**Universidade do Minho**
Escola de Engenharia

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dezembro 2021

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# AFM1 - Aflatoxin M1 in dairy products: causes and strategies to overcome it

Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho realizado sob a orientação do
**Doutor Armando Albino Dias Venâncio**
e da
**Doutora Paula Cristina Azevedo Rodrigues**

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## STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

## AFM1 - Aflatoxina M1 em laticínios: causas e estratégias de mitigação

## RESUMO

As micotoxinas são compostos tóxicos presentes em muitos produtos alimentares. A sua presença e persistência durante o processamento de alimentos representa um desafio para a saúde humana, animal e ambiental. Na cadeia alimentar, a micotoxina original pode transformar-se em outros compostos tóxicos, chegando ao consumidor. Um bom exemplo é a ocorrência de aflatoxina M1 (AFM1) em laticínios, que se deve à presença da aflatoxina B1 na alimentação animal. Este projeto focou-se na avaliação das principais tendências de ocorrência de AFM1, utilizando os dados científicos disponíveis para avaliação de risco e identificação de lacunas existentes com o intuito de gerar novos dados.

Uma revisão sistemática da literatura foi realizada para estudar a possível relação entre a concentração de AFM1 e a década de amostragem, continente, tipo de produto lácteo e espécie animal. Através da metodologia PRISMA, foram identificados 4922 artigos e 329 foram selecionados, incluindo 110 mil dados de ocorrência nas últimas três décadas. Este estudo revelou que (i) o leite apresentou a menor concentração de AFM1, enquanto a manteiga e o queijo apresentaram as maiores; (ii) a ocorrência da AFM1 apresentou uma tendência crescente entre 1990-2019 e (iii) o continente Africano apresentou os níveis mais elevados de AFM1, confirmando as preocupações respeitantes à Segurança Alimentar (*sensu lato*) na região. Foi também realizada, uma avaliação da exposição mundial à AFM1 pela ingestão de leite e iogurte, queijo e manteiga. A Europa e a América Latina apresentaram maior risco que a África e a Ásia, justificado pela maior ingestão do segundo produto lácteo mais consumido – queijo. Grande parte da literatura considera apenas leite na avaliação da exposição, e este estudo evidencia o risco de subestimar o consumo de outros laticínios.

Para avaliar o destino da AFM1 no queijo ao longo do processo produtivo, foram realizadas quatro produções de queijos, utilizando leites de ovelha e de cabra contaminados artificialmente com AFM1. Os resultados mostraram que (i) a coalhada apresentou sempre uma maior percentagem e concentração de AFM1 em relação ao soro de leite (ii) observaram-se concentrações mais baixas de AFM1 no soro de leite de ovelha do que no de cabra e (iii) na fase de maturação foram observadas mudanças significativas em base húmida, mas não em base seca, o que pode estar relacionado com o teor de humidade.

Considerando que (i) o queijo é o segundo tipo de laticínio mais consumido; (ii) o fabrico de queijo concentra a toxina e (iii) há tendência de aumento de AFM1 nos laticínios, o controlo deste perigo é necessário para garantir a segurança do consumidor. Por sua vez, este trabalho levanta a questão da necessidade de adotar limites legislativos em queijo como medida preventiva do risco contra a AFM1.

**PALAVRAS-CHAVE:** Aflatoxinas, Avaliação de Risco, Produtos lácteos, Segurança Alimentar

## AFM1 - Aflatoxin M1 in dairy products: causes and strategies to overcome it

## ABSTRACT

Mycotoxins are toxic compounds present in many food commodities. Their ubiquitous presence and their persistence during food processing represent a challenge to the health of humans, animals and the environment. In the food chain, the original mycotoxin may be transformed in other toxic compounds, reaching the consumer. A good example is the occurrence of aflatoxin M1 (AFM1) in dairy products, which is due to the presence of aflatoxin B1 in animal feed. This project focused on evaluating the principal trends of AFM1 occurrence, making the best use of the available scientific data for risk assessment and identifying the lack of knowledge as a driver to generate new data.

A systematic literature search was performed to study the possible relation between AFM1 concentration and sampling decade, continent, type of dairy product, and animal species. Using the PRISMA methodology, 4922 papers were identified, and 329 were selected for further study, including 110 000 AFM1 occurrence data from the last three decades. This study revealed that (i) milk presented the lowest AFM1 concentration, while butter and cheese the highest ones; (ii) the concentration of AFM1 showed an increasing trend between 1990-2019 and (iii) the African continent was the one with the highest level of AFM1, confirming concerns on food safety and food security in the region. An AFM1 exposure assessment through the consumption of dairy products (milk & yogurt, cheese and butter) was also performed. Europe and America pose a greater risk than Africa and Asia, explained by the higher intake of the second most consumed dairy product, cheese. Most of the literature considers only milk in AFM1 assessment, and this finding demonstrates the risk of underestimating the consumption of other dairy products.

To evaluate the fate of AFM1 concentration in cheese throughout the production process, four productions were carried out, using spiked sheep and goat milks. Results showed that (i) a higher AFM1 percentage and concentration were always observed in curd than in whey samples; (ii) lower AFM1 concentrations were observed in sheep whey than in goat whey and (iii) regarding the ripening stage, significant changes were observed on a wet basis but not on a dry basis, which can be related to the moisture content.

Therefore, considering that (i) cheese is the second most consumed dairy product; (ii) cheese-making concentrates the toxin and (iii) there is a trend for AFM1 increase in dairy products, the control of this hazard is a necessity to ensure consumer's safety. In turn, this finding leads to questioning whether the adoption of legislative limits for cheese could be the approach to minimize the risk against AFM1.

**KEYWORDS:** Aflatoxins, Dairy products, Food Safety, Risk Assessment

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AFs – Aflatoxins

AFB1 – Aflatoxin B1

AFB2 – Aflatoxin B2

AFG1 – Aflatoxin G1

AFG2 – Aflatoxin G2

AFM1 – Aflatoxin M1

ANIL – Associação Nacional dos Industriais de Lacticínios

AOAC – Association of Official Analytical Chemists

$a_w$ – water activity

bw – body weight

CEB – Centre of Biological Engineering

DALYs – Disability-adjusted life years

DNA – Deoxyribonucleic acid

EDI – Estimated Daily Intake

EF – Enrichment Factor

EFSA – European Food Safety Authority

ELISA – Enzyme-linked immunosorbent assay

EU – European Union

FAO – Food and Agriculture Organization

FDA – Food and Drug Administration

FDB – Fat on dry basis

FLD – Fluorescence Detection

FS – Food Safety

FSO – Food Safety Objective

HBV – Hepatitis B virus

HCC – Hepatocellular carcinoma

HI – Hazard Index

HPLC-FLD – High performance liquid chromatography coupled with fluorescent detection

IARC – International Agency for Research on Cancer

INE – Instituto Nacional de Estatística

IR – Ingestion rate per capita per day

JECFA – Joint FAO/WHO Expert Committee on Food Additives

LAB – Lactic acid bacteria

$LD_{50}$ – Dose of any substance tested required to kill half the number (50%) of test animals

LC – Liquid chromatography

LC-MS – Liquid chromatography coupled with mass spectrometry

LOD – Limit of detection

LOQ – Limit of quantification

MFFB – Moisture on fat – free basis

MIP – Molecularly Imprinted Polymers

MPLs – Maximum permissible limits

MSPE – Magnetic-solid phase extraction

MycosepTM – Multifunctional clean-up columns

OECD – Organisation for Economic Co-operation and Development

OTA – Ochratoxin A

($\overline{P_{cancer}}$) – Cancer potency

PRISMA – Preferred Reporting Items for Systematic reviews and Meta-Analyses

QuEChERS – Quick, Easy, Cheap, Effective, Rugged, and Safe method

RSD – Relative standard deviations

SPE – Solid phase extraction

TDI – Tolerable daily intake

TFA – Trifluoroacetic acid

TLC – Thin-layer chromatography

UHPLC-MS/MS – Ultra-high performance liquid chromatography coupled with tandem mass spectrometry

UHT – Ultra High Temperature

USA – United States of America

UV – Ultraviolet

*"If you can dream it, you can do it."*

*Walt Disney*

*Esta tese é dedicada à minha querida irmã Susana*

# CHAPTER 1 – INTRODUCTION

## 1.1. Context and Motivation

Food Safety (FS) is a matter of extreme importance for society. Consumers are increasingly concerned about the products they are provided with, requiring safe and quality food. Food may confer a health risk both for humans and animals. If good hygiene and safety practices are not adopted throughout the food chain, the contamination with toxin producing microorganisms can occur, as it is the case of mycotoxin-producing filamentous fungi (Bhat et al., 2010; Hymery et al., 2014).

Although filamentous fungi are not considered foodborne pathogens, they may produce a number of secondary metabolites (mycotoxins) that have acute or even chronic toxicological effects. Among the various natural toxicants, mycotoxins are considered to be a potential risk to public health, causing adverse and serious effects on the health of animals and humans These effects include: carcinogenicity, genotoxicity / teratogenicity, mutagenicity, nephrotoxicity, hepatotoxicity, immunotoxicity, in addition to other debilitating acute diseases (Benkerroum, 2016; EFSA, 2007, 2012; Ketney et al., 2017).

In the specific case of this study, the mycotoxins to be studied are aflatoxins, more specifically Aflatoxin M1 (AFM1). AFM1 is present in milk from lactating animals that consume feed contaminated with Aflatoxin B1 (AFB1) produced by some *Aspergillus* species such as *Aspergillus flavus, Aspergillus parasiticus* and the rare *Aspergillus nomius*. AFM1, in addition to hepatotoxic and carcinogenic effects, is relatively stable in the processing of various dairy products, such as cheese (Iqbal et al., 2015; Manetta, 2011). Its presence in dairy products poses a high risk to the health of the consumer, as this type of food is part of the daily diet. Given the toxicity of this compounds, it is extremely important to study and understand the trends of AFM1 occurrence in dairy products and study how the different stages of cheese production affect the level of AFM1 in the final product. The cheese will be the food matrix chosen because there is a controversial data about the effect of the productive process on the final levels of this toxin, being cheese the dairy product where a higher concentration of AFM1 is expected (Benkerroum, 2016).

## 1.2 Main Objectives

The main objectives of this work are to determine the main trends of AFM1 occurrence in dairy products, making the best use of the available scientific data, both for risk assessment and to identify the lack of knowledge as a driver to generate new data.

So, the specific objectives to be accomplished of this work were:

- Study and understand the occurrence of AFM1 in dairy products, including milk, cheese, butter and yogurt, in different continents over the years.
- Estimate the human exposure to AFM1 and evaluate the risk to human health through the

ingestion of AFM1 contaminated dairy products by calculating the Hazard Index (HI). Additional risk characterization to estimate human health risk of hepatocellular carcinoma (HCC) was also estimated and evaluated.

- Study and understand the fate of AFM1 during cheese production, using goat and sheep Portuguese milk contaminated with AFM1.

The questions to be answered by this investigation are:

- What is the relation between AFM1 concentration and sampling year, continent, dairy product and animal species?
- How do the different stages of cheese production affect the final levels of AFM1 in cheese?

This research intended to answer this set of questions to obtain new data and generate knowledge in this area of research, filling the existing gaps.

## 1.3 Outline of the thesis

This thesis is divided into 5 chapters, oriented towards presenting the work developed during the doctoral programme.

CHAPTER 1 – presents the context, motivation and structure of the thesis; the scientific outputs are also outlined.

CHAPTER 2 – a concise literature review approaches several fundamental concepts necessary to obtain an overall understanding of the subject. Initially, the topic of FS is addressed to demonstrate its importance. Subsequently, the production of mycotoxins, with special focus on AFM1, is discussed. This section intends to demonstrate where and how this toxin occurs and what is its impact on the consumers' health. Besides that, the general cheese process and the main gaps about the effect of handling and processing on the levels of AFM1 are addressed. The legislation in force worldwide for this toxin in milk and dairy products are also discussed.

Methods to reduce AFM1 content and detection methods in dairy products are also reviewed and discussed. The last topic is presented in the form of a review article: **Vaz, A**., Cabral Silva, A. C., Rodrigues, P., & Venâncio, A. (2020). Detection Methods for Aflatoxin M1 in Dairy Products. Microorganisms, 8(2), 246.

CHAPTER 3 – contains the systematic literature search about AFM1 occurrence in dairy products in the last three decades, at a worldwide level. The estimate of the exposure and of the risk to human health through the ingestion of AFM1 contaminated dairy products can also be seen in this chapter.

CHAPTER 4 – focuses on the experimental results obtained on the distribution and fate of AFM1 during the production of goat and sheep cheeses and their discussion. Also, the materials and methodologies applied in the laboratory execution of this work are described.

CHAPTER 5 – the last chapter presents the overall conclusions and suggestions for future work in this field of research.

Finally, a final section gathers all the references used in the elaboration of this thesis. An Appendix section, to Chapters 2, 3 and 4, with supplementary information, is also included.

## 1.4 Outputs of the thesis

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

**References**

Santos, A. O., **Vaz, A.**, Rodrigues, P., Veloso, A. C., Venâncio, A., & Peres, A. M. (2019). Thin films sensor devices for mycotoxins detection in foods: Applications and challenges. Chemosensors, 7(1), 3.

**Vaz, A.**, Cabral Silva, A. C., Rodrigues, P., & Venâncio, A. (2020). Detection Methods for Aflatoxin M1 in Dairy Products. Microorganisms, 8(2), 246.

**Vaz, A.**, Mendonça, A., Rodrigues, P., & Venâncio, A. Distribution of aflatoxin M1 during production and storage of sheep and goat cheeses (manuscript in preparation).

**Vaz, A.**, Mourão, F..... Rodrigues, P., & Venâncio, A. A systematic review on Aflatoxin M1 occurrence in the last 30 years (manuscript in preparation).

The participation in conferences was used as means of learning new methodologies and to present the work developed along this thesis. The works presented at these events (as poster presentations) are listed below.

**Poster presentation**

**Vaz, A.,** Gomes F., Alves A., Rodrigues P., Venâncio A. Optimization and validation of two methods to determine the levels of AFM1 in milk and cheese samples using IAC columns for extraction and HPLC FLD for quantification. 11th National Chromatography Congress. Costa da Caparica, Portugal, December 9-11, 2019

**Vaz, A.,** Mourão F., Costa P., Rodrigues P., Venâncio A. Aflatoxin M1 in Europe between 1990-2018. 42nd Mycotoxin Workshop. Web conference, May 31st – Jun 2nd, 2021

# CHAPTER 2 – LITERATURE REVIEW

## 2.1 Food Safety and Food Security

Food Safety and Food Security are interrelated concepts with a profound impact on the quality of human life. Food security is frequently mistaken with food safety because they have the same translation in many languages (Robertson et al., 2004).

In 1996, the Food and Agriculture Organization of the United Nations (FAO) stated that food security is established "*when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life.*" On the other hand, food safety "*is the assurance that food will not cause harm to the consumer - through biological, chemical or physical hazards - when it is prepared and/or eaten according to its intended use*" (FAO, 2003; Robertson et al., 2004). Thus, food safety is an essential part of food security and this thesis will focus on Food Safety.

With the evolution of climate change and considering the global trade established, food safety has become a serious issue, that requires transnational solutions (Frazzoli et al., 2017).

To promote a good health and sustaining life it is essential that sufficient quantities of safe and nutritious food are available to all people. According to WHO, more than 200 different diseases, from diarrhea to cancers, can be caused by food contaminated with harmful bacteria, viruses, parasites or chemical substances. It is determined that, worldwide, 600 million people (approximately 10 % of the population), 420 000 deaths and a loss of 33 million healthy life years (DALYs) are caused by people that become ill after eating contaminated food each year (WHO, 2020).

The safety of food products should be controlled in the whole food chain, because the introduction of hazards can occur at any stage of the chain. The occurrence of a contaminant in the field may be carried to the final primary product, and later to the final processed product. In these cases, the final levels of the contaminant will depend on its initial levels at the primary production. Thus, to ensure food safety it is necessary to mobilize all actors in the food chain, from the producer to the consumer. Ensuring food safety means minimizing the risks to an acceptable level as there is no "zero risk" situation (Baptista, 2007).

The risk assessment for mycotoxins is crucial to protect the population from compounds that pose a hazard. Thus, evaluating the exposure to mycotoxins through the ingestion of contaminated food and the corresponding adverse health effects enables the estimation of the risk to human health (Figure1) (Kuiper-Goodman, 1990).

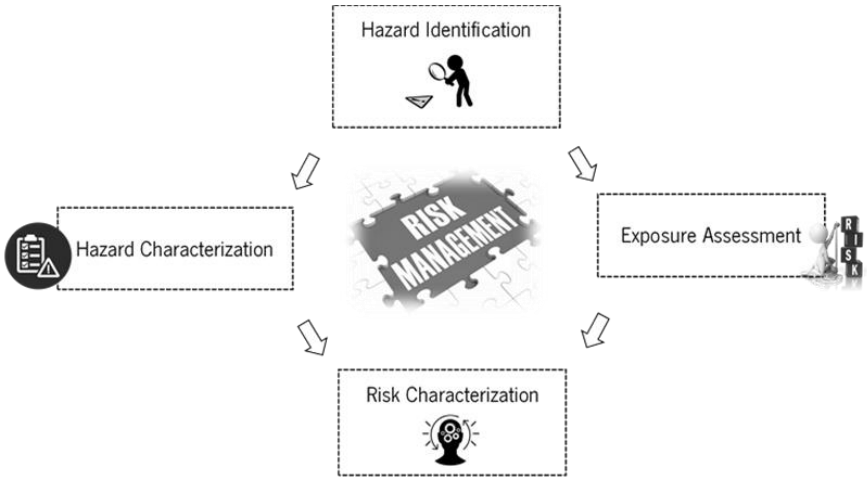


Figure 1. A scheme of a risk assessment adapted from (Kuiper-Goodman, 1990).

The characterization of a toxicological risk assessment in the food chain begins with the identification of the hazard and its characterization. Hazard characterization evaluates the health effects and risk groups, that is, groups that may be more susceptible to be affected. The toxicity assessment, determining the most biologically significant toxic effects and estimating a tolerable daily intake (TDI), that is, the "safe dose" for humans, is also important for hazard characterization. In addition, exposure assessment should also be evaluated to estimate the extent of exposure, based on food products most likely to be contaminated and the most exposed population groups. These latter groups are not necessarily the same as the risk groups previously mentioned. For an accurate risk assessment it is crucial to have precise, comprehensive and comparable data on food consumption (Frazzoli et al., 2017). The final step in risk assessment is the risk characterization, that evaluates if there is a significant risk of illness or disease based on intensity, frequency, and duration of human exposure to a hazard compound (exposure assessment) and the extent and severity of the effects for human health (hazard characterization) ("Risk Assessment and Risk Management of Mycotoxins," 2012).

The risk characterization, which is a worldwide concern, is the base for the risk management of mycotoxins (Kuiper-Goodman, 1990). Although several interventions exist to manage mycotoxin risks in food, until this moment, no single strategy enables that risk be eliminated in any country. Nevertheless, governmental regulations or agricultural and public health interventions could be good ways to control the risk. The imposition of maximum tolerable limits for mycotoxins in food and feed by governments, implementation of good agricultural practices and adoption of methods to reduce the bioavailability of mycotoxins are options to manage the mycotoxin risk in food. Besides that, infrastructures that support technologies for mycotoxin reduction risk and public health interventions, as public education, are also essentials for risk management ("Risk Assessment and Risk Management of Mycotoxins," 2012).

## 2.2 Mycotoxins

The kingdom of fungi includes yeasts and filamentous fungi, such as mushrooms (macrofungi) and moulds (microfungi). Microfungi can invade foods and commodities during pre-harvest (at field level) or during post-harvest in storage, in transport and in processing, and produce mycotoxins (Bhat et al., 2010).

According to Bhat et al. (2010), land-adapted fungi colonize and use solid substrates penetrating into their matrices by secreting enzymes to break down complex products. In most cases, colonizing fungi also produce and secrete compounds with low molecular weight – extrolites – some of which with confirmed toxic properties. These compounds, which result from the secondary metabolism of the fungi, are referred to as "mycotoxins", and are generally considered not essential for the normal growth and survival of the fungi that produce them (Bhat et al., 2010; Hymery et al., 2014).

The term mycotoxin originates from the Greek words "*mykes*" and "*toxicum*". In which "*mykes*" means fungi and "*toxicum*" means toxin. Therefore, the junction of the two independent terms forms the word mycotoxin (toxin produced by fungi). This term was used for the first time in 1962, after a veterinary crisis that resulted in the death of approximately 100 000 young turkeys. These birds had ingested peanut flour contaminated with aflatoxins (Hymery et al., 2014).

Mycotoxin-producing fungi can develop/grow in almost any solid or liquid support if favourable environmental conditions are met. Even if rapid growth of a particular mould occurs on a substrate, this does not necessarily imply that mycotoxins will be produced. However, the fact that there is a big amount of fungi in a given environment means that there is also a greater probability of a large amount of mycotoxins. The nature and quantity of mycotoxins produced are fully influenced by species and by interactions of various factors such as substrate type, available nutrients, moisture content, water activity ($a_w$), temperature, air humidity, fungal colony maturity, biological competition, physical damage of the substrate due to insect activity, among other factors (Bhat et al., 2010; Campagnollo et al., 2016; EFSA, 2012).

Mycotoxins are produced by several strains of filamentous fungi mainly belonging to species of the genera *Aspergillus*, *Penicillium* and *Fusarium*. These invade crops at the field level and can grow in food during storage under favourable conditions of temperature, humidity and $a_w$, and are responsible for their deterioration. For some groups of animals these compounds are toxic when introduced by natural route (food intake) posing a risk to public health (Bhat et al., 2010; Hymery et al., 2014).

Among food chemical contaminants, mycotoxins play an extremely important role. According to Eskola et al. (2019), more than 25 % of the world's agricultural production is contaminated with

mycotoxins above the EU and *Codex Alimentarius* limits. Thus, their ubiquitous presence and their persistence during food processing represent a challenge to the health of humans, animals and the environment (Rahmani et al., 2009; Campagnollo et al., 2016; Ketney et al., 2017).

Until now, over 300 mycotoxins have been identified and reported. However, from the point of view of food safety and regulatory the most important mycotoxins in food and feed are: aflatoxins (AFs), ergot alkaloids (EAs), fumonisins (FBs), ochratoxin A (OTA), patulin, trichothecenes [deoxynivalenol (DON), T-2 toxin (T-2), HT-2 toxin (HT-2)] and zearalenone (ZEN) (Alshannaq & Yu, 2017; Eskola et al., 2019). Maximum levels for all these toxins are regulated according the current legislation of European Commission (EC, 2006b, 2010, 2015). Other mycotoxins that have been suffer structurally modifications are categorized as masked or modified and can also be found in food and feed, representing an additional risk to human and animal health (Rychlik et al., 2014). On the other hand, with the improvement of technology, new molecules, that may have toxic effects, have been detected and identified. These compounds are recognizing as emerging mycotoxins (Marin et al., 2013). Table 1 shows some examples of masked or modified, and emerging mycotoxins.

