development of predictive models for the lag phase of moulds can lead to inadequate predictions and that a validation on the real food matrix is necessary. Application of the developed models is possible in the framework of quantitative risk assessment to develop control strategies against blue mould rot in apple and enables the inclusion of strain variability. However, possible underestimation of the lag phase should be taken into account.

## 1. Introduction

In order to improve the quality and safety of food, a need exists for the development of techniques to predict fungal growth (Dantigny et al., 2002). Moreover, predictive models are necessary to develop quantitative risk assessments (QRA) that follow a farm to fork approach and include mould growth. Despite the fact that for many years the modelling of fungal growth lagged well behind that of bacteria (Gibson & Hocking, 1997), predictive mycology has received more attention the last decade. However, very few studies in scientific literature have validated the models developed to describe fungal growth in real food products (Decker & Nielsen, 2005; Samapundo et al., 2005). For *P. expansum* in particular, two models have been developed (Lahlali et al., 2005; Marin et al., 2006b), but these models were not validated. Validation is essential to know the generalization properties of growth models, or in other words, the performance of the model when confronted with new data and to assess their predictive capacity (Geeraerd et al., 2004). Therefore, the aim of the present study was to develop a predictive model for the growth rate ( $\mu_{\max}$ ) and the lag phase ( $\lambda$ ) of *P. expansum* that was validated on simulation medium and apples. Water activity was not included as variable in the model since *P. expansum* grows on apples and pears, two fruits that have a high water activity (0.98-0.99) (Northolt et al., 1978; Burne, 1987) Atmosphere composition was also not included in the model since previous research has shown that atmosphere composition (20%, 3%, and 1% O<sub>2</sub>; combined with <1% CO<sub>2</sub>) had no effect on growth of *P. expansum* (Chapter 5). In order to include variability of different strains concerning their growth kinetics, six strains were studied. Separate models were developed for each strain and these models can be used in QRA to include strain variability in the model (Chapter 8).

## 2. Materials and methods

### 2.1. Fungal strains

Strains MUM 00.01, MUM 99.23, MUM 99.19, MUM 99.20, MUM 99.22 and MUM 99.24 of *P. expansum* were obtained from the Centro de Engenharia Biológica da Universidade do Minho in Braga (Portugal).

## **2.2. Inoculum preparation**

The inoculum was prepared as described in Chapter 5 – section 2.2. The concentration of the spore solution was adjusted using phosphate buffer, pH 7.4 (Sigma, Germany) until a final concentration of about  $10^5$  or  $10^8$  spores/ml was obtained for experiments with apple puree agar medium (APAM) and apples, respectively.

## **2.3. APAM preparation, inoculation and incubation**

The APAM was prepared and inoculated as described in Chapter 5 – section 2.3. To incubate the APAM plates at different temperatures, the plates were placed in recipients with a perforated, false bottom. Under this false bottom, a 1.5% NaCl solution (VWR, Belgium) was used to keep the water activity of the APAM plates at a constant level ( $a_w = 0.991$ ). The recipients were frequently opened during incubation to avoid the creation of anaerobic conditions. Growth of the six strains of *P. expansum* was studied at 2, 4, 7, 10, 12, 16, 20, 25 and 30°C. For every temperature-strain combination, growth of 15 colonies was followed.

## **2.4. Growth measurements on APAM**

The growth of *P. expansum* on APAM was followed as stated in Chapter 5 – section 2.6.

## **2.5. Inoculation and incubation of apples at different temperatures in air**

Apples (cultivar Boskoop) were inoculated as described in Chapter 5 – section 2.7. To incubate the apples at different temperatures in air, plastic containers with the apples were placed in incubators (2, 4, 7, 12, 20 and 25°C). To evaluate growth at 2, 4, 7 and 12°C, 7 apples were inoculated at 2 opposite sites, so that in total 14 inoculation spots were obtained. To evaluate growth at 20 and 25°C, 7 apples were inoculated at one site on the apple (7 inoculation spots). More inoculation spots were used at the lower temperatures, since germination was not always occurring at lower temperatures. To prevent anaerobic conditions being generated due to the respiration of the apples and fungi, the containers were opened in a sterile environment on a regular basis (every day for apples stored at 20 and 25°C, every 2 days for apples stored at 7 and 12°C and every 5 days for apples stored at 2 and 4°C).

## 2.6. Growth measurements of *P. expansum* on apples

Growth of *P. expansum* on apples was measured as stated in Chapter 6 – section 2.4. Also the diameter of the curved moulded surface was calculated as described in Chapter 6 – section 2.4.

## 2.7. Mathematical and statistical methods

### 2.7.1. Primary modelling

The average diameters (mm) at each time of measurement (h) were plotted and growth rates ( $\mu_{\max}$ , mm/h) were obtained from the slopes by linear regression (Dantigny et al., 2005) using Microsoft Excel (Microsoft Corporation, USA). This method assumes that once the lag phase has passed, growth starts immediately at its maximum rate ( $\mu_{\max}$ , mm/h). The lag phase ( $\lambda$ , h) was cut off by the linear growth zone from the initial inoculum diameter. The linear regression method was preferred over the sigmoidal curve fittings since no stationary phase was observed for all growth curves and high correlations were obtained with this simple method in other studies (Lahlali et al., 2005; Pardo et al., 2005). The uncertainty of the regression was assessed by calculating the adjusted correlation coefficient  $R^2_{\text{adj}}$ .

$$R^2_{\text{adj}} = 1 - \frac{n-1}{n-p}(1-R^2) \quad (7.1)$$

with  $n$  = number of data points

$p$  = degrees of freedom = 2 for linear regression

In addition, 95 % confidence intervals were calculated for each growth curve.

### 2.7.2. Secondary modelling

The growth parameters determined at 2, 4, 10, 16, 25 and 30°C were used for the development of secondary models describing the influence of temperature on the growth rate and lag phase. In a first step, several potentially suitable functions were fitted to the estimated growth rates and lag phases. These equations corresponded to (i) second order polynomial models (with different transformations of the dependent variable, including ln, square-root and inverse values), (ii) extended square-root models for the full biokinetic temperature range (with a transformation, as shown in equation 7.3) (only for the growth rate) and (iii) the Arrhenius-Davey model. The most suitable models were identified on the basis of their mean square error (MSE), and were further evaluated using the determination coefficient  $R^2$  and a

graphical comparison of the observed growth parameters of the primary model and the fitted secondary model. The MSE was defined as:

$$MSE = \frac{\sum (m_{pred} - m_{obs})^2}{n} \quad (7.2)$$

where  $m_{obs}$  = observed value of the dependent variable,  $m_{pred}$  = predicted value of the dependent variable and  $n$  = number of data points.

For the growth rate the extended square root model described by Ratkowsky et al. (1983) did not fit the data properly, but the transformation shown in equation (7.3) provided a better fit.

$$\sqrt{\mu_{max}} = a + b \cdot (T - T_{min}) + [1 - e^{(c \cdot (T - T_{max}))}] \quad (7.3)$$

where  $\mu_{max}$  is the growth rate (mm/h),  $T$  is temperature ( $^{\circ}\text{C}$ ),  $T_{min}$  and  $T_{max}$  are fixed cardinal temperatures representing the cut-off points below and above which, respectively, no growth will be observed, and  $a$ ,  $b$  and  $c$  are coefficients. The optimal temperature for growth was derived from equation (7.3) by calculating the derivative.

For the lag phase the Arrhenius-Davey model (Davey, 1989) was selected (7.4). This equation is an extension of the original Arrhenius model introduced and used by Davey (1989) to model the effect of temperature on bacterial growth.

$$\ln(\lambda) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} \quad (7.4)$$

where  $\lambda$  is the lag phase (h),  $T$  is temperature ( $^{\circ}\text{K}$ ) and  $C_0$ ,  $C_1$  and  $C_2$  are the coefficients which replace the original activation energy of the Arrhenius equation. Finally, the models selected for both lag phase and growth rate were then further evaluated and validated against independently collected data on APAM and apples.

The coefficients of the secondary models were estimated by fitting the models to the predicted growth rates or lag phases using non-linear regression or stepwise linear regression in SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA).

To evaluate the performance of the models in describing the observed growth rate and lag phase;  $R^2$ , root mean square error (RMSE) and standard error of prediction (SEP) were calculated. The determination coefficient ( $R^2$ ) was used as an overall measure of the attained prediction (te Giffel & Zwietering, 1999). The statistical index, RMSE, is a measure of the

residual variability between predicted and observed values of the dependent variable. A lower RMSE value indicates a better suitability of the model to describe the data (Sutherland & Bayliss, 1994). However, if the magnitude of data is larger, RMSE increases proportionally, since residual variability will be higher. In order to facilitate the comparison among different models, the SEP was calculated. SEP is a relative error which makes it irrespective of the magnitude of the variable (Hervás et al., 2001).

$$RMSE = \frac{\sqrt{\sum_{i=1}^n (m_{pred} - m_{obs})^2}}{n} \quad (7.5)$$

$$SEP = \frac{100}{\overline{m_{obs}}} \sqrt{\frac{\sum_{i=1}^n (m_{pred} - m_{obs})^2}{n}} \quad (7.6)$$

where  $m_{obs}$  = observed value of the dependent variable (growth rate and lag phase),  $m_{pred}$  = predicted value of the dependent variable,  $\overline{m_{obs}}$  = mean of the observed value of the dependent variable and  $n$  = number of data points.

## 2.8. Validation

### 2.8.1. Mathematical/statistical validation

According to Ross (1996), the most suitable method for assessing the reliability of predictive models is to use data obtained under well-controlled experimental conditions, such as application of additional conditions in the same way as for the elaboration of the model. Later on, the model must be validated in a food product in order to be applied in food industries. To evaluate the predictive capacity of the developed models, a mathematical validation was carried out by selecting intermediate temperatures inside the experimental range and not included in the development of the model (20, 12 and 7°C). Afterwards, an external validation was performed with observed values on apples for strains MUM 00.01, MUM 99.19 and MUM 99.22 at 25, 20, 12, 7, 4 and 2°C.

To evaluate the performance of the predictive models when confronted with new data;  $R^2$ , RMSE, SEP and accuracy and bias factors were calculated. The bias factor ( $B_f$ ) answers the question whether, on average, the observed values lie above or below the line of equivalence and quantifies the difference. It gives the structural deviations of a model (te Giffel &



Zwietering, 1999). A  $B_f < 1$  indicates that the mould grows slower than predicted by the model. For the growth rate (equation 7.7) and lag phase (equation. 7.8),  $B_f$  values were calculated as:

$$B_f = 10^{\left(\sum \log(\mu_{obs} / \mu_{pred}) / n\right)} \quad (7.7) \qquad B_f = 10^{\left(\sum \log(\lambda_{pred} / \lambda_{obs}) / n\right)} \quad (7.8)$$

where  $\mu_{obs}$  and  $\lambda_{obs}$  are the observed growth rate and lag phase respectively,  $\mu_{pred}$  and  $\lambda_{pred}$  are the predicted growth rate and lag phase respectively, and  $n$  was defined above. Since the  $B_f$  provides no indication of the average accuracy of the estimates (due to the fact that overestimation and underestimation tend to cancel out), the accuracy factor ( $A_f$ ) was also calculated. 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).

$$A_f = 10^{\left(\sum \log(m_{pred} / m_{obs}) / n\right)} \quad (7.9)$$

where  $m_{obs}$ ,  $m_{pred}$  and  $n$  were defined above.

### 2.8.2. Graphical validation

Besides the mathematical/statistical validation, a graphical validation was also performed in order to show the interpolating ability of the models by visually depicting the possible under or overfitting (Geeraerd et al., 2004). Plots of predicted values against observed lag phases and growth rates were used to assess visually the overall reliability of the developed models.

## 3. Results

### 3.1. Effect of temperature on growth rate of *P. expansum*

Fungal growth was characterized by a lag phase followed by linear growth as shown in Figure 7.1. The linear model had a good fit to the growth curves for each strain, as high adjusted correlation coefficients were obtained ( $R^2_{adj} > 0.97$ ). The effect of temperature on the  $\mu_{max}$  of *P. expansum* is shown in Figure 7.2. For all strains, a linear increase was observed from 2 to 25°C, but a decrease was obtained as temperature was further increased from 25°C to 30°C. This decrease was not well described by second order polynomial models ( $MSE = 2.09 \times 10^{-3}$ ) and the Arrhenius-Davey model ( $MSE = 1.27 \times 10^{-3}$ ), in accordance to the MSE values. However, when the temperature range 2-25°C was considered, the natural logarithm

transformation of the polynomial model showed a good fit to the data ( $MSE = 8.84 \times 10^{-5}$ ) in comparison to other transformations of the dependent variable. However, less information was included in the model. The Arrhenius-Davey model clearly showed overfitting at low temperatures, resulting in largely deviating estimations. Therefore, a modification of the extended square root model (Ratkowsky et al., 1983) was selected as it was flexible enough to describe the relationship between temperature and growth rate in the complete temperature range (2-30°C). This provided a good fit between observed and predicted data ( $MSE = 5.38 \times 10^{-4}$ ) as can be seen in Figure 7.2. The coefficients of the developed models, together with the  $R^2$ , RMSE and SEP are shown in Table 7.1. Visual representation of these models is shown in Figure 7.2. Regarding predicted and observed growth rates, differences were noticed between the strains. Strain MUM 00.01 was the fastest growing strain at 25°C (predicted  $\mu_{max} = 0.340$  mm/h) while MUM 99.23 was the strain which showed the slowest growth at 25°C (predicted  $\mu_{max} = 0.212$  mm/h). These results show that the fastest growing strain grew 1.6 and 1.1 times faster than the slowest growing strain at 25 and 2°C, respectively. The optimal temperature for growth varied for the tested strains between 24 and 27°C.

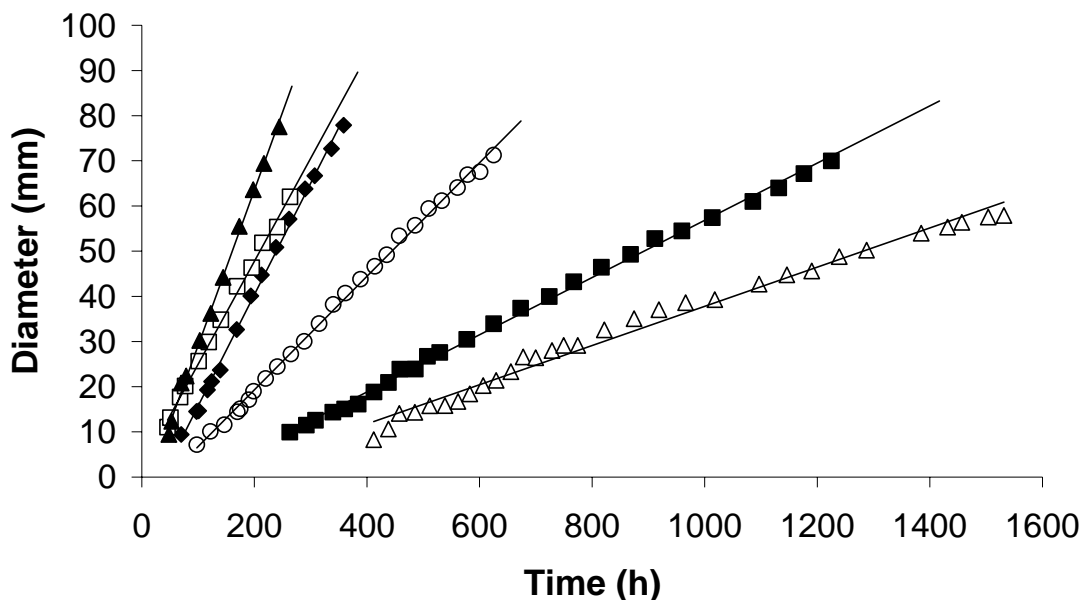


Figure 7.1: Primary modelling (*P. expansum* MUM 00.01) of the diameter (mm) as a function of storage time (h) ( $\square$ : 30°C;  $\blacktriangle$ : 25°C;  $\blacklozenge$ : 16°C;  $\circ$ : 10°C;  $\blacksquare$ : 4°C and  $\triangle$ : 2°C)