Although, among known mycotoxins, the most toxic and with significant impact in agriculture commodities are the AFs (Alshannaq & Yu, 2017; RASFF, 2020).

Table 1. Some examples of masked or modified mycotoxins and the corresponding parent mycotoxin. Examples of emerging mycotoxins produced by different species (EFSA, 2014; Kovač et al., 2018)

| **Masked or modified mycotoxins** | | | | | |
|---|---|---|---|---|---|
| AFs[1] | DON[1] | FBs[1] | OTA[1] | T-2 toxin and HT-2 toxin | ZEN[1] |
| Aflatoxin B1 exo - 8,9 epoxide; Aflatoxin M1 (AFM1) | DON3Glc; 3Ac-DON; 15Ac-DON; Oligoglycosylated forms of DON; D3GlcA; D15GlcA; DON3Sulf; DON15Sulf; DON-GSH | FBs thermal degradation products: NDF and NCM | OTA degradation products: 14R-ochratoxin A, 14-decarboxy-ochratoxin, OTA esters | T-2-Glc; HT-2-Glc; More highly glycosylated forms of T-2 and HT-2 | ZEN14Glc; ZEN14Sulph |
| **Emerging Mycotoxins** | | | | | |
| Produced mainly by *Fusarium* species | | Produced by *Claviceps* species | | Produced by Alternaria species | |
| Fusaproliferin; Mmoniliformin; Beauvericin; Enniatins | | Ergot alkaloids | | Altenuene; Alternariol; Alternariol methyl ether; Altertoxin; Tenuazonic acid | |

[1]AFs – Afltoxins; DON – Deoxynivalenol; FBs – Fumonisins and ZEN – Zearalenone

### 2.2.1 Aflatoxins

The name AF has derived from the combination of "a" for the *Aspergillus* genus and "fla" for the species *flavus*, and toxin meaning poison. Aflatoxins are generally considered the main toxic secondary

metabolites produced by fungi, namely by some *Aspergillus* species such as *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus nomius* (Bhat et al., 2010; Iqbal et al., 2015; Manetta, 2011).

*Aspergillus* spp. affect many foods, food ingredients and feed materials, especially nuts (e.g., peanuts) and grains (mainly corn). The different species of the genus *Aspergillus* producing aflatoxins present different behaviours. *Aspergillus parasiticus* is more adapted to a soil environment, while *A. flavus* is more adapted to the aerial parts of plants (EFSA, 2007, 2012; Frazzoli et al., 2017). *Aspergillus flavus* and *A. parasiticus* are xerophilic fungi. These species can grow at very low $a_w$ (0.77) and at a broad range of temperatures (from 12 °C to 48 °C), even if the proper conditions for growth are: 0.82 $a_w$ at 25.8 °C, 0.81 $a_w$ at 30.8 °C and 0.80 $a_w$ at 37.8 °C (Bhat et al., 2010; EFSA, 2012). Most of the *Aspergillus* species grow above the 25° latitude north and south, but it is expected a high occurrence between the latitudes 26° and 35°. However, these species are uncommon in latitudes above 45° (Campagnollo et al., 2016; EFSA, 2012).

The major AFs are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1). The letters "B" (blue) and "G" (green) indicate the fluorescent colour observed when these mycotoxins are exposed to ultraviolet (UV) light, and the letter "M" refers to the metabolite derived from AFB1 found in milk and dairy products (Campagnollo et al., 2016; EFSA, 2012; Iqbal et al., 2015; Manetta, 2011).

Generally, *A. parasiticus* produces AFB1, AFB2, AFG1 and AFG2 while *A. flavus* only produces AFB1 and AFB2. Aflatoxins are produced in various grains and nuts, e.g., corn, sorghum, cottonseed, peanuts, pistachio nuts, copra, cereals, fruits, oilseeds, dried fruits, cocoa and spices in the field and during storage (Abrunhosa et al., 2016; EFSA, 2007, 2012; Iqbal et al., 2015; Manetta, 2011).

Aflatoxins occur mainly in hot and humid regions where high temperature and humidity are optimal for mould growth and toxins production. Their presence is enhanced by factors like stress or damage to the crop due to drought before harvest, climate change, insect activity, substrate composition, soil type and inadequate storage conditions (Campagnollo et al., 2016; EFSA, 2007, 2012; Espinosa-Calderón et al., 2011; Frazzoli et al., 2017; Iqbal et al., 2015; Manetta, 2011).

The formation of mycotoxins is not a continuous process, but it is presumed that if the filamentous fungus is present in the environment and is capable of producing toxins, mycotoxins may be present in food. In addition, the fungus may be absent, but the toxin may be present, such as the presence of AFM1 in milk and dairy products. The level of contamination is cumulative, so the harvest time, drying conditions and storage may play an important role in the production of AFs (Figure 2) (Campagnollo et al., 2016;

Paterson & Lima, 2010). Biosynthesis of AFs may be completely inhibited, depending on the particular combination of external parameters for fungal growth, even in conditions of normal development. Thus, the knowledge about the relationship fungus/environment is very important since it may allow an assessment concerning the combinations of parameters or factors that can control the biosynthesis of flatoxins and which can promote the appearance and, also, production of aflatoxins (Ketney et al., 2017).

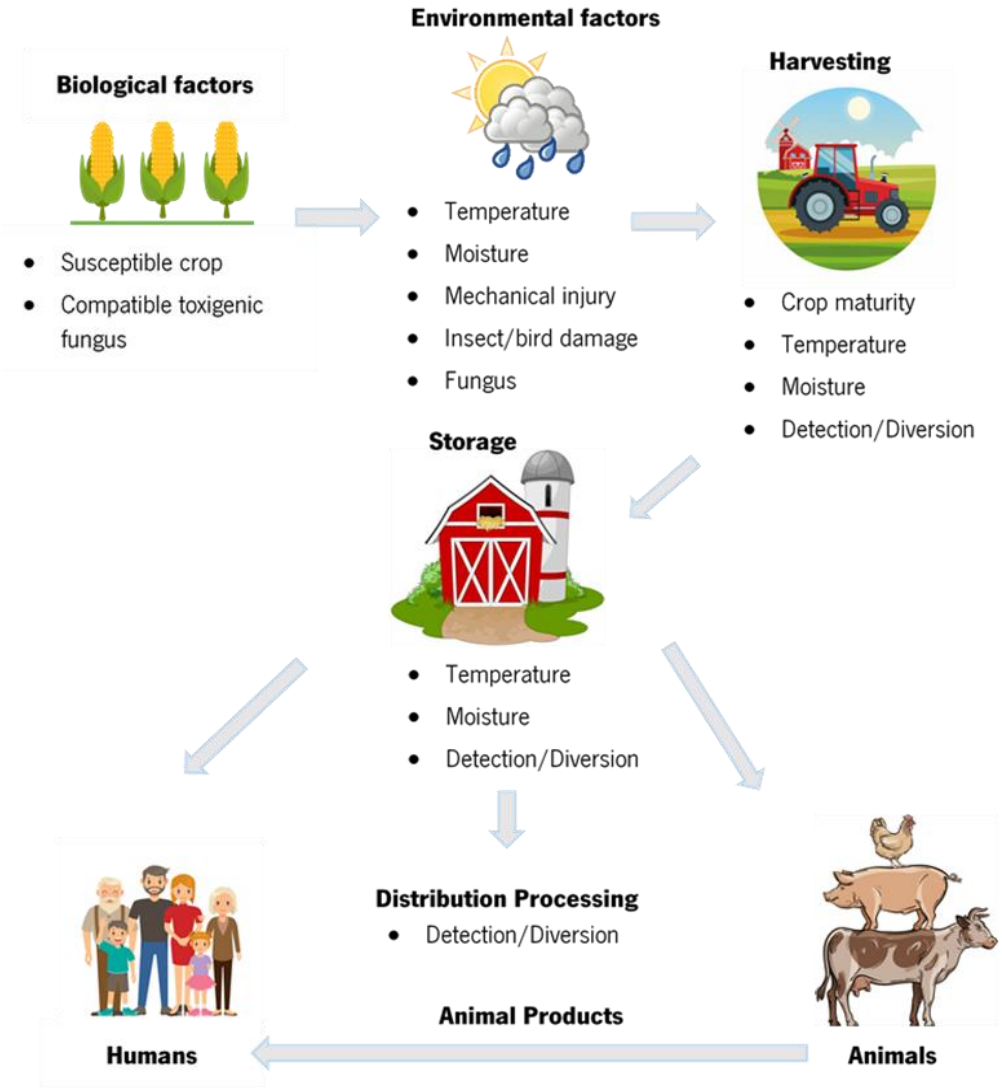


Figure 2. Factors affecting mycotoxin occurrence in the food chain adapted from (adapted from Paterson and Lima, 2010).

Aflatoxins have a low molecular weight and have various chemical structures and properties (Figure 3). They are soluble in solvents such as methanol, chloroform and benzene and they are non-immunogenic. Besides that, they act at low concentrations and are unstable at UV light, but very stable at temperatures above 100 °C. That is why they present small or almost no decomposition when subjected to baking, roasting and pasteurization (Campagnollo et al., 2016).

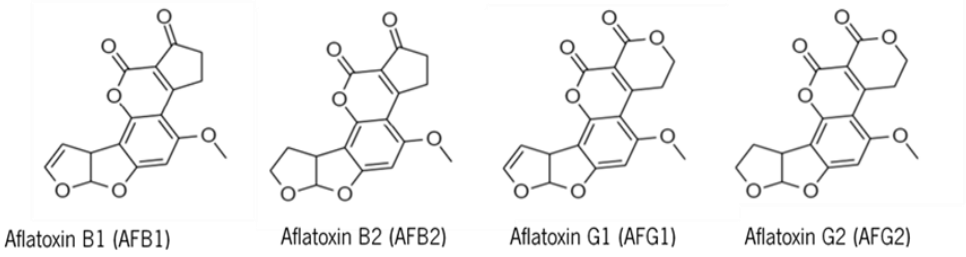


Figure 3. Illustrations of chemical structure of aflatoxins: B1, B2, G1 and G2.

Aflatoxins show a wide spectrum of toxic effects and targets. When ingested, inhaled or absorbed through the skin, they have carcinogenic, hepatotoxic, teratogenic and mutagenic effects in humans and animals even at very small concentrations. They are the most potent, naturally-occurring carcinogens known and have been linked to liver cancer and several other maladies in animals and humans (Ketney et al., 2017; Mosielllo & Lamberti, 2011). The risk of cancer due to the exposure to AFs is well established and is based on the cumulative lifetime dose.

Since the 1960's, due to aflatoxins, several outbreaks of acute or chronic liver failure have been observed in both humans and animals (Newbern & Butler, 1969; WHO, 2018). Chronic aflatoxicosis can result in cancer, immune suppression and other pathologic conditions, while acute aflatoxicosis can ultimately result in death (Zain, 2011).

About 1 to 3 % of the aflatoxins that are absorbed by the organism and transported to the liver through the circulatory system are irreversibly bound to proteins and DNA bases, forming adducts (a segment of DNA bound to a chemical) and causing aflatoxicosis. The symptoms of hepatotoxicity in the early stages of the disease can manifest as anorexia, malaise, and low-grade fever. However, if aflatoxicosis progress to potentially lethal acute hepatitis, the symptoms can get worse and can be: vomiting, abdominal pain and eventually death. Even with supportive care, fatality rates for acute poisoning range from 25 to 40 % (Azziz-Baumgartner et al., 2005).

Among the aflatoxins, AFB1 is the most toxic, carcinogenic, teratogenic and mutagenic AF and is listed as group I carcinogen by the International Agency for Research on Cancer (IARC) (2002). The potency of AFB1 to cause liver cancer is significantly enhanced in individuals carrying the hepatitis B virus (HBV). Due to the consequences of chronic exposure to aflatoxins, epidemiological studies are very complicated (WHO, 2018).

The lethal dose ($LD_{50}$) (the dose of any substance tested required to kill half the number of test animals) of AFB1 for most species is in the range of 1-50 mg/kg, but for some extremely sensitive species, such as pigs, cats, rabbit, dogs and ducklings, the AFB1 has a critical toxicity level ($LD_{50}$<1 mg/kg)

(Applebaum et al., 1982; EFSA, 2007, 2012; Iqbal et al., 2015; Ketney et al., 2017; WHO/IARC, 2002; Wild & Gong, 2010).

Based on JECFA (Joint FAO/WHO Expert Committee on Food Additives) reports, there is strong biological evidence to conclude that AFB1 is a low-dose genotoxic carcinogen. So, even a very low exposure level to aflatoxins (1 ng/kg body weight per day) may induce liver cancer cases (FAO/WHO, 2017). Based on past outbreaks, WHO (2018) estimated that an AFB1 dose of 20–120 μg/kg bw per day, when consumed over a period of 1–3 weeks, is acutely toxic and potentially lethal. In this sense, it is recommended to reduce the dietary exposure to aflatoxins to the lowest practicable levels, so as to reduce the potential risk as far as possible (FAO/WHO, 1987).

#### 2.2.1.1 Aflatoxin M1

Aflatoxin M1 is a mycotoxin derived from the transformation of AFB1 by dairy animals. AFB1 is converted to the monohydroxy derivative AFM1 in the liver of lactating animals by the action of cytochrome P450, more specifically cytochrome P450 1A2 (monooxygenase), when they consume feed contaminated with AFB1 (EFSA, 2004; Jaiswal et al., 2018; Marchese et al., 2018).

AFM1 is structurally similar to AFB1 (Figure 4) and was categorized as group 2B human carcinogen by IARC (IARC, 2002). Besides, according to WHO (2002), AFM1 is cytotoxic and its acute toxicity and genotoxicity in some species (as ducklings and rats) is similar to that of AFB1. AFM1 can also cause cell transformation *in vitro* mammalian cells and DNA damage, inducing gene mutations and anomalies in chromosomes (Min et al., 2021).

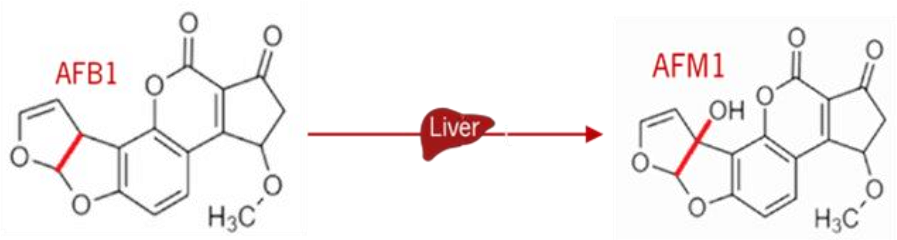


Figure 4. Illustration of the conversion from AFB1 to AFM1.

Depending on factors such as the genetics of the animals, seasonal variation, the milking process and the environmental conditions, AFM1 is eliminated in urine and in milk through the mammary glands of both humans and lactating animals (Campagnollo et al., 2016; EFSA, 2004; Iqbal et al., 2015). Thus, there are various nutritional and physiological factors that can influence the carry-over from feed to milk, which means that this rate can vary between individual animals, from one milking to the next and from day to day (Fink-Gremmels, 2008).

Approximately 1 % for low–yielding cows (milk production < 30 kg /day) to 6.2 % for high–yielding cows (milk production > 30 kg /day), with an average value around 2 %, of the ingested AFB1 is secreted in milk as AFM1 (EFSA, 2004; Iqbal et al., 2015; Ketney et al., 2017). However, according to Britzi et al. (2013), the carry-over rate of the AFB1 in feed to AFM1 in milk is slightly superior for low production cows. They observed that milk production affects the carry-over rate, with an average rate of 2.5 % for low production cows (< 35 kg /day) and 5.4 % for high production cows (> 35 kg /day).

The occurrence of AFM1 in dairy products due to field contamination of feed materials with AFB1 producing fungi (Figure 5) is a good example of the different pathways of mycotoxin occurrence in foods (Benkerroum, 2016; EFSA, 2004, 2007). Thus, the safety of food products should be controlled in the whole food chain. The occurrence of a contaminant in the field may be carried to the final primary product, and later to the final processed product. In these cases, the final levels in the end product will depend on the initial levels at the primary production. For this reason, the carefully control of the harvest and storage conditions is extremely important, in order to reduce the exposure of dairy cattle to aflatoxins and, subsequently, reduce their concentration in milk (Costamagna et al., 2019; Michlig et al., 2016).

The main chemical and physical properties of this toxin are (Schrenk et al., 2020; WHO/IARC, 2002):

- Intensely fluorescent in ultraviolet light, emitting blue–violet fluorescence;
- Unstable to ultraviolet light in the presence of oxygen, to extremes of pH (< 3, > 10) and to oxidizing agents;
- The melting point is 299 °C (decomposition);
- Considered freely soluble in moderately polar organic solvents (e.g., chloroform and methanol). However, it is insoluble in non-polar solvents, and is very slightly soluble in water (10–30 μg /mL), even though it contains 8 oxygen atoms and 7 double bonds;
- The lactone ring is susceptible to alkaline hydrolysis and is also degraded by reaction with ammonia or sodium hypochloride.

Besides that, it is relatively stable during heat treatment, storage, and preparation of various dairy products. As a consequence, it can be found in all types of dairy products like cheese, butter and yogurt (Chavarría et al., 2017; EFSA, 2007; IARC, 2012; Iha et al., 2013; Manetta, 2011; Manetta et al., 2005). Also, humans may be exposed to AFM1 through the breast milk, if the diet of pregnant women is rich with poor quality grain and contaminated with AFB1 (Fakhri et al., 2019; WHO, 2018).

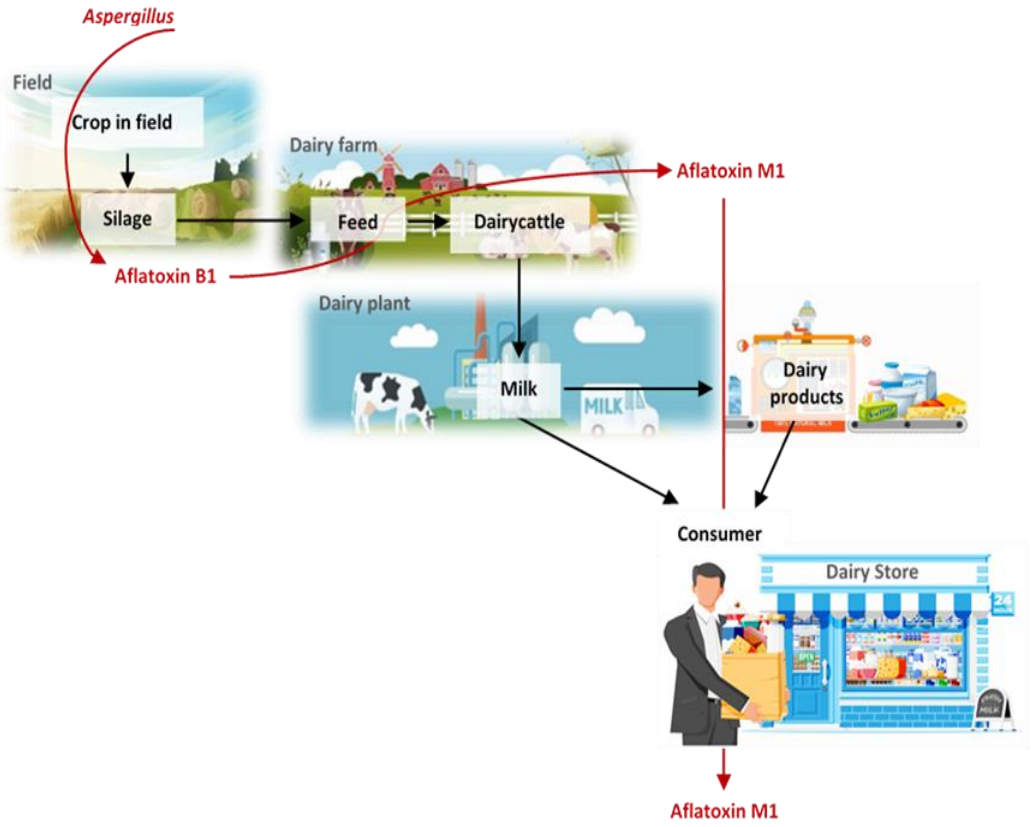


Figure 5. Representative scheme of AFB1 transfer from field to AFM1 in milk and dairy products.

National estimates of dietary exposure to aflatoxins indicate differences between developed and developing countries. In Europe (Van der Fels-Klerx et al., 2019) a tolerable daily intake of AFM1 has been set at 0.2 ng/kg bw. This value was proposed by Kuiper-Goodman (1990), as a safe dose for human health, and according to WHO (2018) it is estimated that the dietary exposure to AFM1 rarely exceeds 1 ng/kg bw per day in any country.

The levels of carry-over of AFM1 from milk to dairy products are controversial, being cheese the product where a higher concentration of the toxin is expected (Benkerroum, 2016; Iha et al., 2013). So, this PhD project is emphasized on cheese production.

## 2.3 Fate of Aflatoxin M1 during cheese processing

### 2.3.1 Cheese processing steps

Cheese is the most diversified group of dairy products, which is widely produced and consumed worldwide (Elsamani et al., 2015). According to the FAO (1978) description, *cheese is the ripened or unripened soft, semi-hard, hard, or extra-hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by:*

(a) *coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from the coagulation, while respecting the principle that cheese-making results in a concentration of milk protein (in particular, the casein portion), and that consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made; and/or*

(b) *processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a).*

Cheese making can be described as a process of milk concentration. In this process, caseins and fat are concentrated in the curd, while whey proteins, lactose and other soluble solids, as the case of some minerals, are removed in the whey (Tetra Pak, 2018). The main ingredients for its production are milk, coagulation agent (rennet), bacteria and salts (Noronha, 2003; Tetra Pak, 2018).

The manufacture of cheese is a process that involves many steps and various biochemical modifications. The main manufacturing steps are coagulation, moulding/pressing, salting and ripening/cure. However, there are numerous variants and there are cheeses that follow alternative production procedures. In Figure 6 the general cheese production flowchart is exhibited.

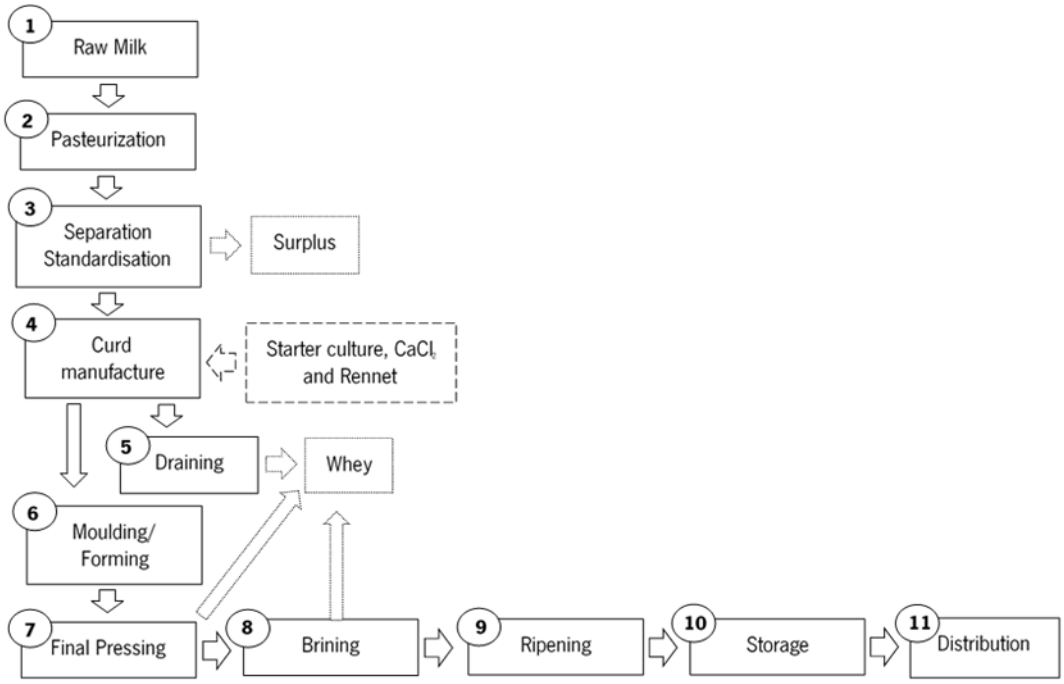


Figure 6. Cheese production flowchart adapted from (Tetra Pak, 2018).

After reception, milk is usually given a heat treatment known as **pasteurization** which aims to destroy the microorganisms present in the milk, thus guaranteeing the conditions of food safety and hygiene. Pasteurization, e.g., at 72–73 °C for 15–20 seconds, permits to inactivate bacteria capable of affecting the quality of the cheese, e.g. coliforms, which can cause early "blowing", a disagreeable taste, and make the product unsafe (Tetra Pak, 2018). This step is followed by the milk **standardisation**, which aims to standardize the fat content needed according to the cheese to be obtained.

For the **formation of curd (coagulation)**, the essential additives in the cheesemaking process are the starter culture and the rennet (enzyme preparation). Besides that, it may also be necessary to add other components such as calcium chloride ($CaCl_2$), to help the curd formation (Tetra Pak, 2018). The addition of starter culture aims on the one hand to restore the microbiota and on the other to lower the pH by the production of lactic acid. This pH reduction, allows for curd formation and inhibits the production of harmful compounds. The rennet addition aims to the hydrolysis of k-casein causing casein micelles coalescence (aided by the addition of $Ca^{2+}$) turning liquid milk into a kind of firm gel. So, the combined result of these three changes results in curd formation (fresh cheese) (Johnson & Law, 2010; Noronha, 2003; Tetra Pak, 2018).

After the milk coagulation, the curd is **cut** with appropriate tools, into small pieces, called 'grains' or 'curd' allowing the expulsion of the whey, a by-product of the cheese making process. This process is known as **draining**. The expulsion of whey can be facilitated by three factors: by lactic acid production, by agitation and by heating of the curd grains. During the curd manufacturing process, the heat treatment and the stir of the curd is important because the growth of acid-producing bacteria is limited by heat, which is used to regulate production of lactic acid and, besides that, the heat also promotes contraction of the curd accompanied by expulsion of whey (syneresis). On the other hand, stirring curd keeps the grains suspended in whey and prevents formation of lumps that may influence the texture of the cheese, as well as causing loss of casein in whey (Johnson & Law, 2010; Tetra Pak, 2018). Then the curd is pressed in moulds and any excess whey is removed.

After having been **moulded or hooped** the curd is subjected to **final pressing**, which aims to assist final whey expulsion, to provide texture and shape the cheese. It is important to note that the rate of pressing and pressure applied are adapted to each particular type of cheese (Tetra Pak, 2018).

In the **brining** step, as in other foods, salt (NaCl) is used to improve cheese flavour. However, salt has other important roles, such as retarding rennet activity and bacterial processes associated with cheese ripening. On the other hand, the application of salt to curd helps in the drying process, because

there will be more expulsion of moisture due to the osmotic pressure (Tetra Pak, 2018).

Generally, the salt content varies between 0.5–2 %. However, blue cheese and white pickled cheese variants (e.g., Feta, Domiati) have a higher salt content (3–7 %). There are different types of salting/brining. If salt is not added to the curd before pressing, it is added to the cheese by soaking it in brine or by rubbing salt onto its surface during ripening (Campagnollo et al., 2016; Johnson & Law, 2010; Tetra Pak, 2018).

Cheese, except for fresh cheese, is now ready for **maturation**, where the flavour and texture develop. The ripening takes place under temperature and humidity conditions that vary according to the type of cheese. This stage is responsible for a series of processes of a microbiological, biochemical and physical nature. These changes affect lactose, protein and fat. The ripening cycle varies from two weeks (semi-hard and semi-soft cheese) to two or more years (some hard cheeses) depending on the cheese variety (McSweeney, 2004; Tetra Pak, 2018).

The purpose of **storage** is to create, as far as possible, the necessary temperature and relative humidity conditions for each type of cheese to control the ripening cycle. Once the cheese production process has been completed, it can then be **distributed** for consumption (Tetra Pak, 2018).

Different manufacture operations, types of milk used, and the addition of other ingredients - extracts like herbs – make it possible to obtain a huge variety of cheeses (Campagnollo et al., 2016; Noronha, 2003). FAO proposed that cheeses can be classified according to moisture content (firmness), fat content and ripening characteristics (Table 2) (*Codex Alimentarius* et al., 1978).

Table 2. Classification of cheese adapted from (Tetra Pak, 2018)

| **According to moisture content** | | **According to fat content** | | **According to ripening characteristics** |
|---|---|---|---|---|
| **MFFB[1](%)** | **Designation** | **FDB[2](%)** | **Designation** | |
| < 41 | Extra Hard | < 10 | Skim | Cured ripened<br>a) Mainly surface<br>b) Mainly interior |
| 49-56 | Hard | 10-25 | Low Fat | Mould cured or ripened<br>a) Mainly surface<br>b) Mainly interior |
| 56-63 | Semi-Hard | 25-45 | Medium Fat | Uncured or unripened[3] |
| 63-69 | Semi-Soft | 45-60 | Full Fat | |
| > 69 | Soft | > 60 | High Fat | |

[1] MFFB: Moisture contents on a Fat–Free Basis; [2] FDB: Fat on Dry Basis; [3]Milk intended for this type of cheese to be pasteurized

Cheese is known to be a suitable substrate for fungal growth. Although, it is not as good for mycotoxin production (Campagnollo et al., 2016). Mycotoxin contamination of cheese can occur in two

ways: (i) directly contamination by producing moulds that growth on cheese and (ii) indirectly contamination by using contaminated milk. The direct cheese contamination can occur via exogenous, if the cheese is contaminated with mycotoxin-producing moulds during cheese-making or via endogenous if commercial fungal cultures that produce mycotoxins are used. *P. roqueforti* and *P. camemberti* are two species used to produce Roquefort and Camembert cheeses, respectively, and with potential for mycotoxin production (Hymery et al., 2014; Kure & Skaar, 2019). *P. roqueforti* produces roquefortine C and mycophenolic acid (MPA) while *P. camemberti* produces cyclopiazonic acid (CPA) (Hymery et al., 2014). However, just a few studies reported the occurrence of these mycotoxins in cheese (Fontaine et al., 2015; López-Díaz et al., 1996; Zambonin et al., 2001).

Although with a low frequency, the most common mycotoxins reported in cheese by direct contamination are: andrastin A–D (Nielsen et al., 2005), citrinin (Bailly et al., 2002), cyclopiazonic acid (Zambonin et al., 2001), mycophenolic acid (Fontaine et al., 2015), OTA (Altafini et al., 2021), patulin (Pattono et al., 2013), and roquefortin C (Fontaine et al., 2015). Some of these mycotoxins are stable under normal processing conditions as CPA and OTA. Therefore, good production practices and storage conditions are crucial to avoid the appearance of toxigenic moulds (Hymery et al., 2014; Kure & Skaar, 2019). Concerning indirect contamination, AFM1 is the main concern and the most frequent toxin reported in cheese (Chavarría et al., 2015; Jiménez-Pérez et al., 2021; Shahbazi et al., 2017), being the target of this thesis.

Studies have reported that AFM1 levels in cheese are influenced by a variety of factors, including the milk used and its origin, production technologies, such as type of unit operations, cheese type, renneting, press time, salt concentration, final pH, temperature and water content (Campagnollo et al., 2016; Cavallarin et al., 2014; Chavarría et al., 2017; Iha et al., 2013).

In dairy processing, the effect of handling and processing on levels of AFM1 is not clear, being cheese the dairy product where more accumulation is expected. Binding to proteins is expected, but AFM1 affinity to different proteins is not consensual. So, in the next sections, the fate of AFM1 in the different steps of cheese making is reviewed and discussed.

### 2.3.2 Effect of pasteurization on AFM1 content

Several studies have addressed the stability of AFM1 during the heat treatment. Dua et al. (2012) reported that neither pasteurization nor boiling influenced the level of AFM1 in milk. Others authors have also observed that pasteurization does not cause statistically significant changes in AFM1 levels (Bakirci, 2001; Dua et al., 2012; Istamboulié et al., 2016; Kafle et al., 2012; Sifuentes dos Santos et al., 2016).

Stability during heat treatment may be due to the fact the aflatoxins are decomposed at a much higher temperature (237–306 °C) (Rustom, 1997) than the typical temperature used for milk pasteurization (72 °C during 15–20 seconds) or for UHT milk (135–140 °C during few seconds) (Tetra Pak, 2018). However, there are also studies reporting that the pasteurization of milk could cause a decrease in AFM1 (Deveci, 2007; Motawee, 2013; Motawee & McMahon, 2009). Deveci et al. (2006) found statistically significant changes in AFM1 content after pasteurization, concentration, and spray drying, in the manufacture of skim milk powder, at different spiked levels of the toxin. They proposed that the losses in AFM1 could be attributed to simultaneous interactions of whey proteins with casein, which may hinder the extraction of casein-bound AFM1. However, if these treatments do not affect the AFM1 structure, the observed reduction is not due to a degradation process, but probably to the inefficiency of the analytical method for the extraction of AFM1. On the other hand, they also suggest that heat treatment destroys the toxin since the extraction method applied before and after treatments is the same. However, as seen before, AFM1 is very resistant to high temperature.

These contradictions could be attributed to natural contamination of milk or spiking, to differences in the initial level of contamination, the range of temperature, and the analytical methods used to extract, clean–up and quantify the toxin. Therefore, pasteurization cannot be used to protect against AFM1 contamination.

### 2.3.3 Effect of standardization on AFM1 content

As seen previously, this step consists in the adjustment of the fat content of milk, to ensure that there is no variability between batches, considering that the amount of fat in the milk varies from animal to animal and also varies with the feed (seasonal variation). The cow is the most widespread milking animal in the world and is found in all continents and in nearly all countries. The content of fat in this milk varies between 2.5 - 6.0 % with an average value of 4 % of the entire milk content (Tetra Pak, 2018).

A study done by Pietri et al. (2016b) demonstrated that AFM1 concentration in raw milk and partially skimmed milk was very similar, possibly due to the semi-polar behaviour of AFM1 which resulted in only a small amount transferred to the surplus cream. There was no significant difference between AFM1 levels in cauldron milk from high and low fat/casein ratio raw milk, as expected, since the milk fat represents only 4 % of the total milk content.

### 2.3.4 Effect of coagulation and draining on AFM1 content

The coagulation occurs with the addition of starter culture and rennet, resulting in curd formation,

followed by curd cut and draining steps that promote the expulsion of whey. So, curd and whey are the products resulting from these steps (Tetra Pak, 2018).

Different studies have been conducted on the fate of AFM1 in different types of cheeses and most of them have reported a higher concentration of AFM1 in curd than in whey. This higher concentration in curd is attributed to the affinity of AFM1 to casein, due to a hydrophobic interaction. Casein is one of the main proteins in milk. Table 3 shows the protein content of milk from different animals species (Tetra Pak, 2018).

Table 3. Percentage of protein fraction in different types of milk adapted from (Tetra Pak, 2018)

| Animal | Total Protein (%) | Casein (%) | Whey protein (%) |
|---|---|---|---|
| Human | 1.2 | 0.5 | 0.7 |
| Horse | 2.2 | 1.3 | 0.9 |
| Cow | 3.5 | 2.8 | 0.7 |
| Buffalo | 4.0 | 3.5 | 0.5 |
| Goat | 3.6 | 2.7 | 0.9 |
| Sheep | 5.8 | 4.9 | 0.9 |

SOFT TYPE OF CHEESE

Cavallarin et al. (2014) investigated the concentration factor of AFM1 in cheese produced with naturally and artificially contaminated milk, following three Italian traditional cheese production methods: Robiola, Primosale and Maccagno. Robiola and Primosole are fresh cheeses (soft-type cheeses), while Maccagno is a hard-type cheese. For these three cheeses, it was possible to observe that the AFM1 concentration was higher in cheese compared to whey, which may be due to the higher affinity of AFM1 for caseins. The AFM1 concentrations of Primosale, Robiola and Maccagno cheeses were 1.4, 2.2 and 6.7-fold higher than in the original milk, respectively. This concentration factor is also known as enrichment factor (EF) and corresponds to the ratio between the concentration of AFM1 in cheese and the concentration in milk. Primosale (soft cheese) and Maccagno (hard cheese) share the same rennet based cheesemaking procedure, but Maccagno was maturated for 3 months, and showed an EF of 6.7. The higher concentration in fresh cheeses related to raw milk was attributed to the affinity to caseins, while the much higher concentration in Maccagno cheese is due to the further processing of cheese, mainly those steps that contribute to the whey and moisture removal. So, these results suggest that the factor responsible for the level of AFM1 concentration from curd to cheese may be related to the loss of moisture during processing. They also observed that a higher AFM1 percentage remains in whey samples, but the highest AFM1 levels were observed in curd samples.

Fernandes et al. (2012) showed that the AFM1 concentration in curd (0.199-0.416 μg/L) was higher than in whey (0.099-0.215 μg/L). Besides that, they also showed that the use of milk containing 0.25 - 0.5 μg/L AFM1 may concentrate the level of AFM1 in Minas Frescal cheeses (a typical Brazilian cheese classified as a semi-fat, non-ripened and soft cheese) up to 2.5-folds, but at levels below the tolerance limit for AFM1 in cheeses as established by Brazilian regulations (section 2.4).

Oruc et al. (2006) also studied the distribution of AFM1 between curd, whey and white pickled cheese. White pickled cheese is a soft cheese mainly consumed during breakfast in Turkey. The results showed that after syneresis the AFM1 concentration was higher in the curd than in whey. The average EF obtained in curd was 3.79.

Costamagna et al. (2019) also studied AFM1 carry-over from milk to cremoso argentine cheese (soft cheese) and observed that AFM1 levels in whey samples were between 55 % and 58 % of the total amount of the toxin present in the naturally contaminated milk, the remaining percentage being in cheese. They also reported that the level of AFM1 was higher in cheese than in the original milk, which results in an EF, for the cheese, between in 5.4 and 5.6. Also, other studies in soft cheeses have found a higher concentration of AFM1 in curd than in whey, and EF were in the range of 1.4 to 8.1 (Table 4).

SEMI HARD TYPE OF CHEESE

Oruc et al. (2007) studied the distribution and stability of AFM1 in Kashar cheese (a semi hard cheese). Raw milk samples were spiked with AFM1 at the levels of 0.050, 0.25 and 0.75 μg/L. The AFM1 concentrations obtained for each contamination level in curds were 2.93, 3.19 and 3.37 times higher than those in milk. The percentage distribution of AFM1, after syneresis, was between 40 and 46 % in curds and in the range of 53–58 % in whey. These results indicate that a higher toxin amount remains in whey, which corroborates the studies mentioned above (Cavallarin et al., 2014; Costamagna et al., 2019).

Pecorelli et al. (2019) also studied the fate of AFM1 during the production of caciotta cheese (a semi hard cheese). To produce the cheese, naturally contaminated cow's milk was used, at levels ranging from 0.020 to 0.148 μg/kg. The distribution of AFM1 in whey and in cheese was between 34-63 % and 42-53 %, respectively. For caciotta cheese an EF of 5 was reported. Just like, Blanco et al. (1988), Pecorelli et al. (2018) and Sakuma et al. (2016) demonstrated a higher concentration of AFM1 in curd than in whey (Table 4).

HARD TYPE OF CHEESE

Manetta et al. (2009), with the objective to study the distribution of AFM1 in whey and in curd in a typical hard and long – ripened cheese – Grana Padano – produced with naturally contaminated milk, reported lower concentration of AFM1 in whey than in the corresponding milk, while the curd samples showed an EF of 3. In Grana Padano cheese the levels of AFM1 were about 1.5-fold higher than those in the corresponding curd and an EF of 4.5, but never exceeding the provisional limit of Italy (section 2.4).

Pietri et al. (2016b) investigated the fate of AFM1 during cheesemaking for the production of parmesan-type cheese (extra hard cheese) and during the ageing time, using naturally contaminated milk. This study also demonstrated that the concentration of AFM1 was higher in curd compared to whey and the average EF was 4.7 for curd and 5.5 for aged cheese.

Thus, in general, an appreciable amount of the toxin present in milk remains in whey, but the concentration of AFM1 in cheese is higher than in whey and in milk. This may be due to AFM1 being a semi polar compound, which may lead to a lower affinity to the serum proteins and a higher affinity to milk caseins. Moreover, it may also be due to the increased dry matter in the cheese (Deveci, 2007; Manetta et al., 2009; Mendonça & Venâncio, 2005; Oruc et al., 2006).

Table 4 provides the data compilation obtained for the different cheese types, the respective milk used to produce them, the type of AFM1 contamination and the enrichment factor obtained for curd and for cheese in each study, when available.

Contrary to what has been stated, Chavarría et al. (2017) reported that whey samples had the highest AFM1 concentration levels than cheese samples and, also, demonstrated that some whey proteins bind AFM1 more strongly than casein. The interaction level of AFM1 with various milk proteins was evaluated using a solution of AFM1 mixed with the main milk proteins (in concentrations resembling milk and whey composition). Then, the AFM1-protein complexes were precipitated and the remnant AFM1 in the supernatant was measured by HPLC-FLD. The results observed indicated that whey and milk proteins with more bound AFM1 molecules were α-lactalbumin and casein, with 88 % and 81 %, respectively, demonstrating a high affinity not only for casein but also for α-lactalbumin. A similar assay for each particular subunit of casein was also performed, being observed a different affinity of each casein to AFM1: α-casein (100 %), β-casein (54.5 %) and κ-casein (21.4 %). Casein have been refered to have a higher affinity to AFM1, but it may suffer hydrolisis during renneting and fermentation. Chavarría et al. (2017) suggested that these changes may cause decreased capacity to bind AFM1, allowing whey proteins to freely associate with AFM1.

The prevalence of AFM1 in cheeses from different animal species can be explained by the affinity of AFM1 to each particular subunit of casein, since the composition of the main protein fractions is different between the species. When compared with sheep and bovine milk, goat milk contains lower levels of $\alpha_{s1}$–casein and β-lactalbumin and higher levels of $\alpha_{s2}$–casein, β–casein and α-lactalbumin (Tamime et al., 2011).

Table 4. Data compilation for the different cheese types, the milk used for production, the type of AFM1 contamination, the Enrichment Factor (EF) obtained and the respective reference

| Cheese type | Cheese name | Milk type | AFM1 contamination | EF in curd | EF in cheese | Reference |
|---|---|---|---|---|---|---|
| **Soft** | Cottage | Cow | Naturally | - | 8.10 | (Applebaum & Marth, 1982) |
| | Mozzarella | Cow | Naturally | - | 8.10 | (Brackett & Marth, 1982) |
| | Telemes | Cow | Artificially | 3.90 | 4.20 | (Govaris et al., 2001) |
| | White Pickled | Cow | Artificialy | 3.19 | 3.80 | (Oruc et al., 2006) |
| | Crescenza | Cow | Naturally | 2.56 | - | (Cattaneo et al., 2008) |
| | Minas Frescal | Cow | Artificially | - | 2.50 | (Fernandes et al., 2012) |
| | Primosale | Cow | Artificially | - | 2.20 | (Cavallarin et al., 2014) |
| | Robiola | Cow | Artificially | - | 1.40 | (Cavallarin et al., 2014) |
| | Cremoso Argentine | Cow | Naturally | - | 5.50 | (Costamagna et al., 2019) |
| | Turrialba | Cow | Artificially | - | - | (Chavarría et al., 2017) |
| **Semi-hard** | Manchego | Sheep (15 %) Goat (35 %) Cow (50 %) | Artificially | 2.14 | - | (Blanco et al., 1988) |
| | Kashar | Cow | Artificially | 3.20 | 3.90 | (Oruc et al., 2007) |
| | Gouda | Cow | Naturally | - | 5.00 | (Sakuma et al., 2016) |
| | Gouda | Cow | Artificially | - | 2.80 | (Sakuma et al., 2016) |
| | Pecorino | Sheep | Naturally | - | 4.13 | (Pecorelli et al., 2018) |
| | Caciotta | Cow | Naturally | - | 5.16 | (Pecorelli et al., 2019) |
| **Hard** | Parmesan | Cow | Naturally | - | 5.80 | (Brackett & Marth, 1982) |
| | Grana Padano | Cow | Naturally | 1.50 | 4.50 | (Manetta et al., 2009) |
| | Maccagno | Cow | Artificially | - | 6.70 | (Cavallarin et al., 2014) |
| | Parmesan | Cow | Naturally | 4.60 | 5.50 | (Pietri, et al., 2016a) |

Alnaemi (2019) studied the fate of AFM1 in soft white cheese made with goat and sheep milk artificially contaminated. Significant differences were observed for the distribution of the toxin between cheese and whey produced with goat and sheep milk. A higher percentage of AFM1 was retained in sheep curd than in goat curd. This result may be explained with higher affinity of AFM1 to the $\alpha_{s1}$–casein fraction.