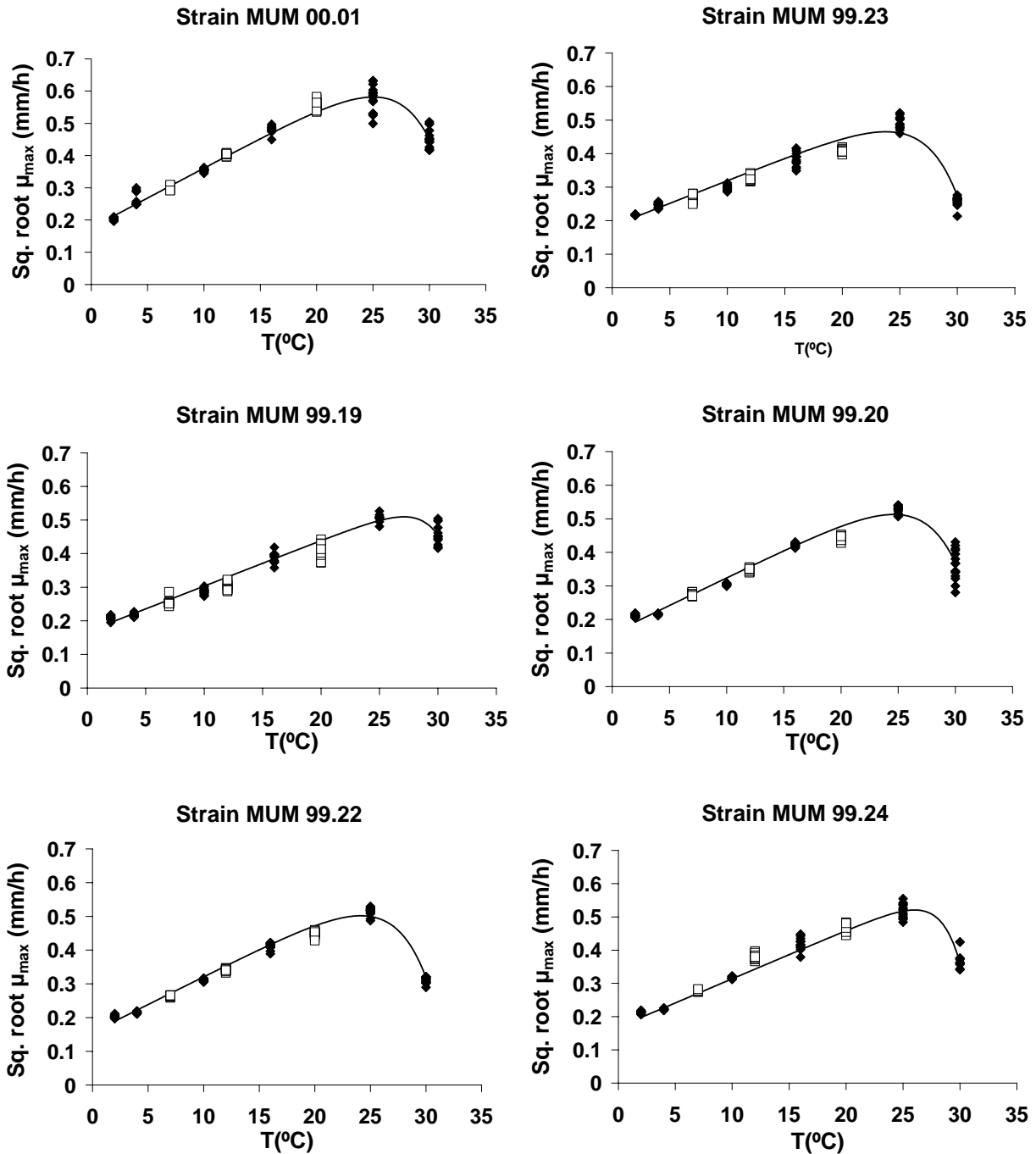


Figure 7.2: Plots of growth rate ( $\mu_{\max}$ , mm/h) versus temperature (°C) (◆: model development data; □: validation data; — modified extended Ratkowsky model)

**Table 7.1: Coefficients, determination coefficient ( $R^2$ ), root mean square error (RMSE) and standard error of prediction (SEP) of the modified extended Ratkowsky model,  $\sqrt{\mu_{\max}} = a + b \cdot (T - T_{\min}) + [1 - e^{(c \cdot (T - T_{\max}))}]$ , describing the effect of temperature on the growth rate of *P. expansum***

Strains	Coefficients <sup>a</sup>					Statistical indices		
	a	b	c	$T_{\min}$	$T_{\max}$	$R^2$	RMSE	SEP (%)
MUM 00.01	$-862.0 \times 10^{-3} \pm 6.4 \times 10^{-3b}$	$186.7 \times 10^{-4} \pm 5.6 \times 10^{-4}$	$31.5 \times 10^{-2} \pm 1.4 \times 10^{-2}$	-2	34	0.967	$2.32 \times 10^{-2}$	13.7
MUM 99.23	$-843.3 \times 10^{-3} \pm 5.8 \times 10^{-3}$	$135.6 \times 10^{-4} \pm 5.0 \times 10^{-4}$	$32.7 \times 10^{-2} \pm 1.3 \times 10^{-2}$	-2	33.5	0.948	$1.74 \times 10^{-2}$	15.9
MUM 99.19	$-859.4 \times 10^{-3} \pm 4.3 \times 10^{-3}$	$135.7 \times 10^{-4} \pm 3.2 \times 10^{-4}$	$53.0 \times 10^{-2} \pm 1.8 \times 10^{-2}$	-2	34	0.974	$1.41 \times 10^{-2}$	11.4
MUM 99.20	$-874.0 \times 10^{-3} \pm 6.3 \times 10^{-3}$	$165.2 \times 10^{-4} \pm 5.4 \times 10^{-4}$	$31.3 \times 10^{-2} \pm 1.3 \times 10^{-2}$	-2	34	0.959	$1.66 \times 10^{-2}$	12.7
MUM 99.22	$-876.8 \times 10^{-3} \pm 3.5 \times 10^{-3}$	$165.0 \times 10^{-4} \pm 3.1 \times 10^{-4}$	$31.4 \times 10^{-2} \pm 0.72 \times 10^{-2}$	-2	33.5	0.986	$1.00 \times 10^{-2}$	8.2
MUM 99.24	$-831.7 \times 10^{-3} \pm 3.6 \times 10^{-3}$	$145.4 \times 10^{-4} \pm 2.8 \times 10^{-4}$	$56.4 \times 10^{-2} \pm 1.3 \times 10^{-2}$	-0.5	32.5	0.975	$1.74 \times 10^{-2}$	13.4

<sup>a</sup>All coefficients were significant ( $p < 0.05$ ); <sup>b</sup>Estimation  $\pm$  standard deviation

### 3.2. Effect of temperature on lag phase of *P. expansum*

Regarding the effect of temperature on the lag phase of *P. expansum*, the same trend was observed as for the growth rate, namely a decrease in the adaptation period as temperature was increased from 2°C to 25°C. However, no relevant differences in the lag phase were observed from 25°C to 30°C (Figure 7.3). In contradiction to the growth rate, strain MUM 00.01 showed the longest adaptation period ranging from 41 h at 25°C to 337 h at 2°C, while MUM 99.19 had the shortest lag phase of 27 h and 204 h respectively. This indicates that the lag phase of strain MUM 00.01 was about 1.6 times longer at 25°C and 2°C than of strain MUM 99.19. The Arrhenius-Davey model was selected as it showed the best correspondence to the observed data over the temperature range investigated (MSE = 136.74). As mentioned above, polynomial models were not able to give suitable predictions at high temperatures (25-30°C) (MSE = 264.39). The coefficients obtained and  $R^2$ , RMSE and SEP values for each strain are shown in Table 7.2. Visual representation of these models relating to the lag phase is shown in Figure 7.3.

### 3.3. Model validation

The results of the mathematical/statistical validation used to determine the ability of the models to describe the influence of temperature on  $\mu_{\max}$  and  $\lambda$  is shown in Tables 7.3 and 7.4, respectively. For the growth rate the SEP was <27% and for the lag phase <26%. The bias factor was close to 1 ( $0.90 < B_f < 1.15$ ) for all models and the  $A_f$  was smaller than 1.2. Figure 7.4 and 7.5 show the plots of the predicted values as a function of the observed values for the growth rate and the lag phase, respectively. Besides a validation on the simulation medium APAM, a validation was also performed on real apples for three strains and six temperatures. The validation indices are shown in Tables 7.5 and 7.6 for the growth rate and the lag phase, respectively. The SEP for the growth rate was <54% and the  $B_f$  and  $A_f$  were >0.63 and <1.4 respectively. For the lag phase larger values were observed for the SEP (<94%) and  $A_f$  ( $\leq 3$ ) while the bias factor was below 1 ( $0.35 < B_f < 0.66$ ). Plots of the predicted values as a function of the observed values for the colony growth rate and the lag phase are shown in Figures 7.6 and 7.7. Moreover, the lag phase was characterized by a large variability between replicates.

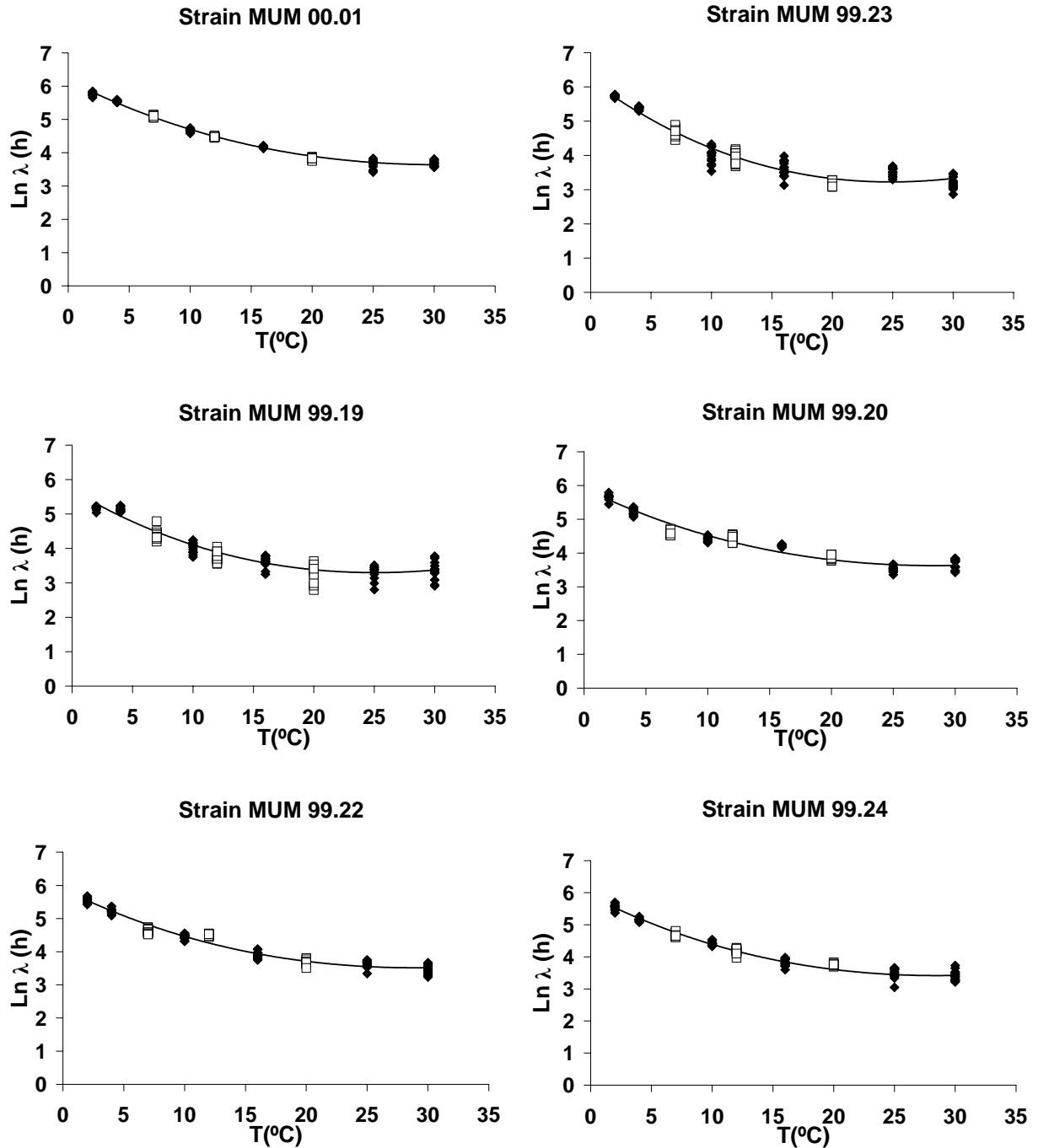


Figure 7.3: Plots of lag phase ( $\lambda$ , h) versus temperature ( $^\circ\text{C}$ ) ( $\blacklozenge$ : model development data;  $\square$ : validation data; —Arrhenius-Davey model)

**Table 7.2: Coefficients, determination coefficient ( $R^2$ ), root mean square error (RMSE) and standard error of prediction (SEP) of the Arrhenius-Davey model,  $Ln(\lambda) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2}$ , describing the effect of temperature on the lag phase of *P. expansum***

Strains	Coefficients <sup>a</sup>			Statistical indices		
	$C_0$	$C_1$	$C_2$	$R^2$	RMSE	SEP (%)
MUM 00.01	$20.8 \times 10^1 \pm 0.8 \times 10^{1b}$	$-12.4 \times 10^4 \pm 0.5 \times 10^4$	$18.8 \times 10^6 \pm 0.7 \times 10^6$	0.990	11.7	7.7
MUM 99.23	$36.1 \times 10^1 \pm 2.7 \times 10^1$	$-21.3 \times 10^4 \pm 1.6 \times 10^4$	$31.7 \times 10^6 \pm 2.2 \times 10^6$	0.947	14.0	12.1
MUM 99.19	$28.9 \times 10^1 \pm 2.4 \times 10^1$	$-17.0 \times 10^4 \pm 1.4 \times 10^4$	$25.3 \times 10^6 \pm 2.0 \times 10^6$	0.940	19.8	21.7
MUM 99.20	$21.5 \times 10^1 \pm 1.8 \times 10^1$	$-12.7 \times 10^4 \pm 1.1 \times 10^4$	$19.2 \times 10^6 \pm 1.5 \times 10^6$	0.962	17.1	15.1
MUM 99.22	$21.5 \times 10^1 \pm 1.2 \times 10^1$	$-12.7 \times 10^4 \pm 0.7 \times 10^4$	$19.2 \times 10^6 \pm 1.0 \times 10^6$	0.982	10.2	8.6
MUM 99.24	$23.1 \times 10^1 \pm 1.3 \times 10^1$	$-13.7 \times 10^4 \pm 0.8 \times 10^4$	$20.7 \times 10^6 \pm 1.1 \times 10^6$	0.981	11.1	10.0

<sup>a</sup>All coefficients were significant ( $p < 0.05$ ); <sup>b</sup>Estimation  $\pm$  standard deviation

## 4. Discussion

The optimal growth temperature of the six strains of *P. expansum* varied between 24 and 27°C, which is close to 25°C reported by Pitt & Hocking (1997). Growth rates between 0.340 and 0.212 mm/h were obtained at 25°C. These values are comparable to the radial growth rate of *P. expansum* reported by Marin et al. (2006b) of about 0.396 mm/h (25°C and  $a_w = 0.98-0.99$ ) and Lahlali et al. (2005) of about 0.160 mm/h (25°C and  $a_w = 0.98$ ).

A modification of the extended Ratkowsky model (Ratkowsky et al., 1983) was selected to describe the growth rate as a function of the temperature. An additional advantage of this model is the fact that it includes biologically significant parameters, giving more insight into the behaviour of the strains (Pitt, 1993). For the lag phase a modified Arrhenius model (Davey, 1989) was selected to describe the lag phase as a function of temperature. Although the model is entirely empirical, it is one of the most studied modifications of the Arrhenius equation to describe the effect of temperature on microbial growth (Ross & Dalgaard, 2003). Moreover, the model was successfully used as a predictor of the lag phase duration of *Aspergillus flavus* and *A. parasiticus* on corn (Samapundo et al., 2006). As seen in Tables 7.1 and 7.2, high  $R^2$  values were obtained for all models ( $>0.93$ ) indicating that at least 93% of the observed variance can be described by the model. In the same way, low RMSE values were obtained ( $<2.33 \times 10^{-2}$  for the growth rate models and  $<19.9$  for the lag phase models), indicating a small difference between predicted and observed values. However, the RMSE values do not allow the comparison of different models as they depend largely on the magnitude of the dataset values; i.e., in the case of the growth rate models, it is supposed that lower RMSE values will be obtained than for the lag phase models because the absolute difference between predicted and observed growth rates is much smaller than the absolute difference between predicted and observed lag phases. Due to this fact, SEP values were calculated, as they are a relative percentage that does not depend on the magnitude of dataset values. It is shown in Tables 7.1 and 7.2 that SEP values were below 22% for all models indicating a good adjustment to data observed. Also the graphical comparison between the models and the observed data (Figures 7.2 and 7.3) show a good fit of the models to the data.