However, there are several factors that can influence the results, such as the composition and quality of milk and the production process. Additional investigation that allows the study of these interactions at the molecular level and also considering different types of milk can be helpful.

So, binding of AFM1 to proteins is expected, but the relative affinity to different proteins is not consensual. More studies are needed to elucidate if the driving force for the distribution of AFM1 between whey and curd is dominated by higher affinity for the caseins, or by the higher amount of caseins in milk (see Table 3, page 21). Assuming this last hypothesis, the highest concentration is not due to the affinity but rather to the total content of each protein fraction.

### 2.3.5 Effect of final pressing on AFM1 content

After the draining step, the curd is pressed into different moulds, which gives it the shape and promotes the expulsion of whey. After being moulded or hooped, the curd is subjected to final pressing, which aims to assist final whey expulsion (Tetra Pak, 2018). This loss of whey leads to the increase in the concentration of the AFM1. As discussed earlier, the moisture content can influence the concentration of toxin (Cavallarin et al., 2014).

### 2.3.6 Effect of salting on AFM1 content

The main functions of salt in cheese are related to conservation and flavour enhancement (Campagnollo et al., 2016). Motawee (2013) studied the effect of salting in Egyptian Domiati Cheese (soft cheese), a type of cheese in which the salt is added to the curd before pressing. The study consisted of analyzing cheeses made from milk containing 6 %, 8 % and 10 % salt. The levels of AFM1 in cheese curd were 2.73 μg/kg, 2.52 μg/kg and 2.31 μg/kg, respectively. On the other hand, the level of AFM1 in the corresponding whey was 0.36 μg/kg, 0.38 μg/kg and 0.40 μg/kg, respectively. So, they observed that the AFM1 level in cheese curd decreased with the increase of salt concentration, while the opposite happened for whey. Therefore, possibly the increase in the concentration of salt in curd can decrease the affinity of AFM1 for caseins and AFM1 remains in solution (i.e., in whey).

Oruc et al. (2006) found that only 2 – 4 % of the initial spiking of AFM1 transferred into the brine solution, after preparation of Turkish white-pickled cheese. Similarly, other studies reported that only a small percentage (about 2-3 %) of AFM1 passed to brine (Deveci, 2007; Oruc et al., 2007).

Motawee and McMahon (2009) studied the salting of feta cheese, which occurred in brine solutions of 8 %, 10 % and 12 % (w/w) salt, at 6 °C and 18 °C, for 60 days. They observed a significant reduction in AFM1 concentration (about 22–27.1 %) during brining, either at 6 °C and 18 °C, on the first 10 days,

keeping constant on the following days. In the other hand, they also observed that an initially higher salt concentration of the brine caused a slightly greater loss of AFM1. However, it is extremely difficult to understand and justify how it was possible to obtain such a high percentage of reduction.

So, the loss of a small percentage of AFM1 during brining of cheese can be explained by the fact of toxin being soluble in water and, as such, the concentration of AFM1 reduces as a result of diffusion into the brine solution (Johnson & Law, 2010; Tetra Pak, 2018).

### 2.3.7 Effect of ripening and storage process on AFM1 content

During the ripening stage physicochemical reactions and microbiological modifications occur with are important to the development of volatile organic compounds, texture and flavour in the cheese. Depending on the type of cheese, the storage can occur at room or refrigerated temperature.

Also, during this step, different findings on the content of AFM1 have been reported. On one hand, due to drying during ripening, increases in AFM1 concentration have been reported (Manetta et al., 2009; Pietri, et al., 2016a). In others studies, a reduction in AFM1 concentration has been reported (Colak, 2007; Govaris et al., 2001; Motawee, 2013), which can be attributed to the activity of enzymes and bacteria and also due to the diffusion to the brining, if the ripening stage occurs in this condition (McSweeney, 2004).

Fernandes et al. (2012) also studied the effect of storage on the AFM1 content and reported that on the 30$^{th}$ day of storage, the EF was 2.14 to 2.60. But during 30 days of storage, AFM1 levels were constant. Also in studies by Oruc et al. (2006), Deveci & Sezgin (2006), Deveci (2007), Iha et al. (2013) and Pecorelli et al. (2019) no changes in AFM1 concentration occurred, possibly due to a compromise between the above two explanations.

In any case, cheese manufacture processes do not eliminate AFM1. The Italian Ministry of Health established a provisional EF of 3.0 and 5.5 for soft and hard cheeses, respectively (Comitato Nazionale per la Sicurezza Alimentare, 2013). However, a wide range of EF values have been described for cheeses in the same hardness categories. The different results obtained for EF may be due to several factors as mentioned in Table 4.

Manetta et al. (2009) found a positive correlation between AFM1 concentration in milk and in Grana Padano cheese and suggested that AFM1 in milk could be a good predictor factor for the presence of this toxin in the final cheese. Also, in a study by Pecorelli et al. (2019), a positive correlation between AFM1 concentration in milk and in cheese was reported. They also studied other parameters [milk AFM1, milk quality parameters (protein, casein and fat contents), cheese AFM1, cheese parameters (protein,

fat, moisture, and cheese yield) and EF] and their correlation, and found that EF was not affected by the AFM1 level in milk. This study demonstrated that the EF is independent of the amount of toxin in milk, but is significantly correlated to milk quality parameters (protein, casein and fat) and cheese yield. Cheese yield corresponds to the cheese quantity produced from a known milk quantity, determined by the overall quality of milk (quantities of the milk constituents, e.g., fat and milk protein). So, it can explain why it is possible to observe different EFs in cheeses which has the same hardness category (moisture content of the cheese), even when the cheese is produced using milk with the same level of contamination.

On the other hand, the use of either naturally or artificially contaminated milk in experimental work, can be another factor that influences AFM1 distribution. According to Manetta et al. (2009), when AFM1 is artificially added to milk, the percentage of AFM1 bound to the protein might be lower than in natural contamination and may establish a different behaviour. This may be related to the fact that the toxin, when added, is not in contact long enough to interact with the caseins and therefore comes out with whey. Sakuma et al. (2016) made Gouda cheese using artificially and naturally contaminated milk to evaluate the EF obtained. The EF in cheese made with naturally contaminated milk was significantly higher than in cheese made using artificially contaminated milk. So, the use of naturally contaminated milk in experiments will possibly produce results closer to real production conditions (Cattaneo et al., 2008).

## 2.4 Legislation and Incidence of AFM1 in dairy products

Demand for dairy products is growing with rising incomes, population growth, urbanization and changes in diets. According to the OECD-FAO Agricultural Outlook 2021-2030 (2021), the world production of milk (approximately 81 % cow milk, 15 % buffalo milk, and 4 % for goat, sheep and camel milk combined) is projected to increase *ca.* 1.7 % yearly until 2030 (to 1 020 Mt), which is faster than for most other important agricultural commodities.

Consumption of dairy products has expanded rapidly over the past decade and constitutes an important source of dietary protein. The *per capita* consumption of fresh and processed dairy products is projected to increase *ca.* 13 to 15 kg on a worldwide basis, mostly due to the strong demand growth expected to occur in India and Pakistan, where the *per capita* consumption is projected to increase 27 and 42 kg/year, respectively, by 2030. On the other side, the *per capita* consumption is expected to be kept low in China and Sub-Saharan Africa (*ca.* 4 kg/year). More than 75 % of the dairy consumed in these countries is fresh milk. In the United States of America (USA) and Europe the consumption is expected

to increase 25 and 30 kg/year, respectively, by 2030, and more than 50 % of this is processed dairy products, mostly cheese. An increase in cheese consumption worldwide is expected, except in Australia and Argentina, and an increase in the consumption of butter is expected in the United States of America (USA), New Zealand, Europe and China (OECD-FAO, 2021).

Every year, approximately 9 tons of cheese are consumed in the EU. According to figures for projected cheese consumption *per capita* in the EU, by 2025 Europeans will eat around 16 kg of cheese (Statista, 2020). In 2020, Europe had the highest *per capita* cheese consumption (average of 18.44 kilograms), followed by the USA and Canada, with 17.4 and 14.3 kilograms of cheese *per capita*, respectively (Statista, 2021). Also in Portugal, the *per capita* consumption of cheese has been increasing since 2012 [10.2 kg/resident (2012) – 13.3 kg/resident (2020)] according to INE (2019).

So, AFM1 presence in milk and in dairy products is a major risk to humans because these products are regularly consumed in daily diet (Iqbal et al., 2015; Ketney et al., 2017). Because of health concerns, several national and international regulatory bodies have established the maximum permissible limits (MPLs) for AFM1 in milk and in dairy products (Jaiswal et al., 2018). The limits can change from country to country, as a result of their stage of development, different perceptions about the levels considered safe for health, or economic interests related to local cultures. Nonetheless, they are very important because they guarantee with more or less efficiency that highly contaminated products are not traded and introduced into the human food chain (Abrunhosa et al., 2016).

Aflatoxins are regulated in more than 80 countries and their legislation is not harmonized at the international level. For the above reasons, and because milk and milk – based products vary with the type of product, origin, storage and processing, this makes it impossible to set a standard legal limit that generally applies to all dairy products. So the maximum limit for AFM1 in milk and in dairy products range from 0 to 1.0 μg/kg (Table 5) (Iqbal et al., 2015; Manetta, 2011; Vaz et al., 2020).

In the EU, AFM1 in raw milk, heat – treated milk and milk for the manufacture of dairy products must not exceed 0.05 μg/kg for adult consumption and 0.025 μg/kg for food products meant for infants and young children (EC, 2010). On the other hand, the *Codex Alimentarius Commission*, and the USA (FDA, 2000) defined the limit of 0.5 μg/kg for milk. The EU has the most stringent regulations concerning mycotoxins in food (Espinosa-Calderón et al., 2011; Iqbal et al., 2015; Jaiswal et al., 2018).

Since the final levels of the toxin in dairy products will depend on the initial levels in milk, which in turn are directly related with the AFB1 levels in the feed, it is also important to consider the legislation regarding the MPLs for AFB1 in feed. In the EU the maximum legal level for AFB1 is 5 and 20 μg/kg, for

complete feeding stuffs for dairy animals and feed raw materials, respectively (EC, 2003). However, these limits are different for other regions as can be seen in

Table 6. Brazil, Barbados, Chile and Mexico are the countries where a higher limit has been established. On the other hand, the UE and Cuba present stricter legislation with lower limits.

Table 5. Regulation on aflatoxin M1 in milk and dairy products in different countries from (Vaz et al., 2020)

| **Country** | **Milk (µg/kg)** | **Dairy Products (µg/kg)** |
|---|---|---|
| Argentina | 0.05 | 0.50 (milk products); 0.25 (cheese) |
| Brazil | 0.5 | 5 (milk powder); 2.5 (cheese) |
| China | 0.5 | 0.5 (milk products) |
| Egypt | 0 | 0 |
| EU | 0.05<br>0.025 (food products meant for infants and young children)<br>Austria<br>0.01 (pasteurized infant milk)<br>France<br>0.03 (for children <3 years) | Italy<br>0.25 (soft cheese); 0.45 (hard cheese)<br>Austria<br>0.020 (butter); 0.25 (cheese); 0.40 (milk powder)<br>The Netherland<br>0.020 (butter); 0.020 (cheese) |
| Honduras | 0.05 | 0.250 (cheese) |
| Iran | 0.05 | 0.50 (milk powder); 0.020 (butter and butter milk); 0.250 (cheese) |
| Nigeria | 1 | - |
| Switzerland | 0.05 | 0.25 (cheese) |
| Turkey | 0.05 | 0.25 (cheese) |
| USA | 0.5 | - |

Considering that the final levels of AFM1 in milk and dairy products depend on the initial levels of AFB1 in the primary production, the exposure levels of dairy animals to AFB1 have been studied by the European Food Safety Authority (EFSA) with the aim of defining the carry-over from feed to milk, to assess the level of feed contamination resulting in unacceptable levels of AFM1 in milk. In 2007, the maximum levels found in animal feed prevented undesirable concentration of AFM1 in milk (EFSA, 2007).

It is well known that this contamination is strictly dependent on the weather conditions during crop growing and can therefore change significantly between years (EFSA, 2007, 2012; Paterson & Lima, 2010). Previous studies demonstrated that there is a trend towards higher levels of AFM1 in milk in winter season, which can be related to the feed administered. During winter season most of the feed administered is stored cereals and not fresh forage, which can increasing the risk of AFB1 contamination (Mahmoudi and Norian 2015; Tomašević et al. 2015; Bahrami et al. 2016). The sources of AF contamination in feed vary by country. For this reason, the incidence and occurrence of AFM1 contamination in milk and dairy products will also depend on the country of origin (Iha et al., 2013).

Table 6. Regulation on total of aflatoxins in complete feedings stuffs and in feed raw materials in different countries

| Country | Complete feeding stuffs Total aflatoxins (µg/kg) | Feed raw materials Total aflatoxins (µg/kg) |
|---|---|---|
| Barbados [1] | 50 | - |
| Brazil [2] | 50 | 50 (Hay cotton peanut, rice, oats, residues of bird bowels, babassu, cocoa, sugar cane [residue-pulp], linseed, dende, manioc, sunflower, crisálidas, malt, wheat, soya, yeast [sugar cane subproduct]) |
| Canada [1] | 20 | 20 (Barley) |
| Chile [1] | 30 (for poultry, pigs and cattle)<br>10 (for animals other than poultry, pigs and cattle) | 50 (All ingredients for use in animal feed except peanuts and derivatives, cottonseed and derivatives, maize and derivatives)<br>200 (Peanuts and derivatives, cottonseed and derivatives, maize and derivatives for animal feeds) |
| China [1] | 10 (Supplementary feeding stuffs for dairy cattle) | 50 (Corn, peanut meal, cottonseed meal, rapeseed meal)<br>30 (Soybean meal) |
| Colombia [1] | 50 (Bovine, pig feeds) | – |
| Cuba [1] | 5 | 5 (all feed ingredients) |
| El Salvador [1] | 10*<br>20* ( supplementary feeds for porcine/poultry/dairy cattle; single composite feedstuffs; bovine/caprine/ovine feedstuffs) | – |
| EU [3] | 5 (for dairy animals) | 20 |
| Japan [1] | 10* (Formula feed for dairy cattle) | 20 (corn) |
| Mexico [1] | 0 (for dairy cattle/poultry) | 200 (cereals for bovine and porcine fattening feedstuffs) |
| Philippines [1] | – | 50 (raw corn grains or corn grits used for feed) |
| Suriname [1] | 30 | – |
| United Arab Emirates [1] | 10 (feeds for dairy cattle and small calves)<br>20 (poultry feeds and other animal feeds) | – |
| USA [4] | 20 | 20 (barley, corn/maize, oat, rice, rye, sorghum and wheat) |
| Venezuela [1] | 20 | – |

* Limit for AFB1; [1] Other data from (VICAM, n.d.); [2] Data from (Diário Oficial República, 2011);[3] Data from (EC, 2010);[4] Data from (FDA, 2000)

While looking at emerging risks in food and feed production, EFSA identified changes in the pattern of mycotoxin occurrence in cereals due to climate change as a relevant topic (EFSA, 2012). With expected increasing temperature and decreasing rain, *Aspergillus* species will find more suitable conditions for their development. Some studies had demonstrated the possible increase of AFB1 on maize and consequently AFM1 on milk due to climate change (Battilani et al., 2016; Van der Fels-Klerx et al., 2019).

Additionally, it is quite difficult to screen all the production with a very high prevalence of contaminated lots. So the toxicological characteristics and potential exposure of the general population, including children, make AFM1 a priority issue for the dairy chains (Frazzoli et al., 2017).

## 2.5 Methods for reducing aflatoxin M1 content in dairy products

The most effective strategies to reduce the accumulation of AFM1 in milk and consequently in milk products are pre- and post – harvest interventions to counteract AFB1 accumulation in crops and raw materials intended for feed production (FAO/WHO, 2014; Pinton et al., 2019).

To prevent the contamination with AFB1, in feed of dairy animal during both crop development and post-harvest storage, biological control (biocontrol) can be implemented as a good way. Biocontrol in the field is possible using different organisms, like bacteria (Nazareth et al., 2019), yeasts (Persons et al., 2013), and nontoxigenic *Aspergillus* strains (Atehnkeng et al., 2008) to reduce the incidence of toxigenic strains of *Aspergillus* in susceptible crops, thus reducing AFs contamination (Mwakinyali et al., 2019). Post-harvest biological control of AF can also be achieved through the use of probiotic microorganisms inoculated into stored goods that will prevent growth and AF production of phytopathogenic fungi and toxin secretion in feed (Giovati et al., 2015). In addition, the accumulation of AFM1 in milk can also be mitigated by reducing the gastrointestinal absorption of AFB1 by lactating animals, by administering enterosorbent agents such as dietary clay minerals through the diet (Rodrigues et al., 2019). Another way to prevent contamination is to vaccinate dairy animals against AFs (Giovati et al., 2014)

Considering the above mentioned evidences, preventative approaches are essential to inhibit the appearance of AFM1 in milk. However, this control is important to reduce the toxin but will not remove it completely. Thus, other methods for mitigation of AFM1 in milk should be considered (Naeimipour et al., 2018). Physical strategies, chemical methods and biological degradation are the three detoxification ways used up to now to reduce AFM1 contamination in dairy products.

Regarding **physical strategies**, most of the studied methods for aflatoxin reduction involve thermal degradation. Several studies have been carried out to evaluate the stability of AFM1 during heat treatment, but aflatoxins showed to be very resistant at high temperatures, turning this type of treatment ineffective in AFM1 control (Bakirci, 2001; Dua et al., 2012; Istamboulié et al., 2016; Kafle et al., 2012; Sifuentes dos Santos et al., 2016).

The use of adsorbents like bentonite and activated carbon have been used to reduce AFM1 in milk. Carraro et al. (2014) studied the detoxification capacity of bentonites in bovine milk, and demonstrated

that contaminated milk with approximately 80 ng/L was purified to safe levels, 50 ng/L (according to the European regulation), with moderate changes in nutritional properties.

**Chemical methods** are other approaches that can reduce AFM1 in milk and include ammoniating, acidic treatment, oxidizing, and reducing techniques. Mohammadi et al. (2017) demonstrated that AFM1 was reduced by 50 % without significant changes of milk composition. However, this type of approach to reduce AFM1 is not currently used or studied.

Physical and chemical methods are rarely applied due to alteration of food composition and palatability and, besides that, their use is expensive (Naeimipour et al., 2018). However, adsorbents are an exception and are currently employed (Assaf et al., 2019; Cha et al., 2021).

**Biological methods** based on bacteria, enzymes and yeasts for detoxification are being widely studied. The AFM1 reduction using lactic acid bacteria (LAB) and/or yeasts is based on an adsorption process instead of degradation (binding between bacteria/yeast cell walls and aflatoxins - reversible bond) (Assaf et al., 2019). On the other hand, enzymes from microbial species can reduce this toxin through degradation (lactone hydrolysis), leading to a structural change in the aflatoxin molecule and, consequently, to the reduction of its biological toxicity (Tran et al., 2020).

Corassin et al. (2013) evaluated the ability of a *Saccharomyces cerevisiae* strain and a pool of three LAB strains (*Lactobacillus rhamnosus, Lactobacillus delbrueckii spp. bulgaricus* and *Bifidobacterium lactis*), alone or in combination, to bind AFM1 in UHT skim milk. The mean percentages of AFM1 bound was higher (100 %) when the yeast and bacteria were used together, during 60 min, comparing to the yeast alone (92.7 %) and the bacteria pool (11.7 %). Yeast, *Saccharomyces cerevisiae,* was also used in a study by Foroughi et al. (2018). They immobilized the yeast on perlite support to detoxify milk contaminated with AFM1. The results obtained were very promising. About 81 % of AFM1 content was reduced from milk that circulated through the biofilter during 80 min, without changes of milk physicochemical characteristics.

Loi et al. (2016) also used a biological method to reduce AFM1. This study elucidated the degrading activity of Lac2 pure enzyme from *Pleurotus pulmonarius* on AFB1 and AFM1. Regarding AFM1, it was completely degraded by Lac2 with all mediators at 10 mM. These results open new perspectives for Lac2 application in food (Cabral Silva & Venâncio, 2020). Sarlak et al. (2017) explored the effects of probiotic strains on the reduction of free AFM1 in fermented milk drinks. Results showed that the probiotic *Lactobacillus acidophilus* was able to reduce free AFM1 in 98 %.

Biological methods have good perspectives, but these practices cannot yet be currently adopted

on a commercial scale for food (Ismail et al., 2018). More studies are needed to explore the binding principle between AFM1 and microorganisms and factors that may enhance the binding capacity and reduce AFM1.

Also, studies in specific food matrices are crucial since the effect of matrix can compromise the action of enzymes or microorganisms. In this sense, the effectiveness of a method in a given product must not be extrapolated to other products, taking into account the intrinsic diversity of the processing steps. Thus, more studies on the effective mitigating strategies are important, being also important to evaluate the organoleptic properties after any treatment. Nevertheless, processing milk with a low content of mycotoxins is the best way to ensure the low contamination levels in the final processed milk products (Tran et al., 2020; Pinton et al., 2019). Therefore, widespread and frequent monitoring programs performed with accurate and reliable analytical techniques still remain the primary means of protecting milk consumers from mycotoxin intake.

## 2.6 Detection methods for Aflatoxin M1 in Dairy Products

Due to the toxicity of AFM1 and considering the current maximum residue levels set, its detection and quantification at very low levels is of the utmost importance. There are many methodologies that have been developed for identification and quantification of AFM1 in milk and dairy products.

The purpose of the aflatoxin analysis restricts the choice of the method, being important to define the level of detail of the analysis:

- if it is intended to detect the presence of the analyte in the sample, rapid methods can be used;
- if it is intended to detect and quantify the analyte, quantitative methods must be used.

Usually, the analytical procedures follow the following steps: sampling, sample preparation (which can include extraction and clean-up (purification)), and analysis (identification and quantification) (Hussain, 2011; Manetta, 2011; Whitaker, 2004).

### 2.6.1 Sampling

Sampling and sample preparation are of utmost importance in the analytical identification of aflatoxins. To determine aflatoxins at the parts-per-billion level, systematic approaches to sampling, sample preparation and analysis are absolutely necessary. The sampling step specifies how the sample will be collected from the bulk lot as well as its size (Whitaker, 2004). It is important to obtain a

representative sample, which involves the collection of different subsamples to obtain an aggregate sample as representative as possible of the entire lot. Increasing sample size, the degree of sample comminution, subsample size, and the number of aliquots quantified can reduce the variability associated with a mycotoxin test procedure (Whitaker, 2004).