To assess the performance of the developed models when they are confronted with extra data, a validation was performed on both the simulation medium and apples. Different statistical



indices (Tables 7.3 and 7.4) were calculated to evaluate the difference between observed and predicted values for growth rate and lag phase models. These indices were the RMSE, SEP,  $B_f$  and  $A_f$ . Low RMSE ( $<2.93 \times 10^{-2}$  for growth rate models, and  $<17.8$  for lag phase models) and SEP values ( $<26.8$  %) were obtained, indicating a good generalization capacity of the models. The  $B_f$  of the growth rate models were close to 1, indicating that the models were good predictors of the true mean colony growth rate. The  $B_f$  was  $<1$  for strains MUM 99.23, 99.19, 99.20 and 99.22, which means that the model is “fail-safe” or that the predicted colony growth rates are higher than the observed ones. For the other strains the predicted growth rates were smaller than the observed ones, but the  $B_f$  was  $\leq 1.1$  indicating that the model is not “fail-dangerous”. The  $A_f$  indicates that the predictions differed by maximum 19% from the observations. For the lag phase models,  $B_f$  values were close to 1 for all strains, and actually  $<1$  for strain MUM 99.24, indicating that the models were adequate predictors of lag phase and “fail safe” for strain MUM 99.24. However, for the other strains the  $B_f$  was  $>1$ , but only for strain MUM 99.19 the model was “fail-dangerous since the  $B_f > 1.1$ . Based on the  $A_f$  the

**Table 7.3: Mathematical indices used to validate the extended Ratkowsky model,  $\sqrt{\mu_{\max}} = a + b \cdot (T - T_{\min}) + [1 - e^{(c \cdot (T - T_{\max}))}]$ , describing the effect of temperature on the growth rate of six *P. expansum* strains on APAM**

Strain	MUM 00.01	MUM 99.23	MUM 99.19	MUM 99.20	MUM 99.22	MUM 99.24
RMSE	$1.58 \times 10^{-2}$	$2.38 \times 10^{-2}$	$2.92 \times 10^{-2}$	$1.84 \times 10^{-2}$	$1.40 \times 10^{-2}$	$1.78 \times 10^{-2}$
SEP (%)	8.4	20.7	26.7	14.0	10.8	12.0
$B_f$	1.02	0.93	0.91	0.94	0.93	1.10
$A_f$	1.05	1.14	1.19	1.07	1.08	1.11

**Table 7.4: Mathematical indices used to validate the Arrhenius-Davey model,  $Ln(\lambda) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2}$ , describing the effect of temperature on the lag phase of six *P. expansum* strains on APAM**

Strain	MUM 00.01	MUM 99.23	MUM 99.19	MUM 99.20	MUM 99.22	MUM 99.24
RMSE	4.09	8.82	9.18	19.56	17.70	8.26
SEP (%)	4.1	14.0	18.0	25.0	22.9	10.9
$B_f$	1.00	1.04	1.14	1.03	1.00	0.96
$A_f$	1.03	1.13	1.18	1.17	1.18	1.12

predicted lag phases differed by a maximum of 18% from the observed values. The plots of the predicted growth parameter as a function of the observed growth parameter (Figures 7.4 and 7.5) show that in general, the validation data points are evenly distributed around the line of equivalence. However, for strain MUM 99.20 and MUM 99.23 higher growth rates are predicted at 20°C than observed and this effect was also observed for strain MUM 99.24 at 12°C. At 7°C overestimation was observed for strains MUM 99.20 and MUM 99.22 at 7°C, while a higher lag phase was observed than predicted at 12°C for strain MUM 99.22.

The observed bias and accuracy factors are comparable to those that have been determined in other studies. Samapundo et al. (2005) obtained values ranging from 0.84 to 1.08 for modelling the growth rate of *Fusarium verticillioides* and *F. proliferatum* on yellow dent corn as function of temperature and water activity. Valík and Piecková (2001) modelled the effect of water activity on the growth rate of three heat resistant fungal isolates at 25°C and obtained bias factors that ranged from 1.007 to 1.014, and accuracy factors that ranged from 1.070 to 1.106.

The most common method for validating models is to carry out experiments directly on the food product of concern. In this study, predictions of the growth parameters of *P. expansum* on simulation medium as a function of temperature were subjected to an external validation of three strains on apples. The validation indices (Table 7.5) for the extended Ratkowsky model ( $\mu_{\max}$ ) showed that the  $B_f$  were between 0.5 and 1, indicating conservative predictions for the growth rate (predicted  $\mu_{\max} >$  observed  $\mu_{\max}$ ). This is also observed on the plots that show the predicted growth rate as a function of the observed growth rate on apple, especially at 25°C (Figure 7.6). The  $A_f$  is  $<1.39$  indicating that the predictions have a deviation below 39% compared to the observed values. For the lag phase, a clear deviation was observed when the predictions are compared to the observed values on apples (Figure 7.7). Longer lag phases were observed on apples than the predicted values, which can also be seen from the  $B_f$  which is  $<1$  (Table 7.6). For strain MUM 99.19 the  $B_f$  was even  $<0.5$  indicating that the model is too conservative and gives no good predictions of the lag phase. The  $A_f$  was  $>1.6$  for the three strains tested.

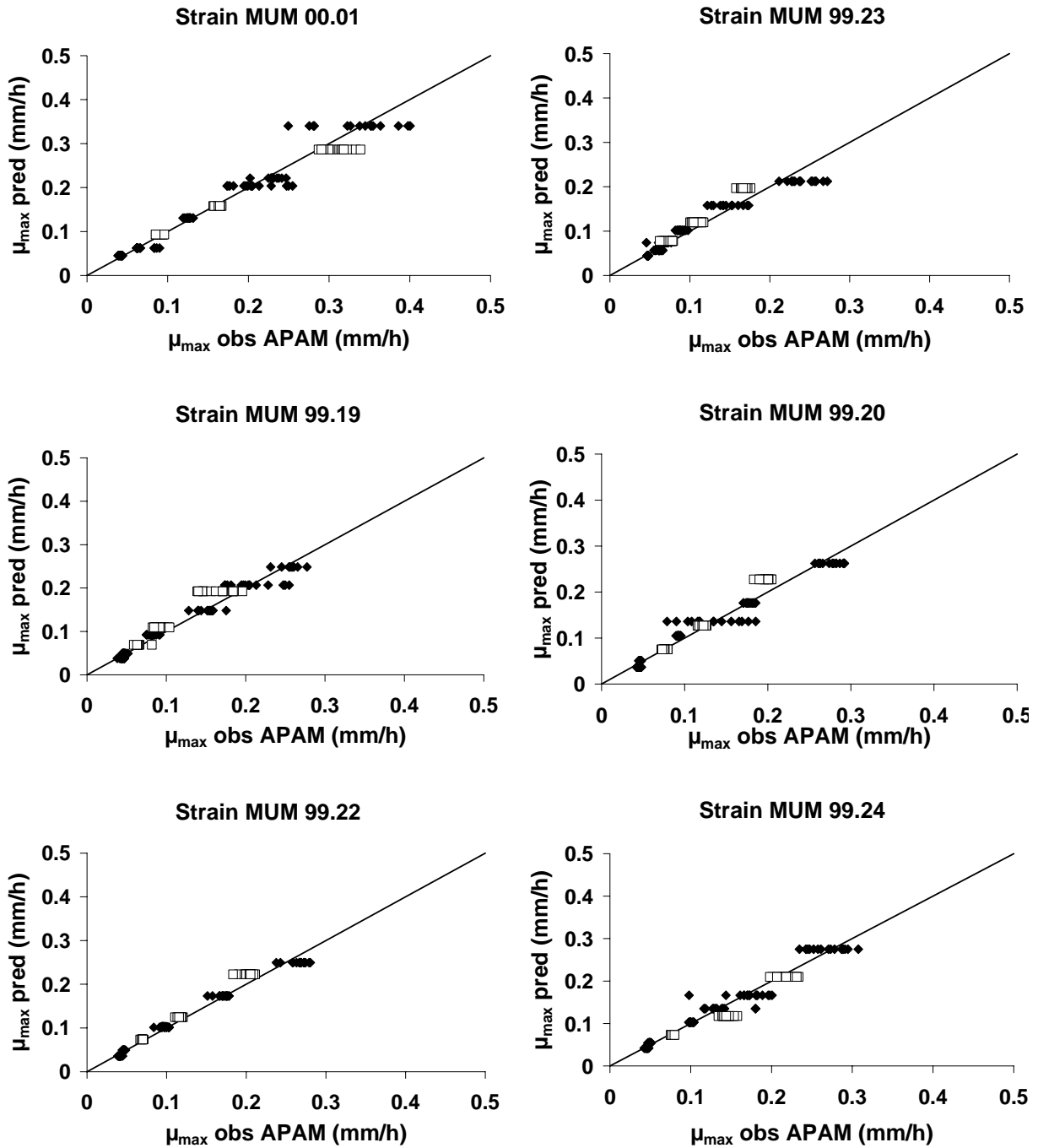


Figure 7.4: Comparison of observed (obs) and predicted (pred) growth rates ( $\mu_{\max}$ , mm/h) (◆: model development data; □: validation data)

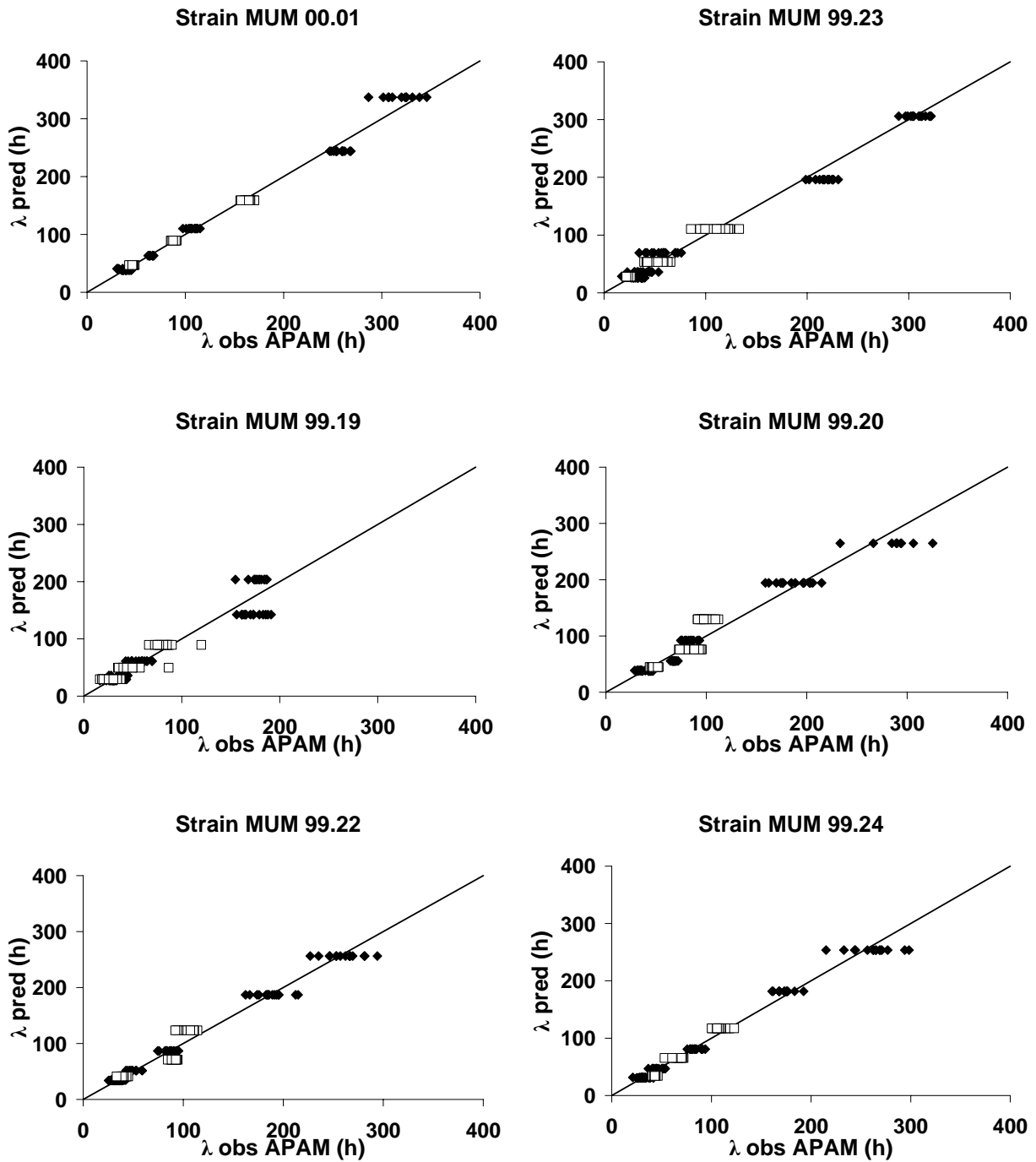


Figure 7.5: Comparison of observed (obs) and predicted (pred) lag phases (h) (◆: model development data; □: validation data)

**Table 7.5: Mathematical indices used to validate the extended Ratkowsky model,  $\sqrt{\mu_{\max}} = a + b \cdot (T - T_{\min}) + [1 - e^{(c \cdot (T - T_{\max}))}]$ , describing the effect of temperature on the growth rate of three *P. expansum* strains on apple**

Strains	MUM 00.01	MUM 99.19	MUM 99.22
RMSE	$5.49 \times 10^{-2}$	$2.90 \times 10^{-2}$	$2.07 \times 10^{-2}$
SEP (%)	53.3	34.8	22.7
$B_f$	0.64	0.75	0.81
$A_f$	1.38	1.29	1.26

**Table 7.6: Mathematical indices used to validate the Arrhenius-Davey model,  $Ln(\lambda) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2}$ , describing the effect of temperature on the lag phase of three *P. expansum* strains on apple**

Strains	MUM 00.01	MUM 99.19	MUM 99.22
RMSE	202	332	247
SEP (%)	68.1	93.0	86.1
$B_f$	0.65	0.36	0.58
$A_f$	1.64	3.00	1.86

The difference between the observed growth rate and lag phase in apples and the predicted values was also observed for the other models that have been developed for *P. expansum*. At 7°C a lag phase of 175 h is predicted ( $a_w$ : 0.98-0.99) by Marin et al. (2006b) while the observed lag phase in apples varied between 270 and 363 h. In correspondence to the presented models in this study, the difference between the observed and predicted values for the growth rate was smaller than for the lag phase. A growth rate of 0.4 and 0.16 mm/h was predicted by Lahlali et al. (2005) and Marin et al. (2006b), respectively at 25°C ( $a_w$ : 0.98-0.99). The observed growth rate in apples stored under these conditions varied between 0.17 and 0.22 mm/h depending on the strain.

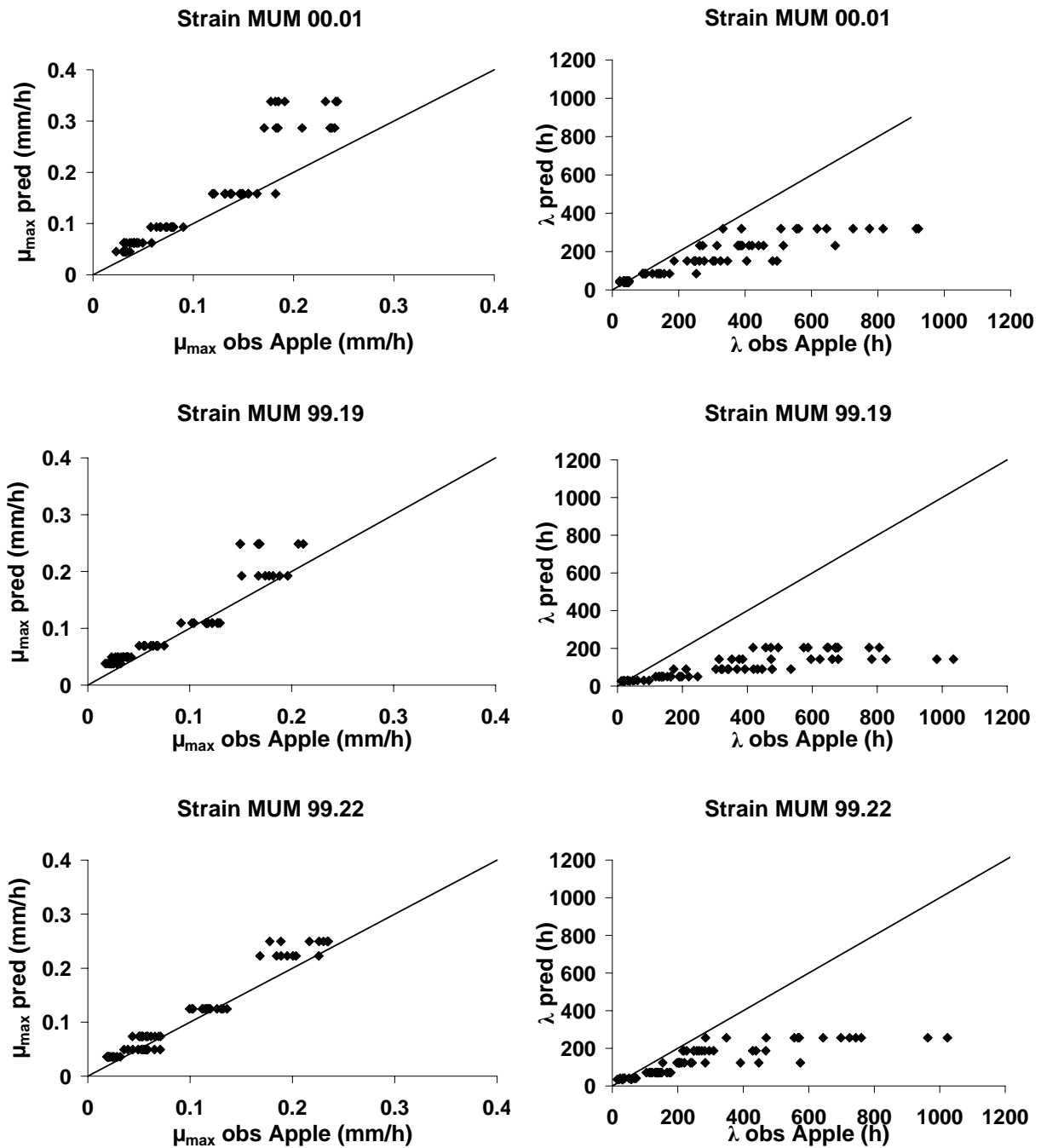


Figure 7.6: Comparison of observed (obs) growth rates ( $\mu_{\max}$ , mm/h) in apples and predicted (pred) growth rates

Figure 7.7: Comparison of observed (obs) lag phases ( $\lambda$ , h) in apples and predicted (pred) lag phases

The lag phase of *P. expansum* on apples was characterized by a large variability which was not observed on APAM (Figure 7.5 and 7.7). Previous research has shown that this variability increases when growth conditions become less optimal or when less spores are used for the inoculation (Chapter 6).

To our knowledge, other models describing fungal growth have been developed for culture media or directly in food products, but none of these models were developed in a culture media and validated on real foods. Nevertheless, differences between predictions in an artificial medium and in the food matrix should be known and taken into account. Giffel and Zwietering (1999) obtained  $A_f$  and  $B_f$  for validation of a growth model for *Listeria monocytogenes* in food products ranging from 1.37 to 4.25 and 0.24 to 2.28 respectively, depending on the food product. Dalgaard and Jørgensen (1998) obtained also similar  $B_f$  and  $A_f$  (1.0 to 3.9) for the validation of a growth model for *L. monocytogenes* in fish products.

The obtained results showed that growth of *P. expansum* on apples is characterized by a longer lag phase and a slower growth rate compared to the predicted values. However, larger deviations were observed for the lag phase than the growth rate. The difference between the predicted and observed lag phase on apple is most probably caused by the apple itself, which indicates that the apple can be seen as a stress factor. This was also observed in another study investigating the effect of environmental conditions on patulin production by *P. expansum* in both simulation medium and apple (Chapter 5). Such stress factors may include the intact tissue structure of the apple, which should be degraded in order to enable mould infection to occur and which causes as well a reduced  $O_2$  availability in the apple structure, compared to APAM. As a consequence, the use of simulation or synthetic media for the development of predictive models for the lag phase of moulds can lead to inadequate predictions and a validation on the food matrix is necessary.

## 5. Conclusion

Secondary models were developed in two steps, a screening of several potential suitable functions by means of the MSE, followed by a more detailed evaluation to select the model that provided the best fit. The developed models described adequately the evolution of growth parameters (growth rate and lag phase) as a function of storage temperature for the different strains of *P. expansum* in APAM medium. However, in order to apply the developed models to a real food product, a validation was carried out on apples. A deviation was observed between predicted and observed growth parameters, which was more pronounced for the lag phase. For one strain (MUM 99.19) the model was evaluated as too conservative and gave no good predictions of the lag phase. These results indicate that the apple itself has a large influence on the adaptation period of the spores, but once germination took place and the

mould started to grow, similar results were obtained on the simulation medium and apple. Therefore, it is necessary to use apples instead of a simulation medium to develop a growth model for the lag phase of *P. expansum* or to include a correction factor. The developed models will be used to predict growth of *P. expansum* in a farm to fork risk assessment (Chapter 8).



### **A farm to fork model for quantitative risk assessment of patulin in apple juice<sup>1</sup>**

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<sup>1</sup> Based on: Baert, K., De Meulenaer, B., Amiri, A., Debevere, J., Devlieghere, F. A farm to fork model for quantitative risk assessment of patulin in apple juice. In preparation for submission.



## Chapter 8

# A farm to fork model for quantitative risk assessment of patulin in apple juice

### Summary

The available reports mainly focus on the different aspects of patulin contamination, like growth of *Penicillium expansum*, patulin production under different conditions and the influence of processing on the patulin concentration in apple juice. The purpose of the present study was to collect this information and to perform a quantitative risk assessment in order to evaluate different strategies to mitigate patulin contamination. Therefore, a quantitative risk assessment model was elaborated describing the complete chain from the picking of apples until storage of produced apple juice. Validation of the elaborated model showed that the simulated mean patulin concentration (6.8 and 3.6 µg/kg for cloudy and clear apple juice, respectively) was in accordance with the mean patulin concentration detected in apple juice in Belgium (6.7 and 4.5 µg/kg for cloudy and clear apple juice, respectively). However, an overestimation was observed for the higher concentrations (95<sup>th</sup> percentile). Different scenarios were evaluated in order to reduce the patulin contamination of apple juice. It was demonstrated that the concentration of patulin in apple juice can be reduced with 50% when less apples are used that were stored in controlled atmosphere. However, when apples are used that were stored in controlled atmosphere, a sorting step should be included to remove apples with an infection spot larger than 10 cm<sup>2</sup>. This measure reduces the patulin contamination in 99.7 to 99.9% of the apple juices to levels below 25 µg/kg. Another critical factor is the duration of deck storage (storage outside at ambient temperature) before the apples are processed.

## 1. Introduction

Previous research has shown that 12% of the apple juices marketed in Belgium are contaminated with patulin, of which 1% has a concentration above 50 µg/kg, the maximum limit allowed in the European Union (Chapter 3). Moreover, a probabilistic risk assessment showed that the current limit can be considered to be insufficient to protect children who consume organic apple juice (AJ), since 0.5% [90% confidence interval: 0.1-1.2%] still exceeds the tolerable daily intake (TDI) when the contamination is below 50 µg/kg. However, when the contamination is below 25 µg/kg, this can be reduced to 0% [90% confidence interval: 0-0.3%] (Chapter 4). Therefore, it is important for public health to reduce patulin contamination in AJ. In this regard the European Commission elaborated the “Code of practice for the prevention and reduction of patulin contamination in AJ and AJ ingredients in other beverages” (European Commission, 2003a).

The aim of the present study was to elaborate a farm to fork risk assessment that describes the complete chain starting from apples until the production of AJ, including growth and patulin production by *P. expansum* and influence of processing. Farm to fork risk assessment is widely used for bacteria but to our knowledge has not yet been used for mycotoxigenic fungi. The advantage of this technique is that different strategies to reduce mycotoxin concentration can be tested, since the microbiological history of the product is taken into account. First, the elaborated quantitative risk assessment model (QRAM) was validated with patulin concentrations analysed in AJ marketed in Belgium. In a second phase, the model was used to evaluate different strategies to reduce patulin contamination of AJ.

## 2. Materials and methods

### 2.1. General overview of the model

The developed QRAM follows a farm to fork approach, starting from the picking of the apples until the manufacture of AJ (Figure 8.1). The QRAM consists of two main parts: the storage of the apples and AJ production.

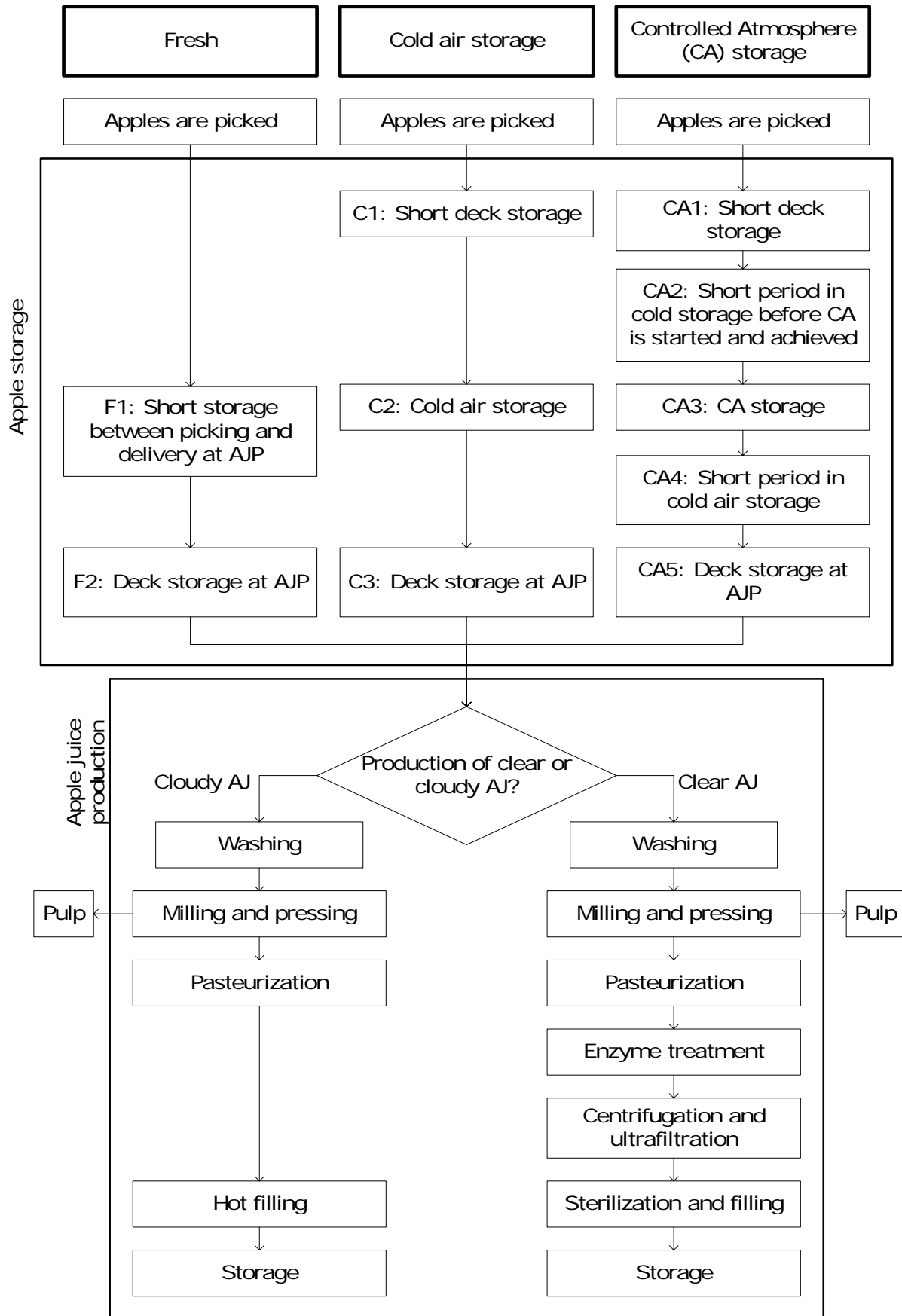


Figure 8.1: Flow diagram of apple juice production (AJP: apple juice producer; AJ: apple juice; CA: controlled atmosphere)

Picked apples can be used directly for the production of AJ or they can be stored until further processing. Refrigerated storage (0.5 to 3.5°C, depending on the cultivar) is used for short storage periods (<3 months), while controlled atmosphere (CA) (0.5 to 3.5°C, 1 to 3% O<sub>2</sub> and <1 to 3% CO<sub>2</sub>, depending on the cultivar) is used for long storage (≥3 months). The storage period consists of different storage steps (Figure 8.1), which are further explained in section 2.2.2. As the storage conditions during every storage step differ, mould growth and patulin production were calculated separately for each storage step. The apples used for the production of one batch of AJ were stored under the same conditions and the length of the storage period was the same. Growth and patulin production were calculated on the level of the individual apples, but the influence of AJ production was calculated on batch level. In order to include strain variability in the QRAM, growth models and data concerning the patulin production of six strains were used. One strain out of six was randomly selected for every apple and the specific growth models and patulin production characteristics of the selected strain were used to calculate the amount of patulin in the studied apple.

For the production of AJ, two production flows were considered: the production of cloudy AJ in glass bottles and the production of clear AJ in Brick carton (Figure 8.1). The flow diagram for AJ production is explained further in detail in section 2.2.6.

## **2.2. Data and parameters of the model**

Information concerning the production of AJ was obtained from a Belgian AJ producer and information concerning the storage of apples was obtained from a Belgian auction. Estimations of the ambient temperature for certain time periods were derived from the reports of the Royal Meteorological Institute of Belgium (Royal Meteorological Institute of Belgium, 2006).

### **2.2.1. Apples and apple juice production**

The weight of an apple used for AJ production varies between 0.08 to 0.4 kg. Based on this information, a uniform distribution between 0.08 and 0.4 kg was used to select the weight of an apple. One thousand kg of apples was used as a typical batch size for AJ production and thus apples were selected until a total weight of 1000 kg was obtained.

AJ production in Belgium starts around half September and fresh apples are used until half November. Between mid November and the end of December, apples are used that were stored in refrigerated storage and from January onwards apples are used that were stored in CA. During September, October and November, AJ production is twice as high as in the months from December till March. From April onwards, the production slows down and the production between April and August is equivalent to one month. The total AJ production in Belgium is equivalent to about 10 months. During 4 months fresh apples are used, 2 months refrigerated storage apples and 4 months CA apples. A discrete uniform distribution was used that generates in 40% of the cases fresh apples, in 20% refrigerated storage apples and in 40% CA apples.

### **2.2.2. Apple storage**

Table 8.1 gives an overview of the different distributions used to model apple storage. Fresh apples are stored after picking during 4 to 14 days (F1) (Figure 8.1). When the storage takes more than 7 days, the apples are stored in cold air (refrigerated storage at 1°C), otherwise apples are stored outside at ambient temperature (deck storage). After delivery at the apple juice producer (AJP), the apples are immediately processed or stored for maximum one day (deck storage) (F2).

Apples for refrigerated storage are first stored during one day outside (ambient temperature) (C1). During refrigerated storage a temperature of 1°C is maintained (C2). This temperature depends on the cultivar but is usually around 0.8 and 1°C. After refrigerated storage, the apples are transported to the AJP. Depending on the production scheme, the apples will be processed immediately or stored for maximum one day (deck storage) (C3).

Apples in CA follow more or less the same route as apples in refrigerated storage. After picking, the apples are stored for 1 day outside (ambient temperature) (CA1). Before the apples are stored under CA, the apples are stored at 1°C for 1 to 6 days to fill the CA storage room and to achieve the CA conditions (CA2). During CA storage, the O<sub>2</sub> level is between 1 and 3% and the CO<sub>2</sub> level between <1 and 3%, depending on the cultivar. In this QRAM, the O<sub>2</sub> level was maintained at 1% and the CO<sub>2</sub> level <1% (CA3). Once the CA storage room is opened, the atmosphere is changed and the storage room needs to be emptied as soon as possible (within maximum 7 days). In this period, the temperature is 1°C and the composition

**Table 8.1: The different distributions used to model the storage of apples**

Storage step	Parameters		
	Duration (days)	Temperature (°C)	%O <sub>2</sub> <sup>h</sup>
Fresh apples			
Storage between picking and delivery at the AJP <sup>a</sup> (F1)	Round(Uniform(4;14);0)	If duration > 7 days => 1 If duration ≤ 7 days => Duniform <sup>b</sup> (14.4;10.4;10.4;6) <sup>c</sup>	20
Deck storage at AJP (F2)	Duniform(0;1)	Duniform(14.4;10.4;10.4;6) <sup>c</sup>	20
Refrigerated storage			
Short deck storage (C1)	1	Duniform(14.4;10.4) <sup>d</sup>	20
Refrigerated storage (C2)	Duniform(24;...;92) <sup>e</sup>	1	20
Deck storage at AJP (C3)	Duniform(0;1)	Duniform(6; 3.4;3.4) <sup>f</sup>	20
Controlled Atmosphere (CA) storage			
Short deck storage (CA1)	1	Duniform(14.4;10.4) <sup>d</sup>	20
Refrigerated storage before CA storage (CA2)	Round(Uniform(1;6);0)	1	20
Storage under CA conditions (CA3)	Duniform(92;...;330) <sup>e</sup>	1	1
Refrigerated storage after CA storage (CA4)	Round(Uniform(1;7);0)	1	20
Deck storage at AJP (CA5)	Duniform(0;1)	Duniform(2.5;3.2;5.7;10.7) <sup>g</sup>	20

<sup>a</sup>AJP: apple juice producer; <sup>b</sup>Duniform: a discrete uniform distribution. A discrete uniform distribution is a special case of the discrete distribution where all possible values have the same probability of occurrence (Vose, 2000); <sup>c</sup>Fresh apples are used from half September until half November. The mean temperature in the month September is 14.4°C, in October 10.4°C and in November 6°C. In September and November fresh apples are used during half a month, while in October during the complete month, therefore 10.4°C was included 2 times in the distribution;



<sup>d</sup>Apples stored in refrigerated storage are picked between half September (mean temperature 14.4°C) and half October (mean temperature 10.4°C); <sup>e</sup>A discrete uniform distribution, of which the numbers are selected to describe the real storage period considering a 5-day work week; <sup>f</sup>Apples stored in refrigerated storage are used between half November (mean temperature 6°C) and the end of December (mean temperature 3.4°C). The temperature 3.4°C was included two times in the distribution since the complete month December is considered while only half November was considered; <sup>g</sup>The mean temperature in January is 2.5°C, in February 3.2°C, in March 5.7°C and in April and May 10.7°C. <sup>h</sup>Fixed values are used for the %O<sub>2</sub> since patulin production in function of the growth is only described for these fixed conditions.

of the atmosphere is equal to that of air (CA4). After delivery at the AJP, the apples are processed immediately or stored for at most one day (deck storage) (CA5).