The European Commission Regulation 401/2006 (EC, 2006a) lays down the methods of sampling and analysis for the official control of the levels of AFM1 in milk. The sampling determines that an aggregate sample of at least 1 liter (or kg) should be prepared, from the collection of 3 to 10 incremental samples of at least 100 g.

In the case of milk, due to the homogeneous distribution of aflatoxins in liquid milk, there is less uncertainty in aflatoxin measurement. In the case of solid commodities, as cheese, mycotoxins are usually not evenly distributed, as they can accumulate in 'hot-spots', contrarily to AFM1. It is thus important to obtain a homogeneous sample. So, the entire aggregate sample must be ground and mixed, so that the analytical test portion has the same concentration of toxin as the original sample.

### 2.6.2 Quantitative Methods

#### 2.6.2.1 Sample Preparation

In dairy products, AFM1 extraction is challenging and, typically, requires pre-treatment steps in order to remove fat and other impurities. So, centrifugation and filtration represent key stages in the extraction process (Al-Mossawei et al., 2016). For milk samples, these two processes are usually sufficient before cleaning and determination, and no use of solvents is required (Shuib et al., 2017; Yoon et al., 2016). For cheese samples, preparation may also involve slurry preparation (Pamela et al., 2019). After proper sampling and sample pre–treatment, different steps of extraction and clean–up are essential to determine and quantify the analyte of interest. Extraction and clean–up may be performed in one step (Nicolás Michlig et al., 2016) or in separate steps (Yoon et al., 2016).

#### 2.6.2.2 Extraction

The purpose of extraction is to remove as much mycotoxin from the matrix as possible, in an adequate solvent (chloroform, dichloromethane) or mixture of solvents (aqueous mixtures of polar organic solvents as methanol, acetone, acetonitrile), for partial purification and later determination. Aqueous mixtures are being increasingly used, not only for environmental reasons, but also because of its higher compatibility with subsequent clean–up steps (Ketney et al., 2017; Manetta, 2011).

The AFM1 extraction from cheese is generally carried out using organic solvents, like an

acetonitrile-water mixture (Škrbić et al., 2015) or a methanol-water mixture (Iha, Barbosa, & Favaro, 2011). But, chlorinated organic solvents like dichloromethane (Al-Mossawei et al., 2016) and chloroform (Fernandes et al., 2012) can also be used. The use of organic solvents usually requires a subsequent clean–up step before determination.

Innovative methods were developed by Pietri et al. (2016a), based on an enzyme assisted extraction using a cocktail of different enzymes (pepsin or pepsin–pancreatin). By using this approach, a liquefied cheese sample was obtained. In the work of Pietri et al. (2016a), this extract was further analysed as a milk sample (applied in Immnunoaffinity Columns (IACs), and determined by HPLC–FLD).

In comparison with the classical extraction with chloroform, extraction with enzymes was simpler, avoiding partition in a separating funnel, solvent evaporation, and dissolution; it resulted in higher recoveries, comparable LOD (limit of detection) and LOQ (limit of quantification), and more accurate results. In addition, the method does not use chlorinated solvents, resulting in considerable environmental advantage.

Extraction with solid sorbents (as solid phase extraction – SPE, magnetic-solid phase extraction – MSPE), or the Quick, Easy, Cheap, Effective, Rugged, and Safe method – QuEChERS – are alternatives to the use of organic solvents.

The SPE is based on the modern chromatography principles: the sample is loaded on a cartridge packed with a selective adsorbent material, on which the analytes to be detected are adsorbed, and then separated by elution with a suitable solvent, usually an organic solvent. This technique provides extraction and purification of the analyte of interest before instrumental analysis (Hussain, 2011; Ketney et al., 2017; Manetta, 2011). Application of SPE column was used by different authors to extract AFM1 from milk and cheese samples as shown in Table A.1 – Appendix to Chapter 2.

A simple and sensitive method using MSPE followed by spectrofluorimetric detection was developed by Hashemi et al. (2014) for separation and determination of AFM1 in milk. This method is based on the extraction of AFM1 using magnetic nanoparticles (MMNPs), coated with 3-trimethoxysilyl-1-propanethiol (TMSPT) and modified with 2-amino-5-mercapto-1,3,4-thiadiazole (AMT).

Alternatively to SPE, Molecularly Imprinted Polymers (MIP) are synthetic materials with recognition sites that specifically bind target molecules in mixtures with other compounds (Regal et al., 2012). In a MIP–based SPE, the imprinted bulk polymer is packed in a cartridge, column, or extraction well plates (for high throughput analysis). This process is fast, consume less solvent and enable selective clean–up of the analytes (Ashley et al., 2017). The MIPs, in contrast to classical SPE sorbents, are more selective

and allow the elution of analytes from the cartridges virtually free from co-extracted compounds (Figure 7) (Regal et al., 2012).

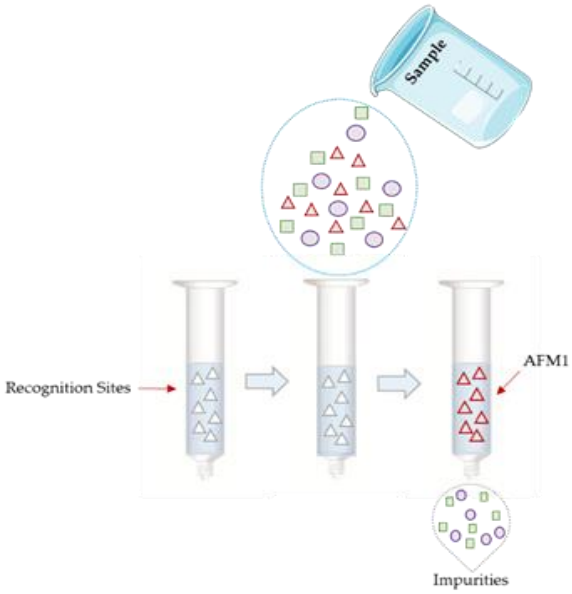


Figure 7. Molecular imprinting process from (Vaz et al., 2020).

Díaz-Bao et al. (2016) developed a quick and easy method for the fabrication of magnetic molecularly imprinted stir–bars (MMIP-SB), using a combination of imprinting technology and magnetite, for the analysis of AFM1 in milk powder (infant formulas). The method had a recovery of 60 %, and a limit of quantification of 0.001 μg/kg. However, this methodology is still recent and more studies about its applicability in dairy products are required.

QuEChERS can be used as an alternative to other extraction methods. It was developed for the extraction and purification of pesticide residues from fruits and vegetables by Anastassiades, Lehotay Stajnbaher, and Schenck (2003). However, due to its simplicity, it has been adapted for other analyses (Arroyo-Manzanares et al., 2014; Nicolás Michlig et al., 2016; Sun et al., 2016).

QuEChERS method includes two steps: an extraction/partitioning step using acetonitrile and salts, followed by a clean–up step based on a dispersive solid–phase extraction (dSPE) (Arroyo-Manzanares et al., 2014; Zhang et al., 2018). Following the acetonitrile extraction step different sorbents such as octadecyl silica (C18), primary secondary amine (PSA), and graphitized carbon black (GCB) can be used for additional purification, which can result in satisfactory recoveries due to the reduced matrix effect (Sun et al., 2016). C18 is used to remove long chain fatty compounds, sterols and proteins; PSA is efficient in the removal of sugars, fatty acids and organic acids; and GCB is a strong sorbent for removing pigments,

as chlorophyll, polyphenols and other polar compounds (Chuang et al., 2015). Therefore, sorbents for clean-up must be selected in accordance with the composition of the sample to be extracted. In some cases, more than one type of sorbent, like PSA and C18, may be applied for sample cleaning, as with milk samples (Nicolás Michlig et al., 2016; Rodríguez-Carrasco et al., 2018).

Michlig et al. (2016), Rodríguez-Carrasco et al. (2018) and González-Jartín et al. (2021) used the QuEChERS method as a preliminary step for AFM1 determination in raw milk and its performance was in compliance with applicable EU validation guidelines and current MRLs of most milk food regulations. Thus, this method could replace or complement existing IAC approaches for enhancing throughput and decreasing costs to improve monitoring of AFM1 in milk.

#### 2.6.2.3 Clean–Up

The main objectives of the clean-up step are the elimination of the matrix interferences and analytes preconcentration (Ketney et al., 2017). Usually, clean–up is applied after extraction to get more accurate and precise results, but it is not always required (Manetta et al., 2005; Škrbić et al., 2015). For most of the rapid methods based on immunochemical techniques, the diluted extracts can be used directly for analysis (Norian et al., 2015; Sarimehmetoglu et al., 2004).

Currently, commonly used purification methods employ IAC or one-step multifunctional clean-up columns (Mycosep™) (Figure 8). These techniques provide several advantages. One example is the analysis of milk samples, after pre-treatment to defat but without any extraction step, directly in the IAC columns for analysis the AFM1 content. However, in case of viscous or solid samples (cheese) it is always necessary to have an extraction step.

Immunoaffinity Columns (IAC) are a very efficient technique of purification: it is based on the recognition of the toxin by a specific antibody. Although IAC are easy to use and have high selectivity, they are single use because of the denaturation of antibodies during the elution step, and as such the costs are high. The SPE is cheaper than IAC (Hussain, 2011; Ketney et al., 2017; Manetta, 2011).

The Mycosep™ multifunctional clean-up columns consist of a number of adsorbents (charcoal, celite, ion exchange resins and others) which are packed in a plastic tube. Almost all interfering substances are retained in the column, whereas the analyte does not show significant affinity to the packing material. Mycocsep™ columns efficiently remove matrix components and can produce a purified extract within a short time, sample purification is achieved in 10–30 s. This rapid and efficient purification supersedes and represents an alternative to conventional SPE or IAC methods which typically require three to four steps (Hussain, 2011; Iqbal et al., 2015).

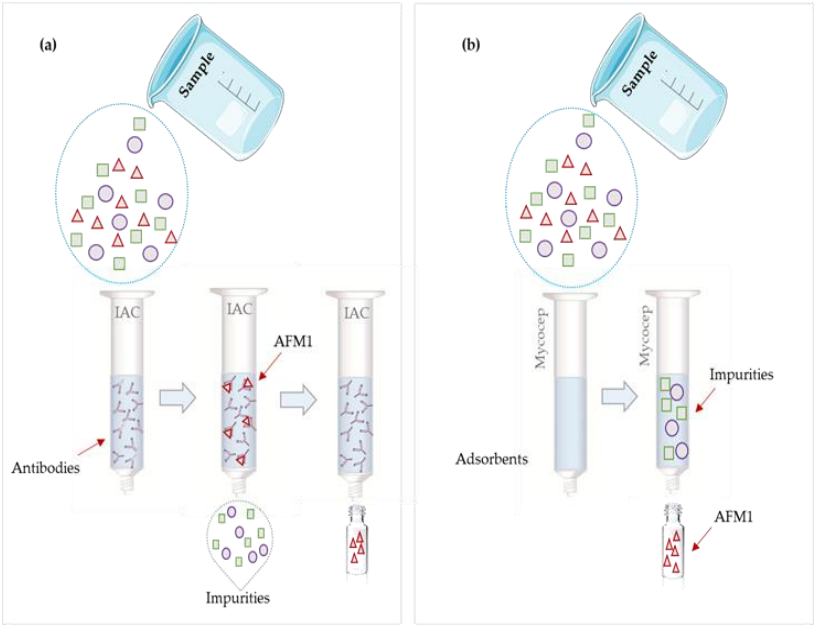


Figure 8. (a) Scheme of immunoaffinity column (b) Scheme of Mycosep TM from (Vaz et al., 2020).

The big difference between the Mycosep™ columns and the SPE columns or IACs is that in Mycosep columns the analyte is eluted and all the other interfering contaminants are retained, while in the other columns the analyte is retained (Figure 8) (Hussain, 2011; Iqbal et al., 2015).

However, the IAC method has proved to be a robust technique for the purification, separation and concentration of AFM1 in milk and dairy products (Grosso et al., 2004; Iha, Barbosa, & Favaro, 2011; Ketney et al., 2017; Öztürk Yilmaz & Altinci, 2019).

Iha et al. (2011) developed and validated a method using IAC as a clean–up column for the determination of AFM1 in cheese, yogurt, and dairy beverages. The recoveries of AFM1 ranged from 61 to 86 % without correction for water content and between 67–101 % with correction for water content. Besides that, the RSDr was in the range of 2–12 %. Thus, the performance of this method was found to be similar to that of the Association of Official Analytical Chemists (AOAC) Official Method for AFM1 in milk (AOAC Official Method 2000.08 - Aflatoxin M1 in Liquid Milk, 2002).

#### 2.6.2.4 Quantification

After the extraction and clean–up steps (when applied), aflatoxin must be quantified. During the past decade, several methods have been used or developed for quantification of AFM1 in dairy products (Mulunda et al., 2013).

TLC is a standard AOAC method for aflatoxin analysis since 1990. It is widely used in laboratories

throughout the world for qualitative analysis and quality control of food products (Espinosa-Calderón et al., 2011; Iqbal et al., 2015; Rahmani et al., 2009).

Improvements in TLC led to the development of high-performance thin-layer chromatography (HPTLC). The most important differences between TLC and HPTLC are: the different particular size of the stationary phase; the care used to apply the samples; and the way to process the obtained data (Espinosa-Calderón et al., 2011; Ketney et al., 2017). Another variation in TLC is the use of overpressured layer chromatography (OPLC), which is a technique designed to integrate the benefits of HPLC and TLC (Saeger, 2011).

The weakness of the TLC method is that it is challenging in the determination of mycotoxin concentrations with exactness (Mulunda et al., 2013). So, despite screening methods based on TLC are applied on a large scale for AFM1 in milk (Fallah, 2010; Kamkar, 2005), these are used in only a few laboratories because they do not provide an adequate LOQ (Espinosa-Calderón et al., 2011; Ketney et al., 2017).

Filazi et al. (2010) analysed fifty samples of cheese for the occurrence of AFM1 using TLC, as a semi–quantitative method. The presence of AFM1 was detected in concentrations between 0.02 to 2 μg/kg in 14 of 50 samples (28 %), being that the lowest detection limit of the method was 0.02 μg/kg and the recovery was 85.6 %. The amount of aflatoxin was estimated visually by comparing with standards, and the identity was confirmed by derivatization with trifluoroacetic acid. Altogether, five cheese samples (10 %) were found to have levels that exceed the legal limits of 0.250 μg/kg established by the Turkish Food Codex.

In case of official control analyses, the methods of analysis for determining AFM1 in milk should be able to detect traces of AFM1 at the level of ng/kg. This performance criterion has been accomplished using the IAC column purification step, followed by LC separation and fluorescence detection. Thus, after numerous interlaboratory testing, this method was published in the standard ISO 14501:2007 (Ketney et al., 2017).

Liquid chromatography (LC) is a technique that provides good sensitivity, high dynamic range and versatility. Detection by LC is usually made by fluorescent detection (FLD), UV absorption or mass spectrometry detection (Espinosa-Calderón et al., 2011).

Liquid chromatographic methods for aflatoxins determination include both normal and reverse–phase separations. However, most current methods use reverse–phase HPLC, with mixtures of methanol, water and acetonitrile as mobile phases (Espinosa-Calderón et al., 2011; Mendonça & Venâncio, 2005).

Reverse–phase eluents quench the fluorescence of mycotoxins like AFM1; for this reason, chemical derivatization can be necessary, using pre– or post-column derivatization. The pre–column approach uses the formation of the corresponding hemiacetals using trifluoroacetic acid (TFA), while post–column derivatization makes use of a reactive halogen like a iodine or bromine (Espinosa-Calderón et al., 2011; Hashemi et al., 2014; Ketney et al., 2017).

A post–column derivatization method analytically equivalent to iodination and bromination is the photochemical one: it is based on the formation of hemiacetals of AFM1 under UV light (Figure 9) (Espinosa-Calderón et al., 2011; Shuib et al., 2017).

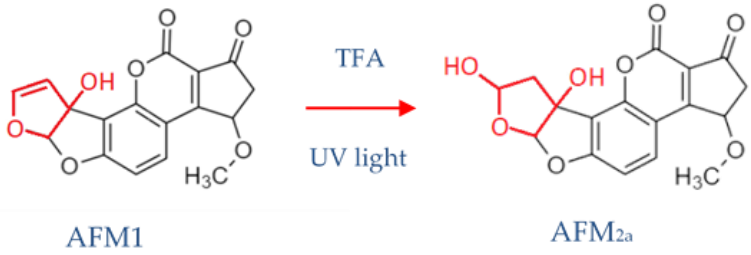


Figure 9. Reaction of AFM1 with TFA and UV light from (Vaz et al., 2020).

Manetta et al. (2005) used an HPLC method with fluorescence detection using pyridinium hydrobromide perbromide as a post-column derivatizing agent to determine AFM1 in milk and cheese. The detection limits for milk and cheese were 0.001 µg/kg and 0.005 µg/kg, respectively; and the average recoveries were 90 and 76 %, respectively. Also, the precision (RSDr) ranged from 1.7 to 2.6 % for milk and from 3.5 to 6.5 % for cheese. The tested method proved to be simple and easily automatable, and therefore useful for accurate and precise analysis of AFM1 in milk and cheese.

Shuib et al. (2017) described the determination of AFM1 in milk and dairy products using IAC and HPLC with photochemical post–column derivatization and fluorescence detection. They reported a reduction in LOD and LOQ of about one third with derivatization, achieving 0.0085 µg/L and 0.025 µg/L, respectively. These limits were further improved when the IAC eluate was evaporated and reconstituted with mobile phase (to 0.002 and 0.004 µg/L, respectively). The method was statistically validated, showing linear response ($r^2$ > 0.999), good recoveries (85.2–107.0 %), and relative standard deviations (RSD) of ≤ 7 %. The proposed method was applied to various types of milk and dairy products. Only 2 samples (10 % incidence) were positive for AFM1, even though at lower levels than the Malaysian and European legislation limits.

Iha et al. (2011) aimed to investigate the incidence of AFM1 in dairy products from Brazil. A total of 123 samples of three different groups of dairy products, including 58 cheese samples, 53 yoghurt

samples and 12 dairy drinks were purchased from grocery stores in the *Ribeirão Preto – São Paulo* state. Cheese samples were classified according to their moisture and fat contents, and were analysed by aqueous methanol extraction, IAC purification, and reverse phase LC with fluorescence detection. AFM1 was detected (> 3 ng/kg (LOD)) in 49 cheese samples. Thirty-nine (39) of the cheese samples were contaminated with AFM1 in the range of 0.010 to 0.304 μg/kg. In yogurt and dairy drinks, AFM1 was detected in 47 yoghurt samples and in 10 dairy drinks, at levels ranging from 0.010 to 0.529 μg/kg, and 0.01 to 0.05 μg/kg, respectively.

The introduction of mass spectrometry (MS) and the subsequent coupling of LC to this very efficient system of detection and identification have resulted in the development of many LC–MS or LC–MS/MS methods for AFM1 analysis in dairy products (Camaj et al., 2018; Campone et al., 2016, 2018; Škrbić et al., 2015). The MS technique is used for confirmation because it has the advantage of producing spectra with characteristic fragmentation patterns (Ketney et al., 2017). There are several types of instruments which can be used: single quadrupole, triple quadrupole, and linear ion trap. Ion trap instruments are more suitable for identification than triple quadruple instruments (higher $MS^n$ power), whereas triple quadruple instruments provide better information for quantification with faster scanning and higher sensitivity (Hussain, 2011; Manetta, 2011).

Many LC–MS or LC–MS/MS methods comprise a single liquid extraction and direct instrumental determination without a clean-up step (Škrbić et al., 2015). This is possible due to the ability of the mass analyser to filter out by mass any co-eluting impurities. However, ionization suppression can occur by matrix effects, and many authors assert that LC-MS analysis would benefit from a sample preparation (clean–up) step (Cavaliere et al., 2006; Chen et al., 2005; Manetta, 2011).

In a recent study by Hung et al. (2014), a sensitive and rapid method was developed for the simultaneous determination of AFM1, ochratoxin A (OTA), zearalenone and $\alpha$-zearalenone in milk by ultra–high performance liquid chromatography combined with electrospray ionization triple quadrupole tandem mass spectrometry (UHPLC–ESI–MS/MS). The LOQ for the mycotoxins were in the range of 0.003–0.015 μg/kg. The high correlation coefficients ($r^2 \geq 0.996$) obtained in the range of 0.01–1.00 μg/kg of the mycotoxins, along with the good recovery (87.0–109 %), repeatability (3.4–9.9 %) and intra-laboratory reproducibility (4.0–9.9 %) at the concentrations of 0.025, 0.10 and 0.50 μg/kg, suggested that the method is adequate for simultaneously determining AFM1, OTA, zearalenone and α-zearalenone in milk (Ketney et al., 2017).

The level of AFM1 was investigated in 54 samples of white and hard type of cheeses produced in

Serbia using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS). Two methodologies were developed for sample extraction, separately for white and hard cheese. White cheese samples were prepared by crude extraction, i.e. the sample extraction was done with acetonitrile / water mixture (86:14, v / v) and the extracts were passed through a syringe filter before injection without sample cleaning step. On the other hand, extraction for hard cheese samples was performed with dichloromethane and acetone, followed by a SPE step prior to analysis by UHPLC-MS/MS. The average recoveries of AFM1 were 73–111 % and the precision (RSD) ranged from 7 to 9 %, for the first method; whilst for the second method the average recoveries were 71-80 %, with RSD ranged from 4 to 10 %. Due to different matrix effects, the LOQ were 0.125 µg/kg and 0.020 µg/kg, for white and hard type of cheeses, respectively. Seven samples (13 %) exceeded the maximum acceptable level of 0.25 µg/kg that has been established for AFM1 in some European countries, as shown in

Table 6 (page 30) (Škrbić et al., 2015).

### 2.6.3 Rapid Methods

Bioassays have become increasingly useful for mycotoxin detection as a rapid screening procedure before chemical analysis (Hymery et al., 2014). Screening assays used for detection of AFM1 are mainly immunochemical methods, including enzyme-linked immunosorbent assays (ELISAs), immunochemical assay involving detection by electrochemiluminicesce (ECL–IA), ELISA using fluorimetric detection, and, more recently, biosensor assays (Matabaro et al., 2017; Santos et al., 2019).

#### 2.6.3.1 Sample Preparation

Immunoassay is an analytical method based on an antibody-antigen (Ab–Ag) reaction, requiring a sample preparation step. The treatment used depends on the matrix and its complexity. In the case of milk, it is only necessary to centrifuge the samples to degrease and the skimmed milk is used directly in the test (Weiss et al., 2016). For cheese, it is necessary, additionally, to use an extraction step with an organic solvents, such as 70 % methanol and hexane to remove interferences of matrix like fat (r-biopharm, 2015).