### **2.2.3. Probability of a diseased apple caused by *P. expansum* and probability of infection**

To determine whether growth of *P. expansum* occurs in an apple during storage, the number of diseased apples caused by *Penicillium* spp. through lenticels and wounds was used to calculate the probability (Amiri & Bompeix, 2005). This probability changes during the storage period. Therefore, the storage period was divided in periods of 30 days and during that period, the probability was considered as constant. Since Amiri and Bompeix (2005) determined the probability for *Penicillium* spp., a correction was included for *P. expansum*. In the mentioned research, it was shown that about 41% of the *Penicillium* spores are those of *P. expansum*. To determine the probability for growth in fresh apples, a random sample (n=50) was taken from fresh apples used for AJ production and was checked for mould growth. It was determined that the probability to have a diseased, fresh apple caused by *P. expansum* was 0.029, seven days after picking. The probability of a diseased apple was used to estimate the probability of infection by taking the lag phase into account.

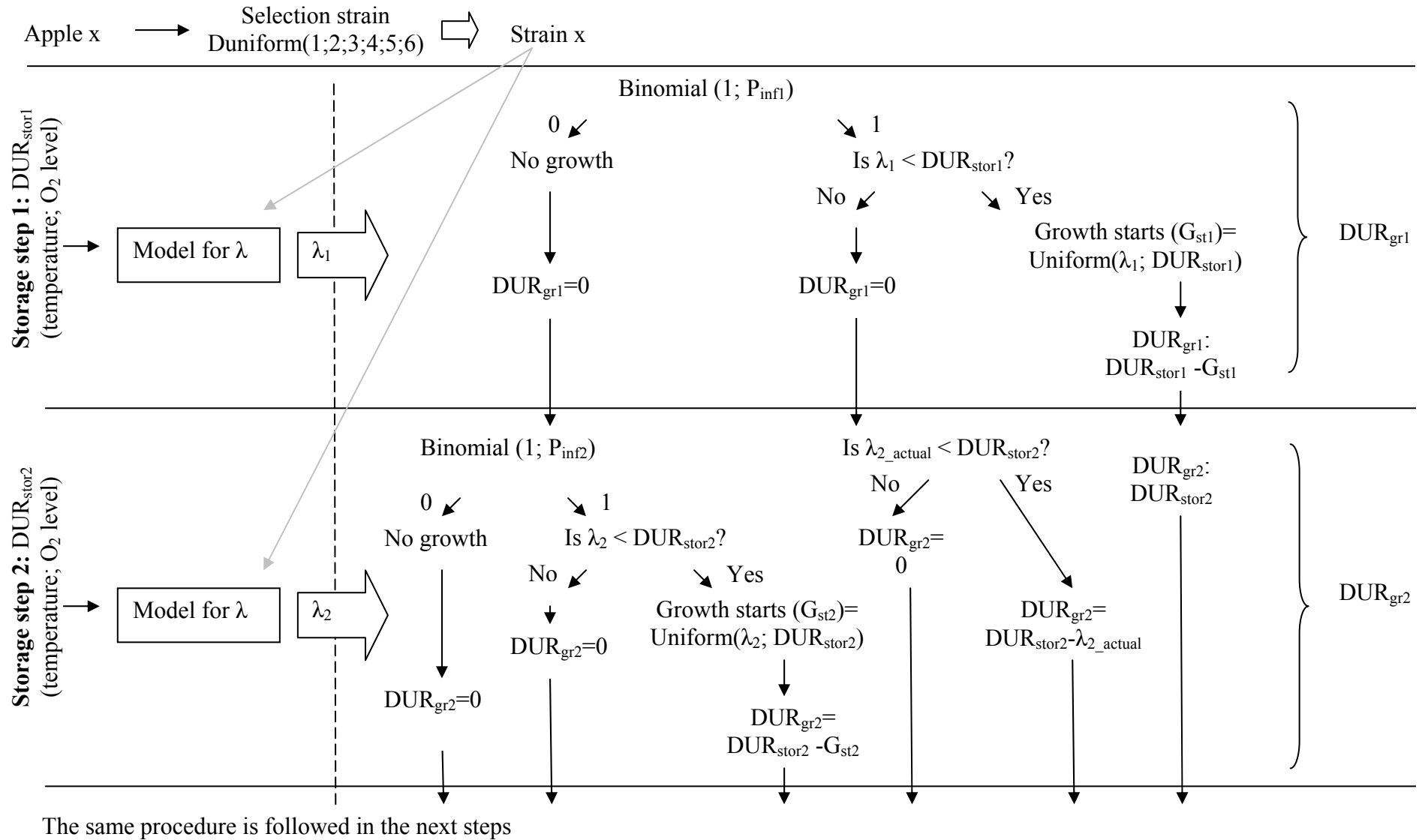
### **2.2.4. Growth of *P. expansum* on apple**

The growth of *P. expansum* was estimated using predictive models for the growth rate and the lag phase. As described in Chapter 7 the growth of six *P. expansum* strains was studied and for each strain a model for the growth rate and the lag phase as a function of the temperature was developed. These models were used to include strain variability of the growth in the QRAM. The models developed on a simulation medium based on apple puree were validated on apples and it was found that the predicted lag phases ( $\lambda_{\text{pred}}$ ) were on average 2.4 times smaller than the observed values on apples. Therefore, the predicted values were corrected with a factor 2.4. Moreover, large variability was observed for the lag phase on apples and this variability was even more pronounced when less spores are inoculated in the apple (Chapter 6). It was estimated that the standard deviation of the lag phase was 0.3 times the mean value. The lag phase ( $\lambda$ ) was calculated using a normal distribution with  $2.4 \times \lambda_{\text{pred}}$  as the mean and  $2.4 \times 0.3 \times \lambda_{\text{pred}}$  as the standard deviation.

Growth of *P. expansum* on apple was calculated for every individual apple. First, one strain was selected out of six using a discrete uniform distribution (Figure 8.2). A discrete uniform distribution is a special case of the discrete distribution where all possible values have the same probability of occurrence (Vose, 2000). In each step, the lag phase was calculated for the selected strain using the temperature of that specific storage step as an input. For the first storage step, it was calculated whether infection will occur using a binomial distribution with the probability of infection ( $P_{inf}$ ) for the specified circumstances and one trial (Figure 8.2). When infection occurs (binomial distribution generates 1) and the lag phase is shorter than the duration of the first storage step, it is calculated when the growth starts ( $G_{st}$ ) using a uniform distribution between the lag phase and the storage duration ( $DUR_{stor}$ ). Based on this result, it is determined how long the mould grows ( $DUR_{gr}$ ) in the first storage step. When the lag phase is longer than the duration of the first storage step or when no infection occurs (binomial distribution generated 0), no growth will occur in the first storage step. Next, the growth was determined for the 2<sup>nd</sup> storage step and 3 cases are possible. (1) When there was no infection in the first storage step, it is determined if infection occurs in the second step in the same way as for the previous period. So when growth started once, it was not possible that growth started a second time on the same apple. (2) When infection occurred in the previous step, but the lag phase was longer than the storage step, the actual lag phase was calculated in step 2 using equation (8.1):

$$\lambda_{2\_actual} = \left(1 - \frac{DUR_{stor1}}{\lambda_1}\right) \lambda_2 \quad (8.1)$$

with  $\lambda_{2\_actual}$ , the actual lag phase in step 2;  $\lambda_1$ , the theoretical lag phase in step 1;  $\lambda_2$ , the theoretical lag phase of step 2 and  $DUR_{stor}$ , the duration of the first storage step. This equation calculates the actual lag phase in step 2 based on the fraction of the lag phase that already passed in step 1 ( $DUR_{stor1}/\lambda_1$ ) and the theoretical lag phase in step 2. For example, when the lag phase in the first step was 2 days and the duration of that storage period is 1 day, then the spore still has to go through 50% of its lag phase. When the lag phase under the conditions of the second storage step is 4 days, the actual lag phase in step 2 will be 2 days. When this actual lag phase is smaller than the duration of the second storage step, growth will occur, otherwise no growth will occur in step 2. (3) When growth was observed in the previous step, growth occurs in the complete second period. This calculation was done for every storage step until the end of the storage period. In this way, the number of days with *P. expansum* growth was calculated for every storage step.

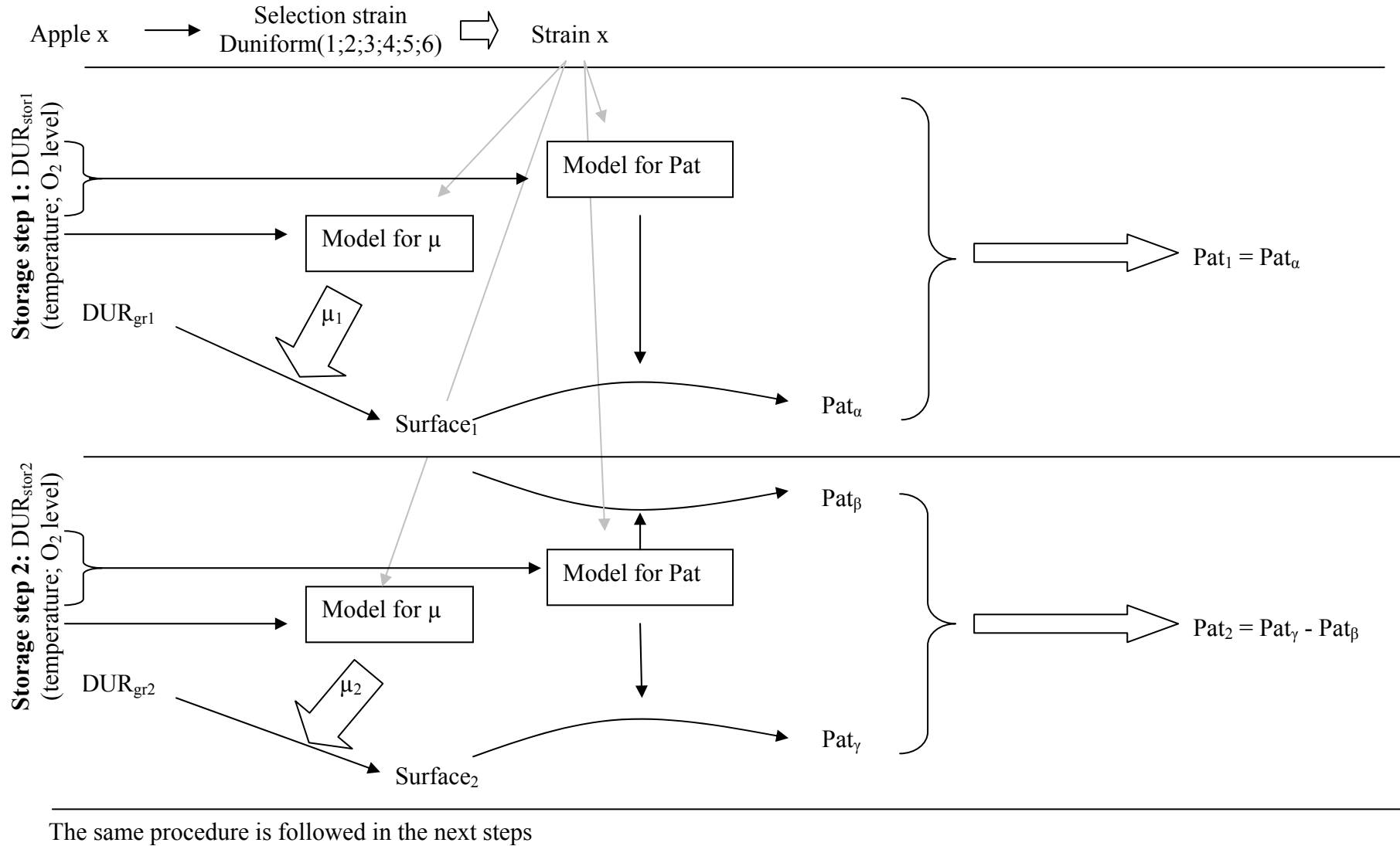


**Figure 8.2: Schematic presentation of the growth duration calculation ( $DUR_{stor}$ : storage duration;  $\lambda$ : lag phase,  $P_{infl}$ : probability of infection,  $G_{st}$ : day when growth starts,  $DUR_{gr}$ : growth duration;  $\lambda_{actual}$ : actual lag phase)**

After calculating the number of days when growth of *P. expansum* is observed, the growth rate ( $\mu$ ) was determined for every storage step, using the growth rate model of the selected strain and the temperature of the specific storage step (Figure 8.3). Based on the growth rate and the duration of growth, the diameter and the surface of the mould spot was calculated.

### 2.2.5. Patulin production

After estimating the growth of *P. expansum*, patulin production was calculated. Not every *P. expansum* strain is able to produce patulin, especially at low temperatures. Based on the data described by Lovett and Thompson (1978) and in Chapter 5, it was determined that at 1°C 83% of the *P. expansum* strains produce patulin, while at 4-7°C and 10-12°C 95 and 100% of the strains produce patulin, respectively. Based on these percentages, it was determined whether the selected strain produced patulin or not. The patulin production by *P. expansum* strain MUM 00.01 as a function of the surface of the mould for different storage temperatures and atmosphere conditions on a simulation medium was studied in Chapter 5. In general, a linear relation was observed between patulin production and the mould surface. Therefore, linear regression was used to describe patulin production as a function of the surface (Table 8.2). The linear regression was forced through 0, since no patulin can be produced when the surface is equal to 0 cm<sup>2</sup>. For 1°C and 1% O<sub>2</sub> a linear relation between patulin production and surface was observed when the surface was <20 cm<sup>2</sup>, but a stagnation was noticed when the surface was ≥20 cm<sup>2</sup>. Therefore, a constant patulin amount of 3 mg was used when the surface was ≥20 cm<sup>2</sup>. However, since *P. expansum* grows in practice on apples and not on simulation medium, the patulin production on both media was compared (Chapter 5). This validation showed that the amounts of patulin produced in simulation medium and apple were comparable except at 1°C, where a clear overestimation of patulin production was observed in simulation medium. At 1°C and 20% O<sub>2</sub> 6 times less patulin was observed in apple than in simulation medium and 4.5 times less at 1°C and 1% O<sub>2</sub>. These values were taken into account in the QRAM.



**Figure 8.3:** Schematic presentation of the surface calculation and the patulin amount produced by *P. expansum* ( $DUR_{gr}$ : growth duration;  $\mu$ : growth rate; Pat: patulin concentration;  $DUR_{stor}$ : storage duration)

**Table 8.2: Linear model to calculate the amount of patulin produced (pat, mg) as a function of the surface (S, cm<sup>2</sup>) (pat = slope x S) for different storage conditions**

Temperature (°C)	O <sub>2</sub> level (%) <sup>a</sup>	Slope
10	20	0.364
4	20	0.720
1	20	0.523
1	1	0.148

<sup>a</sup>CO<sub>2</sub> < 1%; N<sub>2</sub> was used as a filler gas

It was also demonstrated that the amount of patulin produced is strain dependent (Chapter 5). The patulin production of the same strains used for the predictive growth models was determined under different conditions (temperature, % O<sub>2</sub>). With these results, it was calculated how many times a certain strain produces more or less patulin than strain MUM 00.01. These values were used to include strain variability of patulin production in the QRAM, by multiplying the patulin production, determined as described above, with linear regression with the value of the selected strain.

For every storage step, the surface of the mould was calculated at the beginning of the storage step and at the end, except for step 1 where the surface at the beginning is always 0 cm<sup>2</sup> (Figure 8.3). For both surfaces, the patulin production was calculated using the linear regression for patulin production and the difference gives the amount of patulin produced in the studied storage step. When the amount of patulin for each step is summed, the total amount of patulin for an individual apple is known. Once the amount of patulin was calculated for every apple, the total amount of patulin present in the batch was determined and the further calculations were performed on this total amount of patulin.

### 2.2.6. Apple Processing

Two production flows were considered in this risk assessment: the production of cloudy AJ in glass bottles and the production of clear AJ in Brick carton. The different steps for the production of cloudy AJ are shown in Figure 8.1. Between 26 and 55% of the patulin is removed during the washing, milling and pressing (Acar et al., 1998). During milling and before filling, ascorbic acid is added to the mixture to remove the O<sub>2</sub> that is incorporated. Ascorbic acid is added to a concentration of 500 ppm and this results in a removal of 33% of the remaining patulin (Aytac & Acar, 1994). Since patulin is heat stable in an acid

environment such as AJ, the influence of pasteurization on patulin degradation is very limited. A typical pasteurization of AJ is 30 seconds at 96°C. Kadakal and Nas (2003) determined the influence of heat treatments at 90 and 100°C on patulin destruction. Based on their data it was calculated with the Arrhenius equation, that 0.65% of the patulin is removed during the applied heat treatment. Before filling, the AJ is heated at 85°C for 3 minutes. Based on the data described by Kadakal and Nas (2003) it was estimated with the Arrhenius equation, that 2.6% of the patulin is removed. Filling of the AJ itself has no effect on the patulin concentration but a decrease of the patulin concentration is observed during storage of the AJ (Koca & Eksi, 2005). Based on the data described by Koca and Eksi (2005) the influence of storage at 22°C on the patulin concentration was determined using the Arrhenius equation (Table 8.3). The maximum storage time was 12 months, since AJ has mostly a shelf life of one year. A uniform distribution between 1 and 12 was used to determine the storage time.

**Table 8.3: Percentage of patulin degraded during storage of apple juice at 22°C**

Months of storage	Percentage degraded
1	52.9
2	80.8
3	91.8
4	99
5	99.8
6-12	99.9

For the production of clear AJ, a similar flow was used with some changes (Figure 8.1). After washing, milling, pressing, adding ascorbic acid and pasteurization, enzyme is added to the juice in order to aid the clarification. Bissessur et al. (2001) determined that 20.5% of the patulin is removed during this step. During centrifugation, 36.5% of the patulin is removed (Bissessur et al., 2001) and a further reduction between 8 and 17% is obtained during ultrafiltration (Acar et al., 1998). A sterilization is performed for 1 minute at 96°C before the juice is filled aseptically in Brick carton. This causes a reduction of 1.3%, based on the data described by Kadakal and Nas (2003). During storage, between 52.9 and 99.9% of the patulin is degraded (Table 8.3).



The pulp obtained during milling and pressing, is pressed for a second time after adding water and enzyme. The secondary juice which is obtained in this way can be evaporated in order to produce concentrate. However, the production of concentrate was not included in the current QRAM.

### **2.3. Software and simulation settings**

The QRAM was constructed in an Excel spreadsheet (Microsoft, USA) and was simulated using @RISK (Palisade, USA), an Excel add-in program. To run the simulations, Latin Hypercube sampling was used and the random generator seed was fixed at 1. This fixed value was used since, providing the model is not changed, the same simulation results can be exactly repeated. More importantly, one or more distributions can be changed within the model and a second simulation can be run to verify if these changes have an effect on the model's output. In this way, it is certain that any observed change in the result is due to changes in the model and not a result of randomness of the sampling (Vose, 2000). There where  $10^4$  iterations carried out.

### **2.4. Contamination mitigation strategies**

In order to test the influence of different risk management measures, nine different contamination mitigation strategies were simulated and compared to the baseline model (S0).

S1: Influence of storage facility: The model was run four times and the following apples were used:

- 100% fresh apples
- 100% apples from refrigerated storage
- 100% apples from CA
- 50% fresh apples, 30% apples from refrigerated storage and 20% apples from CA

S2: Reduction of wounding: The number of apples that showed growth of *P. expansum* due to wounds was decreased by 10, 50 or 99%.

S3: Deck storage of fresh apples: When fresh apples are used, they are stored for 4 to 14 days before use. When the storage takes not more than 7 days, deck storage is applied. The maximum period of deck storage (7 days) was reduced to 6 or 3 days.

S4: Influence of deck storage of apples stored in refrigerated storage or CA: Apples stored in refrigerated storage and CA are stored first at ambient temperature for 1 day. This period is normally fixed at 1 day but was changed to 0, 2 or 3 days, respectively.

S5: Influence of refrigerated storage before CA is started and achieved: Before the apples are stored under CA, the apples are stored first for maximum 6 days at 1°C. This period was reduced to 3 days and also increased to 12 days.

S6: Influence of refrigerated storage after CA storage: Once a CA store is opened, the atmosphere is changed and the store should be emptied within 7 days. This period was reduced to 4 days and also increased to 12 days.

S7: Influence of deck storage at AJP: The time between delivery at the AJP and the production of AJ varies between 0 and 1 day. This period was changed to a fixed number of days (0, 2 or 4 days).

S8: Influence of sorting: Apples with a mould spot larger than 40, 25 or 10 cm<sup>2</sup> were removed from the batch and were not used for AJ production. A sorting efficiency of 95% was used.

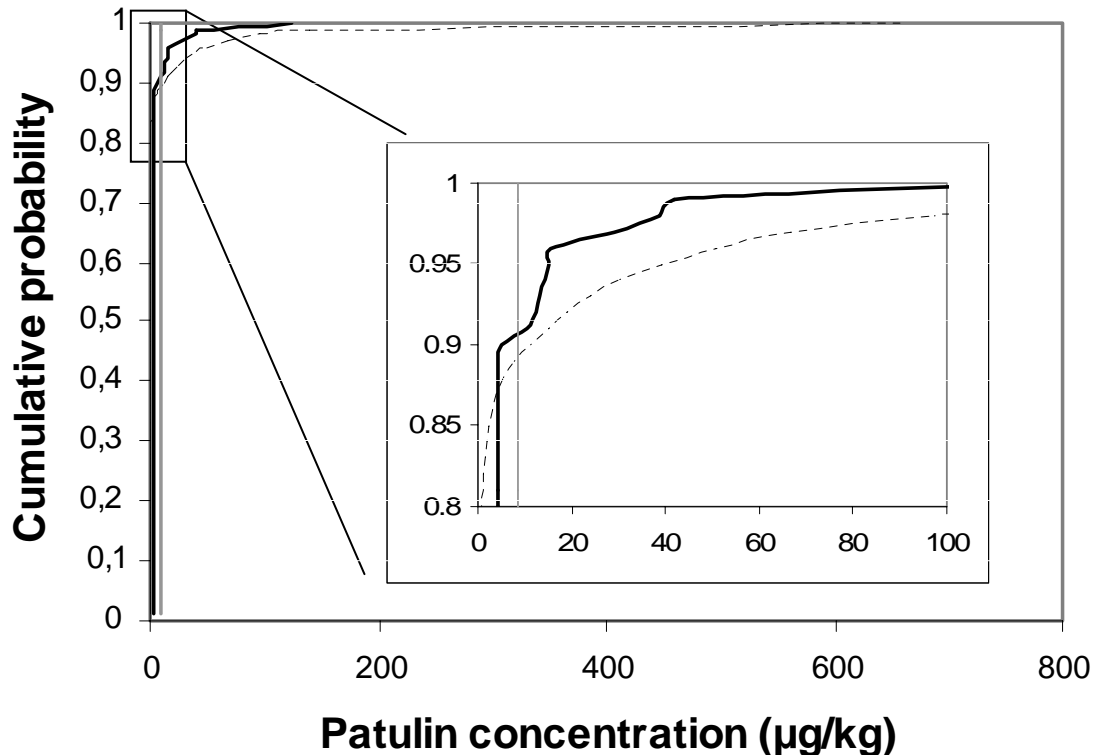
S9: Combination of S1 and S8: 50% fresh apples, 30% apples from refrigerated storage and 20% apples from CA are used and apples with a mould spot larger than 10 cm<sup>2</sup> were removed from the batch and were not used for AJ production. A sorting efficiency of 95% was used.

## **3. Results**

### **3.1. Validation of the baseline model**

The developed QRAM was first validated with patulin concentrations analysed in clear and cloudy AJ in Belgium as described in Chapter 3. Figures 8.4 and 8.5 show the comparison of the simulations and the analyses. For the samples with a concentration below the limit of detection (LOD), a concentration equal to LOD/2 was plotted in the graphs (4.3 µg/kg for cloudy AJ and 2.6 µg/kg for clear AJ). The percentage of cloudy AJ with a concentration above 8.6 µg/kg (the LOD of cloudy AJ) was 11% for the QRAM, which was similar to the

result for the chemical analysis (10%). However, for the higher percentiles, higher patulin concentrations were observed with the QRAM than for the chemical analysis. For clear AJ, the same trends could be determined as for cloudy AJ (Figure 8.5).

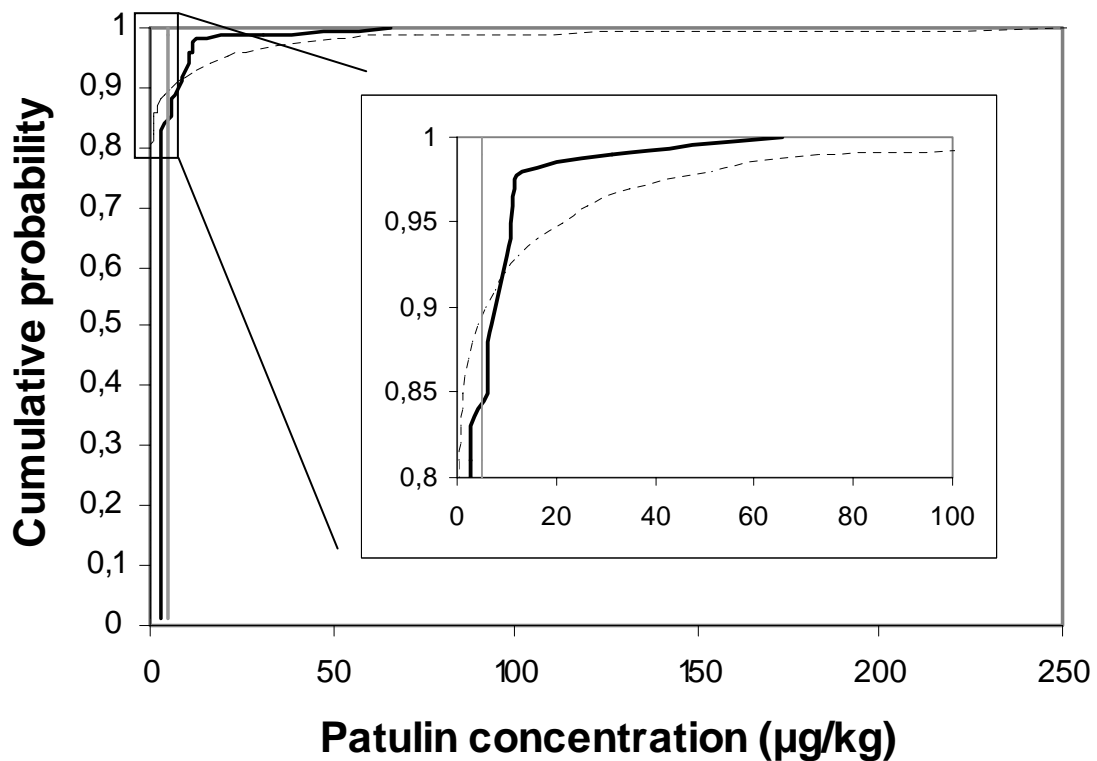


**Figure 8.4:** Distribution of the patulin concentration ( $\mu\text{g}/\text{kg}$ ) in cloudy apple juice determined through chemical analysis (full line) and calculated with the model (dashed line). LOD is indicated with grey line.

### 3.2. Contamination mitigation strategies

In order to evaluate the influence of the storage facility of apples on the patulin concentration, the contamination was simulated when only fresh, refrigerated storage or CA apples were used (S1). Cloudy AJ only made from fresh apples had a mean concentration of  $0.1 \mu\text{g}/\text{kg}$  and clear AJ had a concentration  $<0.1 \mu\text{g}/\text{kg}$  (Tables 8.4 and 8.5). Also AJ made from apples stored under refrigerated conditions had a lower concentration compared to the baseline model, while AJ made from CA apples contained more than two times the patulin concentration in regular juice (S0). None of the juices had a concentration above  $50 \mu\text{g}/\text{kg}$  when fresh and refrigerated storage apples were used and a very low percentage of cloudy juices had a concentration above  $25 \mu\text{g}/\text{kg}$  (0.1% and 0.3% for fresh and refrigerated storage

apples, respectively). A reduced use of CA apples (20% instead of 40% in the baseline model) resulted in a mitigation of the mean patulin concentration of almost 50%.



**Figure 8.5: Distribution of the patulin concentration ( $\mu\text{g}/\text{kg}$ ) in clear apple juice determined through chemical analysis (full line) and calculated with the model (dashed line). LOD is indicated with grey line**

A reduction of the wounds reduced the level of patulin in AJ (S2). A reduction of the wounds by 50% reduced the mean patulin concentration by about 38% (Tables 8.4 and 8.5). However, the influence on the high concentrations was rather limited and even a reduction of the wounds with 99% was not enough to avoid that some juices may contain more than 50  $\mu\text{g}/\text{kg}$  of patulin.

A reduction of the deck storage for fresh apples (S3) had no influence on the patulin concentration in clear and cloudy AJ in comparison to the baseline model (maximum 7 days) (Tables 8.4 and 8.5). Also the deck storage of apples that will be stored in refrigerated storage or in CA had a limited influence on the patulin concentration of AJ (S4).

**Table 8.4: Patulin contamination of cloudy apple juice when different mitigation strategies are applied**

Model	Description	Patulin concentration ( $\mu\text{g}/\text{kg}$ )						Percentage samples above		
		Mean	P25	P50	P75	P90	P95	8.6 $\mu\text{g}/\text{kg}$	25 $\mu\text{g}/\text{kg}$	50 $\mu\text{g}/\text{kg}$
S0	Baseline model	6.8	<0.1	<0.1	0.4	11.1	39.2	10.6	6.8	4.1
S1	100% fresh apples are used	0.1	0	0	<0.1	<0.1	0.4	0.3	0.1	0
	100% apples from refrigerated storage are used	1.3	<0.1	<0.1	0.4	4.0	8.0	4.5	0.3	0
	100% apples from CA <sup>a</sup> are used	15.8	0.2	0.3	5.5	52.4	94.4	23.4	16.2	10.5
	50% fresh, 30% refrigerated storage, 20% CA	3.7	0	<0.1	0.2	2.6	13.1	6.1	3.2	1.9
S2	Reduction of wounding with 10%	6.3	<0.1	<0.1	0.4	7.9	34.3	9.8	6.2	3.5
	Reduction of wounding with 50%	4.3	0	<0.1	0.2	5.4	23.0	8.6	4.7	2.5
	Reduction of wounding with 99%	1.8	0	<0.1	0.1	1.7	9.1	5.2	2.2	0.8
S3	Deck storage of fresh apples max 6 days	6.8	0	<0.1	0.4	8.7	37.4	10.0	6.4	3.9
	Deck storage of fresh apples max 3 days	6.7	0	<0.1	0.3	8.5	37.4	10.0	6.4	3.9
S4	Deck storage of CA and refrigerated storage: 0d	6.8	<0.1	<0.1	0.4	11.1	39.2	10.6	6.8	4.1
	Deck storage of CA and refrigerated storage: 2d	7.4	<0.1	<0.1	0.4	11.1	41.9	10.9	6.8	4.2
	Deck storage of CA and refrigerated storage: 3d	7.8	<0.1	<0.1	0.4	13.1	45.1	11.5	7.3	4.5
S5	Refrigerated storage before CA: max. 12 days	6.8	<0.1	<0.1	0.4	11.2	39.0	10.6	6.8	4.1
	Refrigerated storage before CA: max. 3 days	6.8	<0.1	<0.1	0.4	9.1	38.0	10.1	6.4	4.0
S6	Refrigerated storage after CA: max 12 days	7.1	<0.1	<0.1	0.4	9.1	38.0	10.2	6.5	4.0
	Refrigerated storage after CA: max 4 days	6.7	<0.1	<0.1	0.4	8.9	37.0	10.1	6.3	3.8

**Table 8.4: Patulin contamination of cloudy apple juice when different mitigation strategies are applied (Continued)**

Model	Description	Patulin concentration ( $\mu\text{g}/\text{kg}$ )						Percentage samples above		
		Mean	P25	P50	P75	P90	P95	8.6 $\mu\text{g}/\text{kg}$	25 $\mu\text{g}/\text{kg}$	50 $\mu\text{g}/\text{kg}$
S7	Deck storage at AJP <sup>b</sup> : 0 days	5.0	0	<0.1	0.3	7.8	31.4	9.6	5.8	0
	Deck storage at AJP: 2 days	9.7	<0.1	0.1	0.7	15.7	59.2	12.2	8.3	5.7
	Deck storage at AJP: 4 days	14.7	<0.1	0.2	1.1	25.1	87.7	14.2	10.0	7.2
S8	Mould spot larger than 40 $\text{cm}^2$ removed	3.9	<0.1	<0.1	0.3	7.4	25.6	9.5	5.1	2.0
	Mould spot larger than 25 $\text{cm}^2$ removed	2.7	<0.1	<0.1	0.2	5.7	16.9	8.1	3.2	1.2
	Mould spot larger than 10 $\text{cm}^2$ removed	1.1	<0.1	<0.1	<0.1	2.7	6.7	3.8	0.5	0.1
S9	50% fresh, 30% refrigerated storage, 20% CA and mould spots larger than 10 $\text{cm}^2$ removed	0.7	0	<0.1	<0.1	1.4	4.1	1.9	0.3	0.02