#### 2.6.3.2 Determination

Immunoassays use specific antibodies to detect immunogens, which contain the targeted chemical structures (Matabaro et al., 2017). Among screening methods, the ELISA has been the most used one for AFM1 in different food matrices, such as pasteurized and Ultra High Temperature (UHT) milk, infant

formula, powdered milk, yoghurt, ice cream and cheese, due to its simplicity, sensitivity and adaptability (Table A.1– Appendix to Chapter 2) (Hussain, 2011; Ketney et al., 2017; Mohamadi Sani et al., 2012; Motawee & McMahon, 2009; Sarimehmetoglu et al., 2004). A number of commercially available ELISA kits based on a competitive immunoassay format are widely used.

There are two types of competitive ELISA: direct competitive ELISA and indirect competitive ELISA (Figure 10). In the direct competitive assays, the wells of the microtiter plate are coated with a specific antibody for the analyte under analysis. After addition of sample, the analyte competes with an enzyme labelled analyte to bind with a restricted amount of antibodies. After incubation, unbound compounds are washed off and a chromogenic substrate is added for colour development. The measurement is made photometrically in an ELISA reader. The enzymatic activity in each well is inversely proportional to the aflatoxin concentration in the sample, i.e., the lower the absorbance, the higher the aflatoxin concentration. This happens because the higher the concentration of mycotoxin, the less the conjugate (enzyme labelled analyte) will react with the bound antibody, leading to fainter colour development (Hussain, 2011; Manetta et al., 2005; Saeger, 2011). In the case of the indirect competitive assay the analyte or its analogue, conjugated with a macromolecular carrier (e.g. BSA – Bovine Serum Albumin or OVA – Ovalbumin) is coated onto the well in the microtiter plate during incubation. Then the sample and the specific antibody are added to each well. So, the immobilized analyte and the analyte present in the sample will compete to bind with the antibody in solution. The amount of bound specific antibody is detected, after a washing step, through a secondary antibody, labelled with an enzyme. This approach makes it possible to simplify immunoreagents preparation because there are commercially available enzyme–labelled secondary antibodies (e.g. labelled with horseradish peroxidase (HRP) or alkaline phosphatase (AP)). However, it includes an additional step that can be eliminated by direct labelling of the specific antibody (Saeger, 2011). In aflatoxin analysis, direct competitive ELISA are usually used (Hussain, 2011). Tavakoli et al. (2012) used this method to determine the occurrence of AFM1 in 50 white cheese samples. Aflatoxin M1 was found in 60 % (30/50) of the cheese samples, ranging from 0.0409 to 0.374 μg/kg.

Despite its simplicity, ELISA shows some disadvantages, as long incubation periods and several washing and mixing stages. Based on this, in recent years several modified ELISA methods have been developed for improved detection of AFM1 in milk and dairy products (Ketney et al., 2017; Matabaro et al., 2017). Vdovenko et al. (2014) developed a competitive immunological assay of chemiluminescence (CL ELISA) for detection of AFM1. To improve the method's sensitivity, a mixture of 3-(10-phenotiazine)-

propane-1-sulphonate (SPTZ) and 4-morpholinopyridine (MORPH) was used to increase peroxidase induction. The limit of detection and the dynamic working range were 0.001 and 0.002–0.0075 µg/L, respectively; so, a 20-fold dilution of the milk samples was required. This prevented interferences from the milk matrices and allowed the measurement of AFM1 at concentrations that were below the maximum limit accepted. The recovery range was between 81.5–117.6 % for within assay and 86–110.6 % for between assay (Ketney et al., 2017; Matabaro et al., 2017). Considering that the recovery is higher than the recommended maximum limit of 110 % (EC, 2006a), a method optimization is still required.

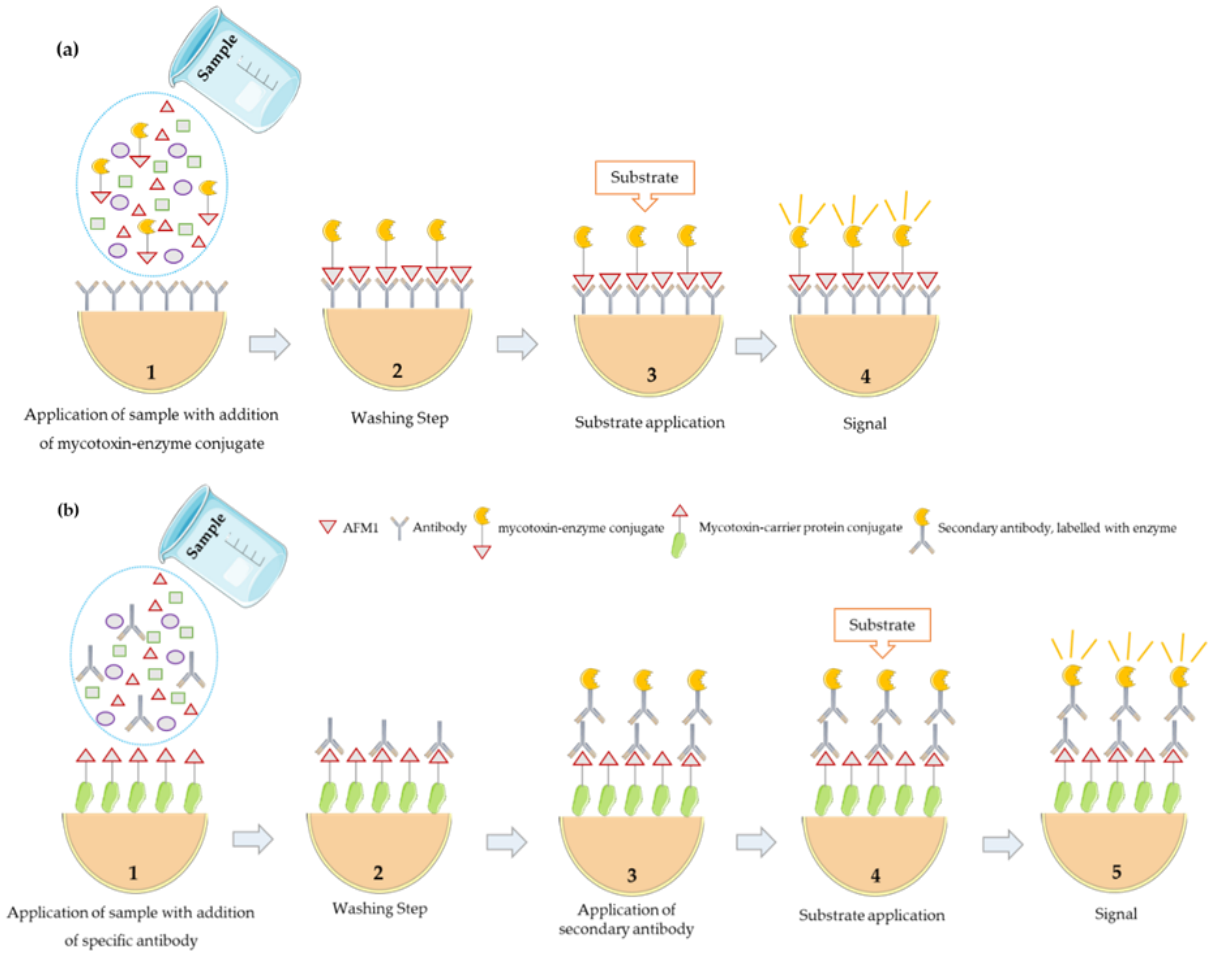


Figure 10. Competitive ELISA principle. (a) Direct format and (b) indirect format from (Vaz et al., 2020).

Chemiluminescent detection allows the use of 384 well plates with an assay volume of 20 µL, when 96 well microtiter format, due to a 5-fold reduction in antibody, labelled probe and chemiluminescent mixture volume that allows to reduce the costs of the assay, maintaining the analytical performance (Manetta, 2011).

Similarly, with the view of increasing advantages such as its high sensitivity, high efficiency and

easiness of manipulation, Kanungo and Bhand (2013) developed an ELISA using fluorimetric detection. This was performed in a 384 well microplate, in which there were AFM1-specific monoclonal antibodies and secondary conjugated antibodies. AFM1 was detected at a level of 0.001 µg/L in a testing volume of 40 µL (Ketney et al., 2017).

Generally, the term biosensors refers to a small, portable and analytical device based on the combination of recognition biomolecules, like antibodies and nucleic acids, with an appropriate transducer, that is able to selectively detect chemical or biological materials with a high sensitivity (Figure 11) (Espinosa-Calderón et al., 2011).

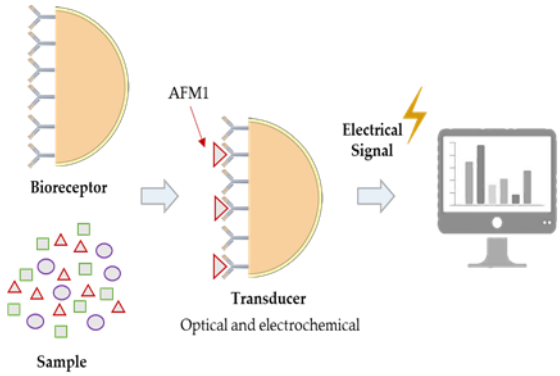


Figure 11. General basis parts of immunosensors from (Vaz et al., 2020).

The detection principle is the binding of the analyte of interest to the complementary bio–recognition element immobilized on a suitable support medium. When the analyte binds the element, a specific interaction occurs which results in a change of one or more physico–chemical properties. Such properties may be: pH, electron transfer, mass, or heat, which are detected with the aid of a transducer. Depending on the method of signal transduction, biosensors can be divided into 3 different groups: electrochemical transducers, which rely on an electrical signal measurement (amperometric, potentiometric, and conductometric) generated by a physico-chemical change; optical transducers, in which an optical signal (colour or fluorescence) changes as a result of formation of a complex; and piezoelectric transducers, which detect changes in mass. The most common transducers in AF detection are the electrochemical and optical ones.

The immunosensors, a type of biosensor, consist of a pair of electrodes (measuring and reference), implemented using the screen-printing technique. The measurement electrode is coated with specificantibodies, which will retain the aflatoxins of interest in the sample, while the other electrode (reference) is commonly made of a combination of Ag/AgCl. The measurement procedure is similar to

that carried out by the ELISA test (Espinosa-Calderón et al., 2011; Ketney et al., 2017; Mosielllo & Lamberti, 2011; Saeger, 2011). In this category of techniques, Rameil et al. (2010) developed a potentiometric AFM1–immunosensor which utilizes 3-(4 hydroxyphenyl)propionic acid (p-HPPA) as electron donating compound for horseradish peroxidase (HRP; EC 1.11.1.7). The assay system consists of a polypyrrole-surface-working electrode coated with a polyclonal anti-M1 antibody (pAb–AFM1), a Ag/AgCl reference electrode and a HRP–aflatoxin B1 conjugate (HRP–AFB1 conjugate). The optimized assay has a detection limit of 0.04 µg/L and allowed the detection of 0.5 µg/L (FDA limit) AFM1 in pasteurized milk and UHT-milk, containing 0.3–3.8 % fat, within 10 min, without any sample preparation. The working AFM1 range was between 0.25 and 2 µg/L. In addition, the use of p-HPPA has the advantage of low toxicity and does not require the presence of organic solvents in the substrate buffer. Other studies on the application of this methodology for the quantification of AFM1 in milk have been performed (Table A.1–Appendix to Chapter 2) (Abera et al., 2019; Micheli et al., 2005).

The aptasensor is a particular class of biosensor and can be a good alternative to immunosensors because it is easier to synthesize and modify with a variety of chemical groups (Danesh et al., 2018; Hosseini et al., 2015). The difference of this sensor is in the biological recognition element that is an aptamer instead of an antibody. The aptamer consists of a synthetic oligonucleotide ligand (either single stranded DNA (ssDNA) or RNA) generally comprising of less than 80 nucleotides and with a size lower than 25 kDa and is known to exhibit high specificity and strong binding affinity (Danesh et al., 2018; B. H. Nguyen et al., 2013; Amit Kumar Pandey et al., 2017; Rhouati et al., 2016). In an aptasensor, the aptamer recognizes the molecular target against which it was previously in vitro selected. So, the aptamer can bind with high affinity and specificity to a wide range of target molecules, such as drugs, proteins, toxins or other organic or inorganic molecules (Moreno, 2014; B. H. Nguyen et al., 2013). Different studies on the application of this methodology for the quantification of AFM1 in milk have been performed (Table A.1 – Appendix to Chapter 2).

Biosensors have the advantage over traditional methods of being simple, rapid, cost effective and portable devices that are specific to the target mycotoxin. However, their sensitivity and stability still need improvement to allow long-term use (Manetta, 2011; Rahmani et al., 2009).

Therefore, there are numerous methods that allow the detection and quantification of this compound, and the most recurrent methods were divided into two groups, chromatographic methods and bioassays. Both allow the detection of AFM1; however, bioassays are techniques commonly used for screening since the immunological methods may give rise to false positives. This is because, although

the antibodies are specific to their antigens, they can react with other substances. Thus, the use of immunological methods, such as the ELISA, could be used at a preliminary stage to select from a wide range of samples those that are contaminated with the toxin under study. Subsequently, other methods are used to confirm the results. Among several methods cited, it has been found that those best suited for AFM1 detection in dairy products are chromatographic ones with fluorescence detection or those coupled to mass spectroscopy.

# **CHAPTER 3 –** SYSTEMATIC REVIEW ON AFM1 OCCURRENCE IN LAST 30 YEARS AND ASSESSMENT OF HUMAN HEALTH RISK

## 3.1 Introduction

The growing demand for dairy products combined with the global geographical distribution of producers and consumers makes the control of a hazard as AFM1 an important and urgent need. Its occurrence in milk and consequently in its derivatives can be related to: (i) the post-harvest storage and handling process of feed, which increase the risk of contamination by AFB1, consequently increasing the risk of dairy animals being feed with poor quality feed and (ii) climate change, that enhances the contamination of feed by AFB1 producing fungi, with a focus on corn, that represents one of the most used cereals in animal feed (Battilani et al., 2016; Mollayusefian et al., 2021; Santos Pereira et al., 2019; Stepman, 2018; Van der Fels-Klerx et al., 2019).

The occurrence of AFM1 in milk has been reported in many studies, however, there is no global study about the occurrence of this toxin in the main types of dairy products, besides milk. Therefore, the objective of this work was to study the trend of AFM1 occurrence in dairy products, including milk, cheese, butter, yogurt, as well as in human milk. To achieve this objective, a systematic literature search on the AFM1 levels was done to evaluate the possible relation between AFM1 concentration and sampling decade, continent, type of dairy product, and animal species. Literature search was performed based on Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guideline that allows to identify, select, evaluate and synthesize studies, providing a systematic review complete and accurate Besides that, AFM1 concentration data, together with the consumption of dairy products data, were used to assess human health risk.

The risk characterization is extremely important to ensure food safety through the adoption and implementation of appropriate control measures, and can be achieved based on human exposure to a certain contaminant and, also, the availability of toxicological reference (Serraino et al., 2019; Udovicki et al., 2019). Simplistically, to determine the risk that a given compound represents to the health of an individual or a population, it is necessary to know the mean daily exposure (EDI) to assess whether this is greater than the recommended daily dose (TDI), representing a danger. One way to express this is the hazard index (HI) ("Risk Assessment and Risk Management of Mycotoxins," 2012). HI is calculated as the ratio of a specific agent's EDI to the TDI of the same agent. For HI > 1, an individual or population may be at risk due to high levels of exposure to the hazardous agent (Pandey et al., 2021). Additionally, the risk characterization can also be evaluated by estimating the predisposition of a certain population to develop cancer (HCC) due to daily exposure to a carcinogenic agent. The carcinogenicity, or cancer potency, of AFs differs in humans with and without chronic hepatitis B virus (HBV) infection. According to

FAO/WHO (1999), AFB1 and the hepatitis B virus (HBV) work synergistically to increase the risk of hepatocellular carcinoma, being this the fifth most frequent cancer in the world (Njombwa et al., 2021).

Given the significance of these parametres in the establishment of AFM1 risk assessment, the HI and the risk of hepatocellular carcinoma (HCC) due to exposure of AFM1 through consumption of dairy products were evaluated for each continent.

## 3.2 Search Strategy (methods)

### 3.2.1 Searching strategy

The international databases Science Direct, Web of Science, Scopus, and B-on were used to collect papers that reported the occurrence of AFM1 in different dairy products in different countries around the world. The search keywords used in each database by the Boolean operation were: "aflatoxin M1" and "occurrence". No time interval was specified, to get the widest possible range of scientific literature in English. The last search was in January 2021. All studies were systematically reviewed according to the PRISMA regulations (Moher et al., 2009). Applying the PRISMA methodology, all papers retrieved from the databases were exported and duplicated papers were excluded in the first stage. At a second stage, papers were screened according to their title and abstract and a double check was done by the two advisors; following a stage where the full text was analyzed. However, this review was not registered.

### 3.2.2 First screening: inclusion and exclusion criteria

To include research articles, the following criteria were defined: (i) original research studies, reporting AFM1 levels in the following dairy products: milk, cheese, butter, and yogurt, (ii) papers with full-text available, and where the range of AFM1 concentration or the positive or total mean concentration were available and (iii) papers written in English. Papers with inconsistent data (e.g., mean AFM1 concentration of positive samples outside the concentration range) were excluded at this stage.

### 3.2.3 Data extraction in the final screening

When provided, the following variables were extracted from screened papers: continent and country; sampling year; sample type; total sample size; positive sample size; range of concentration; mean of positive samples and mean of total samples; method of analysis; and limits of detection (LOD) and quantification (LOQ).

### 3.2.4 Criteria for eligibility

For all the studies found eligible, the occurrence of AFM1 was analyzed based on the number of samples and the AFM1 mean of total samples (including positive and negative samples). Since not all studies provided these data directly, some assumptions and estimates were made.

Considering that the reported values of LOD and LOQ vary widely and were not always provided, it was assumed that negative samples had an AFM1 concentration of zero. AFM1 concentrations were reported in different manners in the eligible studies. Most of them reported the range of AFM1 concentration in positive samples. Still, regarding the mean, different scenarios were observed: the mean concentration of positive samples, or the mean concentration of all samples, or no mean reported. So, a criterion was defined to calculate the AFM1 mean concentration of all samples when it was not provided:

1. When the total number of samples, the positive samples, and the mean of the positive samples was available, the AFM1 mean concentration of all samples was directly calculated (equation 3).

$$\overline{M}_t = \overline{M_+} \times \frac{N_+}{N_t} \quad (3)$$

where $\overline{M}_t$, $\overline{M_+}$, $N_+$, and $N_t$ represent the AFM1 mean of total samples, the AFM1 mean of the positive samples, the number of positive samples, and the total number of samples, respectively.

2. When the total number of samples, the positive samples, and the concentration range was available, a percentile was calculated to estimate the AFM1 mean concentration of the positive samples. This percentile was calculated from data provided in the set of papers mentioned in bullet point 1, as indicated in equation 4.

$$Per = \left( \frac{\overline{M_+} - C_{min}}{C_{max} - C_{min}} \right) \quad (4)$$

where $Per$, $\overline{M_+}$, $C_{min}$, and $C_{max}$ represent the percentile, the AFM1 mean of the positive samples, the AFM1 maximum and minimum in the concentration range, respectively.

After calculating *Per* for all cases, the mean *Per* $\overline{(Per)}$ was used to estimate the AFM1 mean of the positive samples (equation 5), and from this, the AFM1 mean concentration of all samples was estimated using Equation 3.

$$\overline{M_{+,est}} = (1-\overline{Per}) \times C_{min} + \overline{Per} \times C_{max} \quad (5)$$

where $\overline{M_{+,est}}$ represents the estimated AFM1 mean of the positive samples.

### 3.2.5 Data processing and analysis

Data were extracted and analyzed using IBM SPSS Statistics 27.0 statistical software. The trends between AFM1 concentration and different types of dairy products, the origin of samples, continents, and sampling decade were analyzed and discussed using Welch and Brown-Forsythe's methods, followed by post hoc Games-Howell test to evaluate differences between groups. Significance was established at $p < 0.05$ for all tests.

Due to a high number of outliers and extreme values, data were transformed using the function *log(x+1)*, where *x* represents the calculated mean AFM1 concentration of all samples. Figure 12 highlights one example of blox plot graphs obtained with and without using *log(x+1)* function. Further graphics are shown in Appendix to Chapter 3. Considering the type of samples studied and the sample size, and based on the central limit theorem (which describes that large samples tend to show an approximately normal distribution), it was not necessary to verify the normality of the data (Ross, 2021).

The homogeneity of variance was verified using Levene's test ($p < 0.05$). Once this assumption is violated, the robust Brown-Forsythe's test and Welch's test were applied to check for differences between groups, followed by the Post-Hoc Games-Howell test (for three or more groups). The T Test was also used to check for differences between two groups

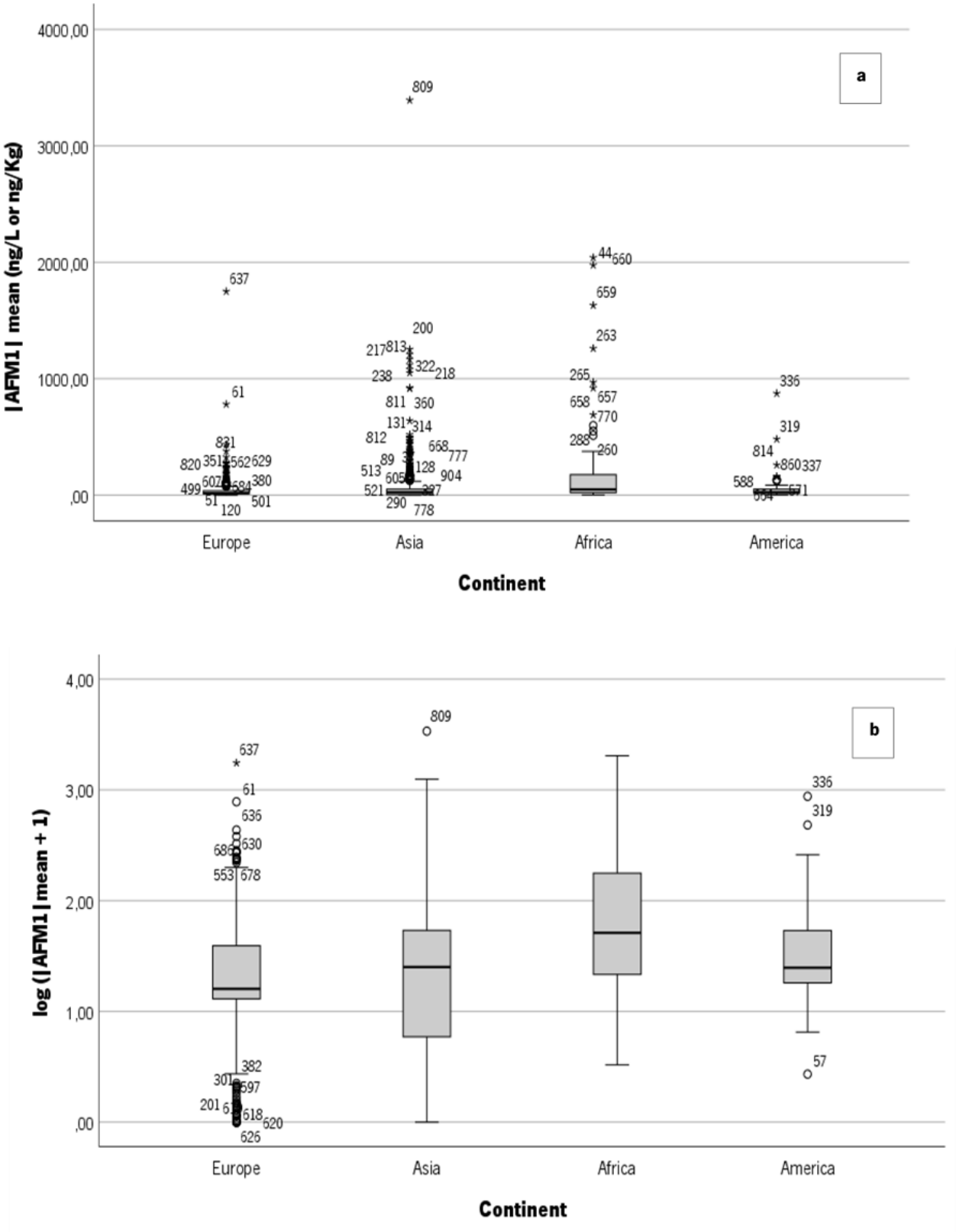


Figure 12. AFM1 mean concentration of all samples by continent (a) expressed in concentration, and (b) after transformation with log(x+1) function.

### 3.2.6 Human health risk assessment

According to FAO and WHO (1995), the exposure assessment is a methodology developed to evaluate the probable intake of chemical substances via food. Thus, the exposure to AFM1 through the consumption of dairy products such as milk, yogurt, cheese and butter, in African, Asian, European and Latin American Continents, was calculated in this study as Estimated Daily Intake (EDI). In the case of the American continent, the data used for mean AFM1 concentration were only from Latin America. The EDI was determined combining three factors for each continent: (i) mean AFM1 concentration, in ng/kg, for the four dairy products (|AFM1|), (ii) consumption of each dairy product *per capita* per day (ingestion rate, IR), and (iii) the mean human body weight in kg (bw), using the equation 6.

$$EDI = \frac{\sum (IR \times |AFM1|)}{bw} \quad (6)$$

The data of dairy consumption and the mean of body weight (Table 7) were obtained from the report of OECD-FAO Agricultural Outlook 2021-2030 (OECD-FAO, 2021) and from Walpole et al. (2012), respectively.

Table 7. Mean of body weight in each continent (bw), the food consumption for the four analyzed continents and the respective percentage contribution of each dairy product

| **Continent** | **bw (kg)** | **Food consumption (Kg/cap)** | | | |
|---|---|---|---|---|---|
| | | Milk and Yogurt | Cheese | Butter | Total of dairy products |
| Africa | 60.7 | 25.8 (96 %) | 0.7 (3 %) | 0.3 (1 %) | 26.8 |
| Asia | 57.7 | 56.1 (96 %) | 0.6 ( 1 %) | 1.5 (3 %) | 58.2 |
| Europe | 70.8 | 101.4 (84 %) | 15.4 (13 %) | 3.9 (3 %) | 120.7 |
| Latin America | 67.9 | 52.7 (74 %) | 17.4 (25 %) | 0.7 (1 %) | 70.8 |

The mean AFM1 concentrations in each continent were obtained from the data extracted in the literature search and correspond to the *x* value calculated through the equation *log(x+1)*. Considering that the report of OECD-FAO (2021) provides the values of food consumption in each continent for the three main dairy groups – milk and yogurt, cheese, and butter – the mean concentration of milk and yogurt was calculated as a mean weighted by the total number of samples. The values used for food consumption represent the mean consumption between 2018 and 2020. However, the values of AFM1 mean concentration collected in the 2010's decade (2010-2019) for cheese and butter samples, in each continent, were not representative. Therefore, the mean occurrence for these groups was taken from the last 30 years. For the milk and yogurt group the mean corresponded to the mean occurrence obtained

in the 2010's decade. Besides that, the Latin American continent lacked butter sampling, and for that reason the global mean occurrence value was assumed.

The hazard index (HI) was used to evaluate the risk to human health through the ingestion of AFM1 contaminated dairy products (Korley Kortei et al., 2019; Teuschler & Hertzberg, 1995). Aflatoxin M1 presents genotoxic and carcinogenic effects and, based on the Joint FAO/WHO Expert Committee on Food Additives (JECFA) reports (FAO/WHO, 2017), there is no intake level that can be considered risk free. Thus, it is recommended that AF' concentration in food should be as low as possible (FAO/WHO, 2017; Sibaja et al., 2021). Kuiper-Goodman (1990) proposed a TDI value of 0.2 ng/kg of body weight. This value results from dividing the TD50 (the daily dose rate in mg/kg bw/day that induces tumors in half of the test animals that would have remained tumor-free at zero doses) by an uncertainty factor of 50 000, a value equivalent to a risk level of 1:100,000. Other studies used the same approach (Kerekes et al., 2016; Ajeet Kumar Pandey et al., 2021; Udovicki et al., 2019). The HI was thus calculated using equation 7.

$$HI = \frac{EDI}{TDI} \tag{7}$$

Additional risk characterization to estimate human health risk of hepatocellular carcinoma (HCC) was performed following an approach described by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 1999). Being a metabolite of AFB1, AFM1 is presumed to have a similar mechanism to induce liver cancer. Therefore, considering, that the carcinogenic potency of AFM1 is one-tenth that of AFB1 (FAO/WHO, 1999), it was estimated to be 0.03 cancer/year/100,000 individuals for hepatitis B surface antigen–positive (HBsAg+) population, and 0.001 cancer/year/100,000 individuals for hepatitis B surface antigen–negative (HBsAg–) population.

The average cancer potency ($\overline{P_{cancer}}$) of AFM1 was estimated taking into consideration the prevalence of both HBsAg+ and HBsAg– individuals in the total population in each continent. Based on the study of Schweitzer et al. (2015), the prevalence of hepatitis B infected individuals (% $HBsAg^+$) in Europe, Africa, Asia and Latin America was 2.06 %, 8.08 %, 2.04 % and 1.10 %, respectively. Thus, following equations 8 and 9 it is possible to estimate the risk of a given population to develop hepatocellular carcinoma.

$$\text{HCC risk} = EDI \times \overline{P_{cancer}} \tag{8}$$

$$\overline{P_{cancer}} = 0.001 \times \%\ HBsAg^- + 0.03 \times \%\ HBsAg^+ \tag{9}$$

## 3.3 Results and Discussion

### 3.3.1 Characterization of the selected studies

In the first screening stage (Figure 13), 4886 documents were identified through database searching, and 36 were articles originating from other sources, which accounts for 4922 articles. From these, 1893 duplicate articles were excluded, and 2617 were discarded after the title and abstract screening. Four hundred and twelve (412) articles were selected as suitable, but 30 of these were excluded due to the lack of critical information and/or to lack of access to the full text. Three hundred and eighty-two (382) papers were kept and considered eligible for quantitative analysis. Finally, after the exclusion of 53 more papers due to inconsistent data, 329 papers remained for further analysis (Appendix to Chapter 3). These 329 papers included data from 112 741 samples of dairy products. The global data is available in Appendix to Chapter 3.

The methods used for the detection of AFM1 in milk samples and milk products were mainly Thin Layer Chromatography (TLC), Enzyme-linked immunosorbent assay (ELISA) and High-performance liquid chromatography (HPLC), wherein ELISA and HPLC were the most used. It is important to note that in some cases ELISA was used for screening and HPLC for confirmation.

From the 329 papers, 154, 106, 38, 30 and 1 papers included data from the Asian, European, American, African and Oceanian continents, respectively. On the other hand, considering decades, 163 papers reported data of the 2010's decade, 106 papers of the 2000's decade and only 13 papers of the 1990's decade. The rest of the papers (47) did not provide the year. To analyze some trends, it was necessary to eliminate some data, as happened in the case of Oceania, because it did not have a representative sample for statistical analysis.

### 3.3.2 General description of the data

From the initial set of data, 2529 samples were excluded from the statistical analysis, since they included some dairy products (dairy desserts, ice creams, curd) where AFM1 concentration could not be confidently assessed due to considerable processing effects (e.g., dilution). The total size of the sample in this study included 110 192 samples of different types of products, collected between 1990 and 2019 worldwide (Table 8). From the total samples analyzed, 47 962 (44 %) had quantifiable levels of AFM1. From Table 8, it can be concluded that most of the samples are milk samples (> 85 %) and originate from Europe and Asia (93 %). Other dairy products and samples from other geographic origins are much less represented, with Oceania representing a small number of samples.

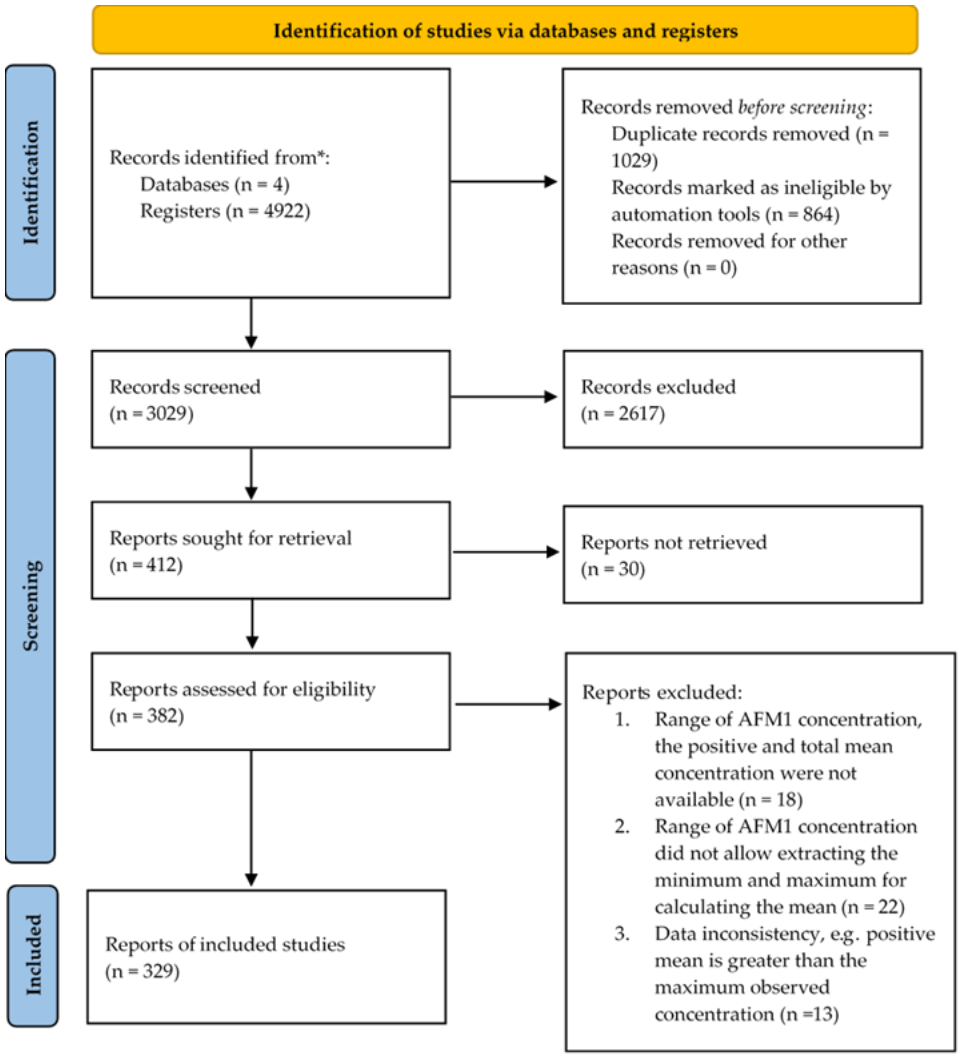


Figure 13. Flow chart indicating the search and selection process of articles.

Table 8. Total number of samples retrieved from the search, between 1990 and 2019 and by continent, for dairy products and for human milk samples

| Continent | Sample type | | | | | Total samples |
|---|---|---|---|---|---|---|
| | Milk | Human Milk | Cheese | Yogurt | Butter | |
| Europe | 58 992 | 511 | 3370 | 915 | 174 | 63 962 |
| Asia | 31 531 | 2065 | 2771 | 1506 | 193 | 38 066 |
| Africa | 3089 | 924 | 390 | 195 | 83 | 4681 |
| America | 2634 | 398 | 314 | 53 | - | 3399 |
| Oceania | - | 84 | - | - | - | 84 |
| Total samples | 96 246 | 3982 | 6845 | 2669 | 450 | 110 192 |
| Positive samples | 40 310 | 2213 | 3525 | 1570 | 308 | 47 926 |

### 3.3.3 Statistic data analysis

#### 3.3.3.1 AFM1 distribution over the decades

The data were organized to evaluate the trend of the AFM1 concentration levels during the last 30 years, by decade, in general and by continent. Table 9 shows the worldwide sampling for each decade, 1990's (1990 – 1999), 2000's (2000 – 2009), and 2010's (2010 – 2019), respective mean of AFM1 concentration and errors. Based on the information available in Table 9, it is possible to observe a tendency for the increase of AFM1 levels over the years, except for the Asian continent.

Table 9. Transformed Mean of AFM1 concentration (± standard error), median and number of samples (N), by continent and by decade

| Continent | Decade | N | Transformed Mean of AFM1 concentration (ng/L or ng/Kg)[1] | Median (ng/L or ng/Kg)[1] |
|---|---|---|---|---|
| Worldwide | 1990 – 1999 | 1882 | 1.0993 ± 0.0164[a] | 11.00 |
| | 2000 – 2009 | 35591 | 1.3117 ± 0.0046[b] | 15.00 |
| | 2010 – 2019 | 62458 | 1.3120 ± 0.0053[b] | 17.57 |
| Europe | 1990 – 1999 | 1486 | 1.1009 ± 0.0182[a] | 10.00 |
| | 2000 – 2009 | 23923 | 1.2073 ± 0.0050[b] | 15.00 |
| | 2010 – 2019 | 36549 | 1.2904 ± 0.0057[c] | 17.57 |
| America | 1990 – 1999 | 190 | 0.9643 ± 0.0532[a] | 13.07 |
| | 2000 – 2009 | 921 | 1.4722 ± 0.0168[b] | 24.91 |
| | 2010 – 2019 | 1583 | 1.5840 ± 0.0220[c] | 21.00 |
| Asia | 1990 – 1999 | 206 | 1.2122 ± 0.0476[a] | 17.27 |
| | 2000 – 2009 | 9658 | 1.5190 ± 0.0089[c] | 38.82 |
| | 2010 – 2019 | 22763 | 1.2859 ± 0.0108[b] | 15.60 |
| Africa | 2000 – 2009 | 1089 | 1.6291 ± 0.0313[a] | 28.25 |
| | 2010 – 2019 | 1563 | 1.9198 ± 0.0266[b] | 81.00 |

[1]Different letters in the mean value indicate significant differences between decades in each continent as determined by the Games-Howell test in case of Europe, America and Asia and by T-test for Africa.

Concerning the European and American continents, AFM1 levels significantly increased over the decades ($p < 0.001$). This increase can be explained by the fact that maize, a crop highly susceptible to contamination by AFB1 (the precursor of AFM1 in milk), is one of the most used crops in dairy cattle feeds and, as reported by Santos Pereira et al. (2019), there has been an increase in its use over the years. Also, in a study by Battilani et al. (2016), a modeling approach demonstrated that climate change might increase the impact of AFB1 on maize, which might have direct consequences on the food safety of dairy cattle. Van Der Fels-Klerx et al. (Van der Fels-Klerx et al., 2019), using a full chain modeling

approach, studied the impact of climate change on the production of AFB1 in maize and in milk contamination with AFM1, more specifically in dairy cow's milk. According to the projected climate change scenario, most calculations suggested an increase in AFM1 in milk by 2030, at least for Europe. The increase in atmospheric $CO_2$ and temperature can influence the quality as well as the availability of water, which can have a direct impact on feed and consequently on milk and its derivatives (Mollayusefian et al., 2021). Therefore, this increase can be explained in part by the climate change suffered in the last years.

Looking at the African continent, statistically significant differences were found between the 2000's and 2010's decades ($p < 0.001$), with the second one showing the highest level of AFM1 concentration. This increased might be related with issues in storage and post-harvest handling process which increased the risk of AFB1 contamination. Besides that, Africa is one of the most vulnerable continent to climate change and some predictions indicate that many African regions will undergo periods of drought, while East Africa may experience a more humid climate but also hotter. These changes can promote the contamination with aflatoxin-producing fungi and, consequently, increasing the risk of AFM1 in dairy products (Stepman, 2018). On the other hand, in the Asian continent, 2000's decade showed the highest AFM1 levels ($p < 0.001$) and 1990's decade presented the lowest levels ($p < 0.001$). According to Streit et al. (2013), an outbreak of aflatoxins in Asian maize between 2005 – 2009 was observed, which may have contributed for the higher value in the 2000's decade. The 2010's decade showed a significantly higher value than the 1990´s decade, which may indiciate a trend for increasing concencentrations as observed for the other continents.

#### 3.3.3.2 AFM1 distribution worldwide

The worldwide occurrence of AFM1 in dairy products was analyzed by comparing the levels between continents (Table 10).

Table 10. Transformed mean of AFM1 concentration levels (with respective standard error) and median in all dairy products and number of samples (N), by continent

| Continent | N | Transformed Mean of AFM1 concentration (ng/L or ng/Kg)[1] | Median (ng/L or ng/Kg) |
|---|---|---|---|
| Europe | 63451 | $1.2540 \pm 0.0039^{a}$ | 15.00 |
| America | 3001 | $1.5230 \pm 0.0160^{c}$ | 24.91 |
| Asia | 36001 | $1.3867 \pm 0.0076^{b}$ | 24.21 |
| Africa | 3757 | $1.7781 \pm 0.0228^{d}$ | 50.25 |

[1] Different letters in the mean value indicate significant differences between continents by the Games-Howell test.

It was possible to observe statistically significant differences ($p < 0.001$) between all continents. The African continent was the continent with the highest toxin content and Europe was the one with the lowest toxin content. These results can be explained by the fact that in the African continent there is no official control and, where available, the legal limit is high (*e.g.*, in Nigeria is 1 µg/L in milk) (Vaz et al., 2020). Although there is an increase in AFM1 levels worldwide, as discussed previously, Europe when compared with other continents it is still the continent with the lowest levels. These results could be attributed to the modern feed processing practices, adoption of food safety systems, strict regulations applied to mycotoxins in food and advanced analytical techniques that allows continuous monitoring of compounds, like aflatoxins (Mollayusefian et al., 2021).

Besides, these results confirm the overall perception of a higher incidence of mycotoxins in Africa (Kebede et al., 2020; Udomkun et al., 2017), indicating the poorer conditions and the food safety and food security concerns in the region. In Sub-Saharan Africa, due to feed and food shortage, maize in bad conditions (spoiled and moulded) is often given as feed to animals. This increases the risk of contamination with mycotoxigenic fungi, putting all dairy farming at risk (Kemboi et al., 2020).

#### 3.3.3.3 Sample type in AFM1 concentration

The trend of the AFM1 concentration in each dairy product over the last 3 decades was also evaluated (Table 11). Among the more than 105 thousand samples, milk represents 90.6 % of the samples, with minor contributions of cheese (6 %), yogurt (3 %), and butter (0.4 %).

Through the statistical analysis, it was possible to observe that, at a worldwide level, AFM1 levels in cheese and butter are similar ($p = 0.372$) and were the groups with the highest toxin content, followed by a significantly lower value ($p < 0.001$) in yogurt, and an even lower level in milk ($p < 0.001$). Increased AFM1 concentration in cheese is well documented (Cavallarin et al., 2014; Fernandes et al., 2012; Manetta et al., 2009; Pecorelli et al., 2019; Pietri et al., 2016a), and the higher level in cheese might be explained by the semi-polar behaviour of AFM1, leading to a lower affinity to the serum proteins and a higher affinity to milk caseins (Rubio et al., 2011). On the other hand, it was possible to observe a significantly higher AFM1 concentration in yogurt and in butter than in milk. Both results are not easy to explain based on available information, and cannot be attributed solely to a yogurt and butter production concentration factor.

To elucidate if the differences were due to different geographic origins of these dairy products, the analysis was repeated per continent. As observed in Table 11, only in the European Continent yogurt is statistically similar ($p = 0.076$) to milk, and butter represents the group with higher AFM1 levels

($p < 0.001$). Regarding the Asian continent cheese was the group with highest levels of AFM1 ($p < 0.001$). On the other hand, in the African and American continents the AFM1 concentration in milk is significantly lower ($p < 0.001$) than in cheese. Cheese always shows significantly higher values of AFM1 ($p < 0.001$) than milk, but butter has a discrepant and unpredictable behaviour in Europe (significantly higher concentration). This result has to be carefully analyzed, as the sample size is small, all data are from the same country and includes a study where reported concentrations are quite high (up to 7 µg/L) (Tekinşen & Uçar, 2008), possibly due to the use of milk contaminated with high AFM1 levels for butter production, which may have biased this conclusion. Besides that, in Africa continent yogurt had higher AFM1 levels than cheese ($p = 0.033$) which can be explained by the high AFM1 levels observed in a study from Ethiopia (1.63 µg/kg) (Tadesse et al., 2020). The trend for lower AFM1 levels in milk confirms the stability of the toxin, which is not easily removed or destroyed during processing and will maintain its levels or concentrate in different dairy products.