<sup>a</sup>CA: Controlled atmosphere; <sup>b</sup>AJP: apple juice producer

**Table 8.5: Patulin contamination of clear apple juice when different mitigation strategies are applied**

Model	Description	Patulin concentration ( $\mu\text{g}/\text{kg}$ )						Percentage samples above		
		Mean	P25	P50	P75	P90	P95	8.6 $\mu\text{g}/\text{kg}$	25 $\mu\text{g}/\text{kg}$	50 $\mu\text{g}/\text{kg}$
S0	Baseline model	3.6	<0.1	<0.1	0.2	5.8	21.3	10.3	4.2	2.2
S1	100% fresh apples are used	<0.1	0.0	0	<0.1	<0.1	0.2	0.5	0	0
	100% apples from refrigerated storage are used	0.7	<0.1	<0.1	0.2	2.1	4.0	3.4	0	0
	100% apples from CA <sup>a</sup> are used	8.3	<0.1	0.2	3	25.8	52.3	22.9	10.4	5.3
	50% fresh, 30% refrigerated storage, 20% CA	1.9	0	<0.1	<0.1	1.4	7.7	6.1	1.9	1.1
S2	Reduction of wounding with 10%	3.2	<0.1	<0.1	0.2	5.4	19.4	10.2	3.7	1.9
	Reduction of wounding with 50%	2.2	0	<0.1	0.1	3.4	13.1	8.7	2.7	0.9
	Reduction of wounding with 99%	0.9	0	<0.1	<0.1	1.0	5.0	4.8	0.6	0
S3	Deck storage of fresh apples max 6 days	3.4	0	<0.1	0.2	5.4	21.0	10.1	4.0	2.0
	Deck storage of fresh apples max 3 days	3.4	0	<0.1	0.2	5.2	21.0	10.0	4.0	2.0
S4	Deck storage of CA and refrigerated storage: 0d	3.6	<0.1	<0.1	0.2	5.8	21.3	10.3	4.2	2.2
	Deck storage of CA and refrigerated storage: 2d	3.8	<0.1	<0.1	0.2	7.1	23.2	10.9	4.5	2.4
	Deck storage of CA and refrigerated storage: 3d	4.0	<0.1	<0.1	0.4	13.1	45.1	11.3	5.1	2.5
S5	Refrigerated storage before CA: max. 12 days	3.6	<0.1	<0.1	0.2	5.8	21.4	10.3	4.2	2.2
	Refrigerated storage before CA: max. 3 days	3.5	<0.1	<0.1	0.2	5.8	21.1	10.3	4.2	2.2
S6	Refrigerated storage after CA: max 12 days	3.6	<0.1	<0.1	0.2	6.0	21.9	10.4	4.3	2.2
	Refrigerated storage after CA: max 4 days	3.4	<0.1	<0.1	0.2	5.8	20.4	10.3	3.9	2.1

**Table 8.5: Patulin contamination of clear apple juice when different mitigation strategies are applied (Continued)**

Model	Description	Patulin concentration ( $\mu\text{g}/\text{kg}$ )						Percentage samples above		
		Mean	P25	P50	P75	P90	P95	8.6 $\mu\text{g}/\text{kg}$	25 $\mu\text{g}/\text{kg}$	50 $\mu\text{g}/\text{kg}$
S7	Deck storage at AJP <sup>b</sup> : 0 days	2.6	0	<0.1	0.2	3.8	16.5	9.1	3.0	0
	Deck storage at AJP: 2 days	5.2	<0.1	<0.1	0.4	7.7	28.8	11.4	5.6	3.1
	Deck storage at AJP: 4 days	7.9	<0.1	<0.1	0.6	12.2	44.4	13.0	7.2	4.6
S8	Mould spot larger than 40 $\text{cm}^2$ removed	2;1	<0.1	<0.1	0.1	3.7	13.1	8.8	2.5	0.6
	Mould spot larger than 25 $\text{cm}^2$ removed	1.5	<0.1	<0.1	<0.1	3	8.8	7.4	1.6	0.2
	Mould spot larger than 10 $\text{cm}^2$ removed	0.6	<0.1	<0.1	<0.1	1.4	3.6	3.7	0.1	0
S9	50% fresh, 30% refrigerated storage, 20% CA and mould spots larger than 10 $\text{cm}^2$ removed	0.4	0	<0.1	<0.1	0.7	2.1	1.8	0.02	0

<sup>a</sup>CA: Controlled atmosphere; <sup>b</sup>AJP: apple juice producer



Before apples are placed under CA conditions, a short storage step at 1°C is used. When this period was made shorter or longer (S5) the patulin concentration was not influenced (3.6 µg/kg with 6 days and 3.5 µg/kg with 3 days for clear AJ). Also after the CA storage, a short storage at 1°C is applied. This step had hardly an effect on the patulin concentration as seen in Tables 8.4 and 8.5 (S6).

On the contrary, the time between delivery at the AJP and processing of the apples had a large influence on the patulin concentration (S7). Normally, this time varies between 0 to 1 day (baseline model) but an increase to 2 days resulted in a mean patulin concentration of 5.2 and 9.7 µg/kg in clear and cloudy AJ, respectively. When apples were processed immediately after delivery, the patulin concentration was reduced to 2.6 and 5 µg/kg in clear and cloudy AJ and the limit of 50 µg/kg was not exceeded anymore.

When apples are removed (S8) with a mould spot larger than 10 cm<sup>2</sup> and a sorting efficiency of 95%, the mean patulin concentration was reduced to 0.6 µg/kg for clear AJ and 0.1% of the samples exceeded 25 µg/kg. For cloudy AJ, the patulin concentration was reduced to 1.1 µg/kg (reduction of 84%) and 0.5% of the samples exceeded 25 µg/kg.

Based on the obtained results, an additional scenario combining S1 and S8 was evaluated (S9). This resulted in the use of less CA apples (20% instead of 40%) and a sorting step in which apples with a mould spot larger than 10 cm<sup>2</sup> were removed. For cloudy AJ, a mean concentration of 0.7 µg/kg was obtained compared to 6.8 µg/kg for the baseline model. The patulin concentration of the 95<sup>th</sup> percentile was reduced from 39.2 µg/kg to 4.1 µg/kg and the percentage exceeding 25 µg/kg was reduced from 6.8 to 0.3%. Also for clear AJ, a clear mitigation of the patulin contamination was observed (0.4 µg/kg for the mean patulin contamination compared to 3.6 µg/kg for the baseline model) and the percentage exceeding 25 µg/kg was reduced to 0.02%.

## 4. Discussion

Mycotoxin risk assessment has focused mainly on the chemical aspects of mycotoxins. Mycotoxin concentrations are measured in the product as it is consumed and an estimation of the intake is made (Kroes et al., 2002). The advantage of this methodology is that a clear estimation of the intake is obtained, however, it is not possible to evaluate different strategies

to reduce the mycotoxin formation and concentration. In order to do this, the microbiological history of the product needs to be taken into account together with the influence of processing on the mycotoxin concentration. Therefore, the present study described the transmission of the mould *P. expansum* and the mycotoxin patulin through the whole chain from the harvest of the apples to the consumption of the AJ.

In this study, the picking of the apples was chosen as the first step of the QRAM, since *Penicillium* spores are seldom found on the apple surface during the pre-harvest period (Amiri & Bompeix, 2005). The picking of the apples has a large influence on the infection of apples, since careless handling can lead to bruises, stem end injuries or finger-nail scratches (Doores, 1983; European Commission, 2003a; Janisiewics & Peterson, 2004). The influence of apple handling on the patulin contamination of AJ was included via the probability of infection (Amiri & Bompeix, 2005). In this probability, wounds and lenticels were included. However, infection can also occur via an open calyx (Archer, 2002) or via stems (Rosenberger et al., 2001). The latter two infection routes were not taken into account in the QRAM, since no data were available on the extent of their occurrence during the storage of apples.

Growth of *P. expansum* was included in the model using the percentage of diseased apples as described by Amiri and Bompeix (2005) and models for the growth rate and lag phase (Chapter 7). However, the percentage of diseased apples was only available for refrigerated storage until 150 days of storage, while in the present study, longer storage periods and CA storage were used. It was assumed that the increase of the percentage diseased apples between 120 and 150 days, was the same as after 150 days. Since it was observed in Chapter 7 that the lag phase and growth rate of apples under CA was not different from air, the data for refrigerated storage were also used for CA storage. Strain variability of both growth and patulin production was included in the model. Delignette-Muller and Rosso (2000) observed that the biological variability has a great impact on the accuracy of the result of a quantitative risk assessment and should not be systematically neglected.

Patulin production was included in the QRAM using linear regression for some specific temperature – O<sub>2</sub> combinations, since a model that predicts patulin production as a function of the mould surface, temperature and % O<sub>2</sub> is not available. The availability of models predicting mycotoxin formation is still very limited and to our knowledge, models are only available for aflatoxin production (Pitt, 1993; Molina & Giannuzzi, 2002) and fumonisin B<sub>1</sub>

by *Fusarium moniliforme* and *F. proliferatum* (Marin et al., 1999). Availability of these models is of great importance for the development of farm to fork QRAM for mycotoxins and is one of the new challenges in predictive mycology. Also temperature cycling has an influence on patulin production (Lin et al., 1981) however, it was not possible to include this in the model.

The validation of the QRAM showed that realistic concentrations of patulin in AJ are obtained, although an overestimation of the higher concentrations needs to be taken into account. The QRAM gives concentrations which are 2.5 and 2.0 times higher than the results of the chemical analysis for cloudy and clear AJ respectively (based on the 95<sup>th</sup> percentile). For the lower percentiles, the QRAM predictions are within the 0-LOD range, which is the expected range based on the observed data. It can also be assumed that some of the AJs do not contain patulin and the QRAM estimated this to be around 20%.

Previous research has shown that AJ in Belgium sometimes contains more than 50 µg/kg (Chapter 3). Moreover, the current maximum limit of 50 µg/kg does not protect children for exceeding the TDI (Chapter 4), while a reduction of the contamination below 25 µg/kg gives a significant reduction of the percentage of children exceeding the TDI. Therefore, the present study aimed to evaluate different strategies for contamination mitigation. From the data, it can be observed that the use of CA apples contributes to a large extent to the patulin contamination of AJ. This phenomenon is confirmed by earlier observation of juice producers in the UK who saw dramatic rises of the patulin content in juice produced in June, July and August – the months prior to the new harvest season where apples have been stored for almost a year (Moake et al., 2005). The present study shows that a reduction of the use of CA apples from 40 to 20% reduces the mean patulin concentration in AJ with about 50%. These results indicate that the storage of apples should be as short as possible, since growth and patulin production by *P. expansum* is still possible during the applied storage conditions (Rosenberger et al., 2001; Chapter 5). An increase of the AJ production in the months September to December is not always possible from an economical point of view, taking the production capacity into consideration. However, these data show that AJ with high patulin concentrations can be produced from January onwards and it would be useful to take this into account when sampling plans are made by AJP in the framework of their HACCP-system. Also the government and control agencies should take this into consideration when monitoring programs are elaborated.

During harvest and transportation, the apples are prone to physical damage. Wounds are an important infection route for *P. expansum* and a reduction of the wounds of apples reduces the patulin concentration in apples. However, the effect of a reduction of the wounds by for example 10% was rather limited. A higher reduction had more effect, but is very difficult to achieve in practice. The limited effect of a reduction of the wounds is a consequence of the fact that wounds are only one of the four infection routes of *P. expansum*. Lenticels are an important infection route, especially late in the storage season, when apples have been weakened by ripening and aging (Amiri & Bompeix, 2005). Sorting on the other hand, was more effective to reduce the patulin concentration of AJ, because sorting enables not only the removal of apples that are infected via wounds but also via other routes. When the apples are removed which contain an infected spot larger than 25 cm<sup>2</sup>, 1.2% of the apples are removed on average. In this way, the percentage of juices that exceed 25 µg/kg is reduced from 6.8 to 3.2% for cloudy AJ and from 4.2 to 1.6% for clear AJ. A removal of the apples with an infected spot larger than 10 cm<sup>2</sup> (on average 2.1% of the apples) reduces this even further to 0.5 and 0.1%, respectively. Since the results showed that the use of CA apples and sorting were two critical factors to reduce the patulin contamination, a combination of both scenarios was evaluated. The use of only 20% CA apples in combination with a removal of the apples with a mould spot larger than 10 cm<sup>2</sup> resulted in a patulin concentration which was on average 89% lower compared to the baseline model and that exceeded 25 µg/kg in 0.3-0.02% of the cases. Taking the overestimation that is made for the higher concentrations into account, it can be concluded that reduced use of CA apples in combination with a sorting step will enable AJP to reduce the patulin concentration below 25 µg/kg. However, it should be mentioned that during sorting, it is not possible to distinguish between apples infected with *P. expansum* and apples infected with another mould, so in practice more apples will need to be removed. The effect of removing rotten and damaged apples has also been described by Sydenham et al. (1997) and Leggott et al. (2000), who observed reductions of the patulin concentration of 83 to 90%. Also trimming has been proven to be an efficient measure to mitigate the patulin concentration since reductions of 93 to 99% are obtained (Lovett et al., 1975). However, trimming is a very expensive and labour intensive method which makes it inapplicable in the AJ producing industry. Moreover, patulin is able to diffuse 1 to 2 cm from the rotten core in apples (Taniwaki et al., 1992; Rychlik & Schieberle, 2001; Marin et al., 2006a), which indicates that more than the rotten spot needs to be removed.

A reduction of deck storage for fresh apples had no effect on the patulin contamination, although it is recommended to keep this period as short as possible (European Commission, 2003a). Also for apples that are stored under CA and refrigerated storage, the deck storage had a limited influence. A reduction had no effect. The time before the CA conditions are achieved and after the CA store is opened again, had no influence on the patulin concentration. This is in contradiction with the recommendations of the European Commission to keep this period as short as possible (European Commission, 2003a).

It is recommended to keep the time between removing the apples from the cold storage room and the processing as short as possible and ideally <24 hours (European Commission, 2003a). This was also observed in the present study where immediate processing of the apples resulted in 0% of the juices exceeding 50 µg/kg and a reduction of the percentage of juices exceeding 25 µg/kg with 15 and 30% for cloudy and clear AJ, respectively.

The observed data indicate that further refining of the model is necessary in order to decrease the overestimations at higher concentrations. Therefore, more accurate data are necessary, including data on organic and conventional production methods, since previous research has shown that higher patulin concentrations are observed in organic AJ compared to conventional (Chapter 3). Availability of data is a general problem within farm to fork risk assessment and has been pointed out by other researchers (Notermans & Batt, 1998; Anderson et al., 2001; Hartnett et al., 2001; Bemrah et al., 2002; Duffy & Schaffner, 2002; Lindqvist et al., 2002; Oscar et al., 2004; Uyttendaele et al., 2006).

## 5. Conclusion

The validation of the elaborated model showed that similar results were obtained from the QRAM and the analysis, although an overestimation at high concentrations was observed. From the presented results it can be concluded, that fresh and refrigerated storage apples should be used as much as possible for the manufacture of AJ. When CA apples are used, a sorting step should be included to remove apples with an infection spot larger than 10 cm<sup>2</sup>. In this way, the patulin contamination will be below 25 µg/kg in almost all AJs, which is necessary to protect young children. Another critical factor is the duration of the deck storage before the apples are processed. This point should be taken into account when a HACCP plan for the AJ processing industry is made.



## Conclusions and perspectives

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## Conclusions and perspectives

From the literature review presented in Chapter 1, it is clear that there is a need for risk assessments of the hazards that occur in the food chain. Patulin is one of these hazards and although the toxicological characteristics are not as clear as for other mycotoxins (e.g. aflatoxins) due to the reaction with proteins and glutathione (Fliege & Metzler, 1999b), research has shown that the exposure should be limited (Alves et al., 2000; Liu et al., 2003; Schumacher et al., 2003). The main aim of the present study was to evaluate the risk of patulin in apple juice and if necessary to formulate recommendations to reduce this risk.

### **Are children consuming apple juice at risk?**

An analytical method for patulin detection in apple juice using HPLC-UV was implemented and validated. A time dependent decrease of the patulin recovery was observed in cloudy apple juice (Chapter 2). This effect was caused by an interaction of patulin with the solid particles of cloudy apple juice and most probably with the proteins of these solid particles. It was observed that the fraction of patulin that interacts is not detected using HPLC-UV leading to an underestimation of about 20%. If this 20% needs to be taken into account, depends on whether the bound patulin can be released in the gastro-intestinal tract. Further research in this respect is necessary. However, based on the precautionary principle it is advisable to include this bound fraction in the reported results until further scientific information is available.

Since apple juice and apple nectar are the main sources of patulin intake in most countries, the validated method (Chapter 2) was used to study the occurrence of patulin in apple juice marketed in Belgium (Chapter 3). Although, the incidence of patulin in organic apple juice (12%), conventional apple juice (13%) and handcrafted apple juice (10%) was not significantly different, the mean level of patulin in contaminated samples was significantly higher in organic apple juice (43.1  $\mu\text{g}/\text{kg}$ ) than in conventional apple juice (10.2  $\mu\text{g}/\text{kg}$ ) and in handcrafted apple juice (10.5  $\mu\text{g}/\text{kg}$ ). From these results, it could be concluded that patulin did not occur more frequently in organic apple juice compared to other apple juices, but when it occurred, higher concentrations were detected. Whether these higher concentrations in organic apple juice are caused by the reduced use of pesticides or other differences in the

production method is a matter of debate and the present work has not investigated this. However it is clear that the organic industry should take measures to reduce these high contamination levels since it is possible to produce apple juice with patulin concentrations below the maximum limit, as was observed for conventional apple juice. The study has also shown that people buying cheaper apple juice e.g. in discounters, are not more exposed to higher concentrations. On the contrary a positive correlation exists between the price of apple juice and the patulin concentration, mainly due to the high price of organic apple juice.

A probabilistic exposure assessment was performed to link the consumption of apple juice by preschool children in Flanders with the observed concentration of patulin in apple juices (Chapter 4). It was observed that 0.9% [90% confidence interval: 0.3 and 1.8%] of the children consuming organic apple juice exceed the TDI. For consumers of conventional and handcrafted apple juice, this was respectively 0.1% [90% CI: 0-0.3%] and 0% [90% CI: 0-0.2%]. The study also showed that the current maximum limit of 50 µg/kg is not sufficient to protect children from exceeding the TDI. A decrease of the contamination to levels below 25 µg/kg reduced the percentage of children consuming organic apple juice that exceed the TDI, to 0% [90% CI: 0-0.2%]. A reduction of the apple juice consumption to 200 ml on the other hand reduced this to 0.6% [90% CI: 0.1-1.4%], indicating that a reduction of the apple juice consumption is less effective than a reduction of the patulin concentration in apple juice.

## **Development of a farm to fork risk assessment model**

Based on the observed exposures it can be concluded that a decrease of the contamination level from 50 to 25 µg/kg is important to protect the health of preschool children. However, before the maximum level of a contaminant in legislation can be reduced, it is also necessary to provide tools for apple juice producers to reduce the patulin contamination of apple juice. Therefore, a farm to fork risk assessment was elaborated. This technique includes the microbiological history of the products and allows the evaluation of different measures including measures that affect the growth and mycotoxin production of the mould. Germination, growth and patulin production of *Penicillium expansum* were studied in order to develop the quantitative farm to fork risk assessment model.

The inoculation level has a profound effect on the lag phase of *P. expansum* (Chapter 6). The use of low inoculation levels resulted in longer lag phases (up to 81% longer) and a larger

variability of the lag phase, compared to high inoculation levels. The growth rate however was not significantly influenced by the inoculation level. Temperature had a large influence on the growth parameters of *P. expansum* with the optimum temperature for growth being between 24 and 27°C (Chapters 5 and 7). The used oxygen level (20%, 3% and 1% O<sub>2</sub>) on the other hand had no influence on the lag phase and the growth rate. In order to predict the influence of storage conditions and strain variability on the growth of *P. expansum*, predictive models were developed and validated (Chapter 7). The obtained models described the growth rate as a function of the storage temperature adequately, but the models predicted shorter lag phases than these observed in apples. These data indicate that further research concerning modelling the lag phase of *P. expansum* in real food products is necessary. Until now, a large number of models have been developed and are used to predict the lag phase of a mould without a proper validation. The present research showed that models which were not validated in the real food matrix should be used with caution, since underestimations of the lag phase are possible. When these models are used for example for estimating the shelf life of a product, clear underestimations can be obtained.

The influence of the extrinsic factors, temperature and oxygen level, on the patulin production by *P. expansum* is clearly strain dependent (Chapter 5). However in general, it can be stated that intermediate stress levels stimulate patulin production, while increasing the stress (lowering the temperature to 1°C and the O<sub>2</sub> level to 1% O<sub>2</sub>) reduces the patulin production. The reduction of patulin production at 1°C was even more pronounced in apples than in simulation medium suggesting that apple as a food matrix is an additional stress factor. From these results it can be concluded that the conditions applied in practice for the storage of apples, allow growth and patulin production. Based on this information it is important that the starting quality of the apples is as good as possible in order to avoid growth and patulin production during storage.

The developed growth models and the data describing patulin production as a function of growth, strain, temperature and O<sub>2</sub> level were combined in a farm to fork risk assessment. The risk assessment model described the complete chain starting from picking apples until storage of the apple juice. The validation of the elaborated model showed that the obtained results were similar compared to the concentration of patulin observed in apple juice in Belgium, although an overestimation at the high concentrations was observed (95<sup>th</sup> percentile).

## Recommendations to reduce the risk

Different mitigation strategies were evaluated and it was observed that the storage conditions of apples had a large influence on the concentration of patulin in apple juice but also on the prevalence of contaminated juice. This is probably the reason why no difference is observed in the prevalence of patulin in organic, conventional and handcrafted apple juice (Chapter 3), since apples of the three storage types (fresh, refrigerated and controlled atmosphere) are used for the three production methods. It is advisable to reduce the use of apples stored in controlled atmosphere for apple juice production, and when they are used, a sorting step should be included to remove apples with an infection spot larger than 10 cm<sup>2</sup>. In this way the patulin contamination will be below 25 µg/kg in almost all apple juices, which was proven to be necessary to protect young children. Another critical factor is the duration of the deck storage before the apples are processed. This point should be taken into account when a HACCP plan for the apple juice processing industry is made. The farm to fork risk assessment confirmed also the concern formulated in Chapter 5 that controlled atmosphere storage does not avoid patulin production. On the contrary, controlled atmosphere showed to be responsible for high concentrations found in apple juice.

## Recommendations to risk assessment of mycotoxins

In the present work, two different approaches have been used to assess the risk of a mycotoxin. The first approach (Chapter 4) started from the concentration of patulin in apple juice and determined the exposure of preschool children in Flanders. The second approach (Chapter 8) took the complete chain starting from the picking of the apples into account. Which approach is more appropriate to use depends on the overall goal of the study. If the aim is to estimate the actual risk of the population or a population group, it is advisable to select the starting point of the risk assessment as close as possible to the consumption (approach 1) to reduce the uncertainty. Indeed, every step included in the risk assessment leads to the incorporation of uncertainty. However, when the aim is to test different strategies in order to reduce the risk it is advisable to use a farm to fork risk assessment which incorporates the complete chain. This will enable the risk assessor to compare different risk mitigation strategies and to evaluate the relative reduction of the risk.

## Perspectives

The present study gives indirect proof that patulin binds to proteins present in apple juice. In order to gain direct proof further research using LC-MS is necessary to determine the patulin-protein adducts in apple juice. It should also be investigated whether the bound patulin is released in the gastro-intestinal tract, for example as a result of the action of pepsin. It would also be interesting to know the influence of drinking contaminated apple juice in combination with a snack (e.g. a biscuit) that contains proteins. A possibility to investigate this further would be the experimental design used by Rychlik et al. (2004) to study the absorption of patulin from the rat stomach.

This doctoral dissertation focused on the exposure of children, since this group is known as having the highest intakes. However, it is also necessary to consider other population groups in order to evaluate their risk as well.

The presented work suggests further research concerning the modelling of the lag phase of *P. expansum*. The approach used by Samapundo et al. (2006) to study the individual lag phase of *Aspergillus flavus* and *Fusarium verticillioides* would be useful in this respect. However, it is crucial that apples are used as a growth medium instead of simulation or synthetic medium.

The conducted farm to fork risk assessment enabled the evaluation of different risk management options, but is still limited in its possibilities. A clear need exists for a model that predicts the patulin production as a function of temperature and atmosphere composition. This would allow testing the effect of deviating temperatures and oxygen levels during the storage of apples on patulin production and the final concentration in juice. However, patulin production is characterized by a large variability which hampers the development of a model. Furthermore, the model should be further elaborated in order to determine the uncertainty of the obtained results, especially the model uncertainty.



## List of abbreviations

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## List of abbreviations

ADI	Acceptable daily intake
$A_f$	Accuracy factor
AJ	Apple juice
AJP	Apple juice producer
ALARA	As low as reasonably achievable
ALOP	Appropriate level of protection
ALR	Acceptable level of risk
APAM	Apple puree agar medium
$a_w$	Water activity
$B_f$	Bias factor
BMD	Benchmark dose
BMDL	Benchmark dose lower confidence limit
bw	Body weight
C	Conventional
CA	Controlled atmosphere
CAC	Codex Alimentarius Commission
CFU	Colony forming unit
CI	Confidence interval
CM	Control measure
CRA	Chemical risk assessment
cv	Cultivar
$DUR_{gr}$	Growth duration
$DUR_{stor}$	Storage duration
EDR	Estimated diet record
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FFQ	Food-frequency questionnaire
FSO	Food safety objective
GATT	General agreement on tariffs and trade
$G_{st}$	Day when growth starts

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H	Handcrafted
HACCP	Hazard analysis critical control point
HFS	Health food shop
HMF	5-(hydroxymethyl) furfural
HPLC-UV	High performance liquid chromatography-ultra violet
IARC	International Agency for Research on Cancer
ICMSF	International Commission on Microbiological Specifications for Foods
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid chromatography – mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MC	Microbiological criterion
MEA	Malt extract agar
ML	Maximum limit
MOE	Margin of exposure
MPRM	Modular process risk model
MRA	Microbiological risk assessment
MRL	Maximum residue level
MSE	Mean square error
n	Number of samples or repetitions
n.d.	not determined
NOAEL	No observed adverse effect level
NRC	National Research Council
O	Organic
Pat	Patulin concentration
PC	Performance criterion
$P_{inf}$	Probability of infection
PO	Performance objective
PRP	Prerequisite program
QRA	Quantitative risk assessment
QRAM	Quantitative risk assessment model
$R^2$	Determination coefficient
$R^2_{adj}$	Adjusted correlation coefficient
$RfD_{acute}$	Acute reference dose

RH	Relative humidity
RMSE	Root mean square error
S	Surface
SCOOP	Scientific cooperation
SD	Standard deviation
SEP	Standard error of prediction
SPS	Sanitary and phytosanitary measures
T25	Carcinogenic potency index
TD50	Tumorigenic dose rate
TDI	Tolerable daily intake
TLR	Tolerable level of risk
TTC	Threshold of toxicological concern
TWI	Tolerable weekly intake
WHO	World Health Organization
WTO	World Trade Organization
$\lambda$	Lag phase
$\lambda_{\text{actual}}$	Actual lag phase
$\lambda_{\text{pred}}$	Predicted lag phase
$\mu_{\text{max}}$	Growth rate



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# Curriculum vitae

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## Curriculum vitae

Katleen Baert werd geboren op 5 oktober 1978 te Sint-Niklaas en behaalde in 2001 het diploma van bio-ingenieur scheikunde aan de Universiteit Gent. Van augustus tot en met oktober 2001 was zij werkzaam als assistente aan de Vakgroep Farmaceutische Analyse (Faculteit Farmaceutische Wetenschappen, Universiteit Gent) binnen het vakgebied analytische chemie. Sinds november 2001 is zij aangesteld als assistente in het vakgebied kwaliteitszorg en risicoanalyse aan de Vakgroep Voedselveiligheid en Voedselkwaliteit (Faculteit Bio-ingenieurswetenschappen, Universiteit Gent). Tijdens deze periode werkte zij aan dit doctoraat onder leiding van haar promotoren Prof. dr. ir. Bruno De Meulenaer en Prof. dr. ir. Frank Devlieghere. In april 2005 behaalde ze het getuigschrift van de doctoraatsopleiding in de toegepaste biologische wetenschappen aan de Universiteit Gent. Zij was verantwoordelijk voor het uitwerken, organiseren en begeleiden van de practica 'Kwaliteitszorg en risicoanalyse in de agro- en voedingsindustrie' in het kader van de opleiding 'Bio-ingenieur' en 'Food microbiology and analysis' in de opleiding 'Master in Food Science and Technology'. Daarnaast verzorgde zij practica in de opleidingsonderdelen 'Levensmiddelenchemie', 'Food chemistry and analysis', 'Food chemistry', 'Levensmiddelenmicrobiologie- en conservering', 'Food microbiology', 'Technology of fishery products', 'Fisheries and fishery products' en 'Meat and meat products'. Zij heeft ook als lesgever deelgenomen aan verschillende intensieve programma's (Erasmus), Tempusprojecten en opleidingen georganiseerd door de overheid en het Instituut voor Permanente Vorming (IVPV). Zij heeft ingestaan voor de begeleiding van 8 thesisstudenten. Verder nam zij deel aan de dienstverlening van de vakgroep door het uitvoeren van analyses en literatuurstudies voor industriële partners en door het meewerken aan adviezen van de Hoge Gezondheidsraad met betrekking tot risicobeoordeling.

## List of publications

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## **Conferences**

### **Conferences with oral presentation**

De Meulenaer, B., Lanckriet, H., Baert, K., Devlieghere, F., Huyghebaert, A. Development of an immunological method to assess bisphenol A migration, 1<sup>st</sup> EPIFO symposium, Ghent, Belgium, September 24-27, 2000.

De Meulenaer, B., Lanckriet, H., Baert, K., Devlieghere, F., Huyghebaert, A. Development of an immunological method to assess bisphenol A migration from food packaging materials, 6<sup>th</sup> FLTBW PhD symposium, Ghent, Belgium, October 11, 2000.

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Baert, K., De Meulenaer, B., Devlieghere, F., Debevere, J. Mycotoxins: current and future perspectives, Ninth Conference on Food Microbiology, Merelbeke, Belgium, September 16-17, 2004.

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Devlieghere, F., Samapundo, S., Baert, K., De Meulenaer, B., Debevere, J. Effect of atmosphere on growth and mycotoxin production of moulds, Journées du réseau de mycologie, Société Française de Microbiologie, Marseilles, France, February 2-3, 2005.

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Uyttendaele, M., Baert, K., Grijspeerdt, K., De Zutter, L., Devlieghere, F. Comparing the effect of imposing various microbiological criteria for Salmonella in poultry based meat preparations on the probability of infection, FoodMicro 2006, Bologna, Italy, August 29 – September 2, 2006.

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children in Flanders, Workshop Sci Com, Toepassing van risico-evaluatie in de voedselketen, Brussels, Belgium, October 20, 2006.

Baert, K., De Meulenaer, B., Amiri, A., Debevere, J., Devlieghere, F. A farm to fork model for risk assessment of patulin in apple juice. XIIth International IUPAC Symposium on Mycotoxins and Phycotoxins, Istanbul, Turkey, May 21-25, 2007 (accepted for oral presentation).

### **Conferences with poster presentation**

De Meulenaer, B., Lanckriet, H., Baert, K., Huyghebaert, A. Development of an immunological method to assess bisphenol A migration, 2<sup>nd</sup> International Symposium on Food Packaging: ensuring the safety and quality of foods, ILSI, Vienna, Austria, November 8-10, 2000.

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Mamiro, P.R.S., De Meulenaer, B., Van Camp, J., Baert, K., Devlieghere, F., Meghji, W., Opsomer, A., Kolsteren, P., Huyghebaert, A. Exposure of fumonisins in finger millet, kidney beans and peanuts, which are ingredients used for complementary food in Tanzania, 2<sup>nd</sup> World Mycotoxin Forum, Noordwijk, the Netherlands, February 17-18, 2003.

Mamiro, P.R.S., De Meulenaer, B., Van Camp, J., Baert, K., Devlieghere, F., Meghji, W., Opsomer, A., Kolsteren, P. Exposure study of fumonisins in a Tanzanian complementary food based on finger millet, kidney beans and peanuts, EURO FOOD CHEM XII: strategies for safe food, Bruges, Belgium, September 24-26, 2003.

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Baert, K., Kasase, C., De Meulenaer, B., Huyghebaert, A. Incidence of patulin in organic and conventional apple juices marketed in Belgium, 9<sup>th</sup> PhD Symposium on Applied Biological Sciences, Leuven, Belgium, October 16, 2003.

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Baert, K., Kamala, A., De Meulenaer, B., Huybrechts, I., De Henauw, S., Devlieghere, F. Exposure assessment of patulin in apple juice for Flemish young children, The third international symposium on applications of modelling as an innovative technology in the agri-food chain, Leuven, Belgium, May 29 – June 2, 2005.

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