Table 11. Transformed mean of AFM1 concentration levels (with respective standard error), median and number of samples (N), by dairy product and continent

| Continent | Dairy product | N | Transformed Mean of AFM1 concentration (ng/L or ng/Kg)[1] | Median (ng/L or ng/Kg) |
|---|---|---|---|---|
| Worldwide | Milk | 96246 | $1.2865 \pm 0.0038^{a}$ | 15.00 |
| | Cheese | 6845 | $1.8089 \pm 0.0131^{c}$ | 65.00 |
| | Yogurt | 2669 | $1.4034 \pm 0.0256^{b}$ | 23.25 |
| | Butter | 450 | $1.7613 \pm 0.0565^{c}$ | 69.70 |
| Europe | Milk | 58992 | $1.2274 \pm 0.0039^{a}$ | 15.00 |
| | Cheese | 3370 | $1.7048 \pm 0.0200^{b}$ | 56.00 |
| | Yogurt | 915 | $1.1816 \pm 0.0371^{a}$ | 14.43 |
| | Butter | 174 | $1.9421 \pm 0.0882^{c}$ | 236.0 |
| Asia | Milk | 31531 | $1.3432 \pm 0.0083^{a}$ | 22.30 |
| | Cheese | 2771 | $1.8875 \pm 0.0161^{b}$ | 85.00 |
| | Yogurt | 1506 | $1.3809 \pm 0.0295^{a}$ | 23.86 |
| | Butter | 193 | $1.3428 \pm 0.0632^{a}$ | 21.60 |
| Africa | Milk | 3089 | $1.6635 \pm 0.0232^{a}$ | 48.00 |
| | Cheese | 390 | $2.2382 \pm 0.0750^{b}$ | 282.1 |
| | Yogurt | 195 | $2.4281 \pm 0.1146^{c}$ | 234.5 |
| America | Milk | 2634 | $1.4903 \pm 0.0174^{a}$ | 21.00 |
| | Cheese | 314 | $1.6990 \pm 0.0311^{b}$ | 71.19 |

[1] Different letters in the mean value indicate significant differences between dairy products in each continent by the Games-Howell test in case of Europe, Asia and Africa and by T-test for America.

#### 3.3.3.4 Influence of animal's species in AFM1 concentration

The data collected were organized to evaluate the trend of the species origin of the milk samples in AFM1 concentration. For the study of animal species, only milk samples were considered, and human milk was also considered for this analysis.

Table 12 shows the sampling for each species in each continent. Concerning the total number of milk samples collected in the literature search (100 254 samples), only 57 811 indicated the animal species. Most of the samples were from cow, and from Europe and Asia. According to the OECD-FAO Agricultural Outlook 2020-2029 report (2020), around 81 % of the world's milk production is cow milk. Samples from donkey were not considered for the statistical analysis due to low representativeness (five samples from one study). The statistical analysis indicated significant differences ($p < 0.001$) between most species, and only for sheep there were no statistical differences with cow ($p = 0.335$) and human ($p = 0.107$) milk samples. Camel milk samples were the ones with the lowest toxin content, while buffalo milk samples had the highest toxin content. Regarding cow samples, they presented significantly higher concentrations than human ($p < 0.001$), goat ($p < 0.001$), and camel ($p < 0.001$) milk samples.

Human milk is a particular case; however, the AFM1 levels observed in this milk are also directly related to the ingestion of poor quality grain contaminated with AFB1 (Fakhri et al., 2019; WHO, 2018).

The low incidence of AFM1 in camel milk can be related with the fact that this species is usually feed with graze and rarely with compound and concentrated feeds. Besides that, on the deserts, where usually camels graze, the environmental conditions are not good for the growth of mycotoxigenic fungi and production of aflatoxins (Fallah et al., 2016; Mollayusefian et al., 2021). On the other hand, one possible reason for the high incidence of AFM1 in cow and buffalo milks is the fact that these species are frequently fed with forage and silage, which can be a main source of AFB1 contamination, contrary to species that consume this type of feed less frequently (Bahraman et al., 2020; Khaneghah et al., 2021; Mollayusefian et al., 2021). In fact, one of the main causes of AFM1 contamination in dairy products is the use of feed contaminated with AFB1. Britzi et al. (2013) studied the carry-over rate of the AFB1 in feed to AFM1 in milk and they observed that milk production affects the carry-over rate, with an average carry-over rate of 2.5 % for low production cows (< 35 L/day) and 5.4 % for high production cows (> 35 L/day). Thus, it is crucial to ensure the good harvest and storage conditions of animal feed, since ambient temperature and relative humidity are important factors for the development of mycotoxin-producing fungi like *A. flavus* and *A. parasiticus*, the main AF producers (Cotty & Jaime-Garcia, 2007; Medina et al., 2014; Oldham et al., 1991).

Table 12. Transformed mean of AFM1 concentration levels (with respective standard error), median and number of samples (N), by animal species (including human milk) and continent

| Continent | Animal species | N | Transformed Mean of AFM1 concentration (ng/L or ng/Kg)[1] | Median (ng/L or ng/Kg) |
|---|---|---|---|---|
| Worldwide | Human | 3898 | $1.1628 \pm 0.0283^{c}$ | 4.680 |
| | Cow | 49446 | $1.2341 \pm 0.0055^{d}$ | 17.57 |
| | Goat | 1159 | $1.0288 \pm 0.0273^{b}$ | 9.531 |
| | Sheep | 1774 | $1.2106 \pm 0.0209^{cd}$ | 18.00 |
| | Buffalo | 1155 | $1.3276 \pm 0.0207^{e}$ | 27.00 |
| | Camel | 290 | $0.8081 \pm 0.0699^{a}$ | 2.375 |
| Europe | Human | 511 | $0.7732 \pm 0.0381^{b}$ | 3.371 |
| | Cow | 28632 | $1.3082 \pm 0.0067^{d}$ | 17.57 |
| | Goat | 316 | $0.5982 \pm 0.0370^{a}$ | 4.055 |
| | Sheep | 1049 | $1.1938 \pm 0.0311^{c}$ | 27.56 |
| | Buffalo | 128 | $0.5927 \pm 0.0132^{a}$ | 3.000 |
| Asia | Human | 2065 | $1.0764 \pm 0.0355^{bc}$ | 4.632 |
| | Cow | 17376 | $1.0491 \pm 0.0097^{b}$ | 11.98 |
| | Goat | 729 | $1.1092 \pm 0.0236^{c}$ | 11.26 |
| | Sheep | 725 | $1.2350 \pm 0.0240^{d}$ | 14.00 |
| | Buffalo | 1027 | $1.4192 \pm 0.0160^{e}$ | 27.00 |
| | Camel | 290 | $0.8081 \pm 0.0698^{a}$ | 2.375 |
| Africa | Human | 924 | $1.8933 \pm 0.0599^{b}$ | 217.6 |
| | Cow | 2264 | $1.6710 \pm 0.0272^{a}$ | 49.48 |
| America | Human | 398 | $0.4153 \pm 0.0436^{a}$ | 0.9091 |
| | Cow | 1174 | $1.3212 \pm 0.0169^{b}$ | 21.00 |

[1] Different letters in the mean value indicate significant differences between species in each continent by the Games-Howell test in case of Europe and Asia and by T-test for Africa and America.

In addition, previous studies demonstrated that seasonal variation has a strong impact on AFM1 contamination in dairy products, since in winter there is a trend towards higher levels of AFM1 in milk. During winter season most of the feed administered is stored cereals and not fresh forage, thereby increasing the risk of AFB1 contamination (Bahrami et al., 2016; Mahmoudi & Norian, 2015; Tomašević et al., 2015). Bahraman et al. (2020) studied the incidence and the levels of AFM1 in milks from five animal species and an higher prevalence of AFM1 was observed in samples of northern coastal in comparison to mountainous regions. Geographical and environmental conditions can have impact on the formation of AFB1 in stored animal feedstuffs and consequently in milk and its derivatives. According to FAO and International Dairy Federation (2014), roughage represents a major part of the feed consumed

by dairy animals (around 80 %). The rest of the diet is composed of concentrated and compound feed. However, for cow, buffalo, sheep and goat species, the inclusion of the concentrate and compound feed is higher during the lactating stage than roughage, which is higher during the dry stage.

The AFM1 concentration trend in each continent was also evaluated. Compared to cow milk, human milk had significantly lower values ($p < 0.001$) in Europe and America, and significantly higher values ($p < 0.001$) in Africa. The latter finding demonstrates the higher exposure of Africa population to aflatoxins in their diet. For the other animal species, data are available only for Europe and Asia. In Europe, higher levels ($p < 0.001$) of AFM1 were observed for cow samples than for any other animal species. On the contrary, in the Asian continent cow samples showed the lowest AFM1 levels ($p < 0.001$), whereas buffalo milk was the type of milk with the highest level.

Cow's milk is one of the most consumed and produced types of milk in Europe (*Milk and Milk Product Statistics.*, 2020.), while in Asia, buffalo milk is one of the most consumed (Siddiky & Faruque, 2017). Therefore, the fact that there is a higher concentration of AFM1 in cow's milk in Europe and in buffalo's milk in Asia may be due to the fact that concentrate feed is incorporated in the animals' diet to increase the production yield and to combat the shortage of fresh feed at certain times of the year, such as winter. As discussed earlier, concentrate feed is more susceptible to contamination by AFB1, the precursor of AFM1.

Thus, the levels of AFM1 in different species are probably related to different factors, as the genetics of the animals, seasonal variation, the milking process, the environmental conditions, and most importantly the intake of contaminated food or feed.

### 3.3.4 Human health risk assessment

The risk for human health due to exposure of AFM1 through the ingestion of dairy products was evaluated. This risk was evaluated by: (i) considering the ingestion of four dairy products (milk, yogurt, cheese and butter), and (ii) considering the ingestion of just milk and yogurt (as discussed in the majority of available reports).

Table 13 summarizes the mean AFM1 concentration calculated and food consumption for each dairy product in each continent. These values were used for the calculation of the EDI of AFM1 and for the risk characterization per continent, presented in Table 14. Table 15 shows the contribution of each dairy group for the EDI values in each continent.

Table 13. Mean of AFM1 concentration in each dairy product (|AFM1|) and the respective ingestion rate (IR) for the four analyzed continents

| Continent | $\|AFM1\|^{1}_{milk\ and\ yogurt}$ ng/kg | $IR_{milk\ and\ yogurt}$ Kg/cap/day | $\|AFM1\|^{1}_{cheese}$ ng/kg | $IR_{cheese}$ Kg/cap/day | $\|AFM1\|^{1}_{butter}$ ng/kg | $IR_{butter}$ Kg/cap/day |
|---|---|---|---|---|---|---|
| Africa | 50.15 | $7.07 \times 10^{-2}$ | 172.07 | $1.92 \times 10^{-3}$ | 225.54 | $8.22 \times 10^{-4}$ |
| Asia | 21.13 | $1.54 \times 10^{-1}$ | 76.18 | $1.64 \times 10^{-3}$ | 21.02 | $4.11 \times 10^{-3}$ |
| Europe | 15.85 | $2.78 \times 10^{-1}$ | 49.67 | $4.22 \times 10^{-2}$ | 86.52 | $1.07 \times 10^{-2}$ |
| Latin America | 30.80 | $1.44 \times 10^{-1}$ | 49.00 | $4.77 \times 10^{-2}$ | 56.71 | $1.92 \times 10^{-3}$ |

[1]Represents the |AFM1| calculated (x) through the equation of log (x+1)

Table 14. Estimated daily intakes (EDI) of dairy products and percentages contribution of each group in each continent

| Continent | EDI (ng/kg bw/day) | | | |
|---|---|---|---|---|
| | Four dairy products | Milk and yogurt | Cheese | Butter |
| Africa | 0.067 | 0.058 (87 %)[1] | 0.0054 (8 %)[2] | 0.0031(5 %)[3] |
| Asia | 0.060 | 0.056 (94 %)[1] | 0.0022 (4 %)[2] | 0.0015 (2 %)[3] |
| Europe | 0.105 | 0.062 (59 %)[1] | 0.030 (28 %)[2] | 0.013 (12 %)[3] |
| Latin America | 0.101 | 0.065 (64 %)[1] | 0.034 (34 %)[2] | 0.0016 (2 %)[3] |

[1]Represents the percentage contribution of milk and yogurt group calculated as: EDI milk and yogurt / EDI four dairy products

[2]Represents the percentage contribution of cheese group calculated as: EDI cheese / EDI four dairy products

[3]Represents the percentage contribution of butter group calculated as: EDI butter / EDI four dairy products

Table 15. Human health risk assessment for AFM1 - hazard index (HI) and risk of hepatocellular carcinoma (HCC) – by continent, considering the ingestion of four dairy products (milk, yougurt, cheese and butter) or the ingestion of milk and yougurt only

| Continent | Ingestion of four dairy products | | Ingestion of milk and yogurt | |
|---|---|---|---|---|
| | HI | HCC Risk cases/year/$10^5$ | HI | HCC Risk cases/year/$10^5$ |
| Africa | 0.334 | $2.236 \times 10^{-4}$ | 0.292 | $1.952 \times 10^{-4}$ |
| Asia | 0.300 | $9.541 \times 10^{-5}$ | 0.281 | $8.958 \times 10^{-5}$ |
| Europe | 0.524 | $1.675 \times 10^{-4}$ | 0.311 | $9.936 \times 10^{-5}$ |
| Latin America | 0.507 | $1.339 \times 10^{-4}$ | 0.327 | $8.638 \times 10^{-5}$ |

For the worldwide most consumed group of dairy products – milk and yogurt (Table 7 and Table 13) – Europe is the continent with the lowest AFM1 levels (Table 13), possibly due to the strict regulations applied and to the strong concern with food safety. Contrarily, the African continent presents the highest AFM1 levels which may be related to the poorer conditions and the lack of official control. FAO/WHO (2001) reported the EDI of AFM1 through milk consumption for the European, Latin American, Far Eastern, Middle Eastern and African diets as being 0.11, 0.058, 0.20, 0.10 and 0.002 ng/kg bw/day, respectively. The values reported for African and American continents tend to be lower than the ones in

this study, considering the ingestion of milk and yogurt. On the other hand, for the European and Asian continents the values were similar and higher, respectively, than the ones in this study. WHO (2018) states that the dietary exposure to AFM1 rarely exceeds 1 ng/kg bw per day in any country. A study on the risk of AFM1 exposure of Latin American populations through milk consumption reported a mean EDI of 1.00 ng/kg bw/day (Sibaja et al., 2021), which is a ten-fold higher than the value obtained in this study. Higher values than reported in this study were also observed in studies conducted in Kenya (Ahlberg et al., 2018) and in Iran (Nejad et al., 2019), who reported EDI values of 0.8 and 0.107 ng/kg bw/day, respectively. Contrarily, other studies developed in some European countries, such as Portugal (Duarte et al., 2013) and Italy (Serraino et al., 2019), and also in the Asian continent, like India (Sharma et al., 2020), reported 0.08, 0.02 – 0.08 and 0.001– 0.002 ng/kg bw/day, respectively. When milk and yogurt ingestion is considered, the EDI values for Europe are of the same magnitude as those found for Africa, Asia and Latin America. This demonstrates the risk of underestimating the intake of AFM1 by disregarding dairy products other than milk. It is therefore vital to take into consideration that other types of dairy products which are consumed on a regular basis can also be contaminated with considerable levels of AFM1 (Table 13).

Looking at the values reported in Table 15, there is no evidence of risk (HI < 1), but there is clearly a trend to higher HI values for European and Latin American continents. Furthermore, for Europe and America, the HI values reduce to about half if only milk and yoghurt consumption is considered, which emphasizes the importance of considering other dairy products to estimate the risk, in particular cheese. In some countries of Europe, like Serbia (Milićević et al., 2017) and Italy (Serraino et al., 2019) it was reported HI values similar to this study, considering the ingestion of the milk and yogurt group. In opposite, in Argentina (Costamagna et al., 2021) and India (Pandey et al., 2021) lower and higher HI values were reported, respectively, than the ones in this study.

The risk of hepatocellular carcinoma per year per 100 000 individuals was also estimated considering the ingestion of the four dairy products and only milk and yogurt (Table 15). The obtained levels ranged from $9.541 \times 10^{-5}$ to $2.236 \times 10^{-4}$ and $8.638 \times 10^{-5}$ to $1.952 \times 10^{-4}$ cases per year per 100,000 individuals, respectively, showing a low risk of cancer due to AFM1 exposure from dairy products' consumption when compared with the values reported for AFB1, 3.0 – 54.0; 3.0 – 30.0; 0.09 – 1.2 and 6.0 – 15.0 cases per year per 100 000 individuals for African, Asian, European and Latin America continents, respectively (Liu & Wu, 2010). However, an increased risk of HCC is clearly observed for the European and Latin American continents, when the four dairy products are considered. Besides that,

comparing Latin America with Asia, both have a similar consumption of milk and yogurt group (Table 13), but the cheese consumption is much higher in the Latin American continent leading to an increase of risk to develop cancer. On the other hand, Africa, despite being the continent with lower dairy products consumption, the HCC risk value is the highest in both scenarios since predisposition to develop cancer ( $\overline{P_{cancer}}$ ) in this continent is also higher. Other studies from Italy (Serraino et al., 2019), Greece, (Udovicki et al., 2019), Serbia (Udovicki et al., 2019), Argentina (Costamagna et al., 2021), and Kenya (Ahlberg et al., 2018) reported higher HCC values ($4x10^{-4}$ – $8x10^{-4}$; $5x10^{-4}$ – $7x10^{-4}$; $1.7x10^{-3}$ – $2.2x10^{-3}$; $4.9x10^{-3}$ and $4x10^{-3}$) than the values observed in this study. Contrarily, one study from India (Pandey et al., 2021) reported a lower value.

This study demonstrates the potential contribution of cheese to the AFM1 exposure risk. It leads also to the question if there is a need to adopt legislative limits for cheese, since the forecast consumption of this dairy product tends to increase (OECD-FAO, 2021).

For the risk estimations, it was assumed that AFM1 presents a carcinogenicity potency 10 times lower than AFB1 and this value was based on data from studies in rodents (FAO/WHO, 1999). Thus, the estimates about the carcinogenicity of AFM1 are not as well established as for AFB1. Besides that, a cumulative effect with other factors, such as the combined exposure to different AFs, other food contaminants, poor diet and consumption of alcohol, can cause unknown and eventually even more significant risks due to synergistic effects between risk factors (Ahlberg et al., 2018).

## 3.4 Conclusions

This study demonstrated an increasing trend of AFM1 levels between 1990-2019 worldwide and that the African continent presented the highest AFM1 content in dairy products, while Europe is the continent with the lowest AFM1 levels. Focusing on dairy products, milk was the product with the lowest AFM1 concentration and butter and cheese the ones with the highest AFM1 levels, which proves that AFM1 is a stable and resistant compound. The risk for human health due to AFM1 exposure through the ingestion of all dairy products and of milk and yogurt alone was also evaluated. This study, demonstrated the importance of taking in consideration widely consumed dairy products other than milk, such as cheese, to estimate the risk. It also raises the issue of the need to adopt legislative limits for cheese, the second most consumed dairy group, as a preventive to ensure ensuring consumers' safety.

Dairy products are healthy and nutritious foods, so it is important to include them in the daily diet. Therefore, it is essential to reduce the exposure of dairy cattle to AF, to prevent milk contamination with

AFM1, ensuring the food safety even at increased dairy products' consumption. This can be achieved through the careful control of the feed harvest and storage conditions. Besides that, regular analytical control of animal feed before administration to dairy cattle associated with strict legislation may prevent the presence of AFM1 in milk and its derivatives.

# CHAPTER 4 – DISTRIBUTION OF AFLATOXIN M1 DURING THE PRODUCTION AND RIPENING OF SHEEP AND GOAT CHEESES

## 4.1 Introduction

AFM1 can appear in dairy products as a result of milk contamination with this toxin, being cheese one of the dairy products with the highest levels, as reported in Chapter 3. AFM1 concentration in cheese may increase up to 8 times when compared with the origin milk (Brackett & Marth, 1982). Most of the studies about the fate of AFM1 in different types of cheeses, like soft (Costamagna et al., 2019), semi-hard (Pecorelli et al., 2018) and hard cheese (Pietri et al., 2016a), reported a higher concentration of AFM1 in curd than in whey. This higher concentration in curd was mainly attributed to the affinity of AFM1 to casein, due to hydrophobic interactions. Furthermore, the same studies reported a continuous increase in concentration throughout the cheese making process, which was mainly attributed to moisture loss (Cavallarin et al., 2014). However, in many of these studies, AFM1 concentration in each step of production process was not evaluated in wet and dry basis to determine the contribution of the moisture loss to the increase in AFM1 concentration. Also, most studies on AFM1 distribution in cheese focus on cow milk cheeses (Cavallarin et al., 2014; Chavarría et al., 2017; Costamagna et al., 2019; Pietri et al., 2016a), and just a few studied sheep and goat cheeses (Alnaemi, 2019; Pecorelli et al., 2018).

To better understand the AFM1 behaviour throughout processing, it is of major importance to study the correlation between the casein content and the increase of AFM1 in curd cheese for cheeses from different milks. Therefore, the present research aimed to clarify the contrasting information from existing research reports, by investigating the fate of AFM1 along the goat and sheep cheese production process using artificially contaminated milk.

## 4.2 Experimental Design

### 4.2.1 Cheeses production and sampling

Milk and cheese samples were obtained during two cheese production processes performed for sheep and goat cheeses in July and December of 2020, in the Mountain Research Center and Escola Superior Agrária, *Instituto Politécnico de Bragança*, Portugal.

Regarding the origin of the milk for cheese production, sheep's milk was obtained from a farm in the municipality of *Macedo de Cavaleiros*, with 400 heads and producing approximately 80,000 L per year. Regarding goat's milk, it was obtained from a producer's cooperative association with around 250 members. The annual production exceeds 300,000 L and the collection area is vast, from *Valpaços* to *Bragança* and *Mogadouro*. Both types of milk were pasteurized at 72 °C for 15 to 20 seconds. After

pasteurization, milk was spiked with AFM1 at a level between 0.05 and 0.1 µg/L and mixed to ensure a good homogenization. Milk was sampled for AFM1 analysis before and after the spiking procedure. After pasteurization, milk was cooled in a water bath until around 35 °C, and diluted microbial liquid rennet – Reniplus *(Luso Coalho, Portugal)* – was added (33 mL/100 L) to coagulate the milk in 30 to 45 minutes. When the gel became sufficiently firm, it was cut into small pieces and allowed to rest for 5–10 min to allow the expulsion of the whey. The curd was then transferred to stainless steel molds and pressed until whey drainage stopped. This step lasted for 3 hours and afterwards the cheeses were immersed in a 20 % NaCl brine solution for 2 hours. The cheeses where then incubated in a maturation chamber with 85 % relative humidity and 10 °C for 3 to 6 weeks. During the production, three samples were collected at each step and all samples were stored in the freezer in airtight containers until analysis. Figure 14 shows the scheme of cheese production with the corresponding sampling points.

### 4.2.2 Chemicals and apparatus

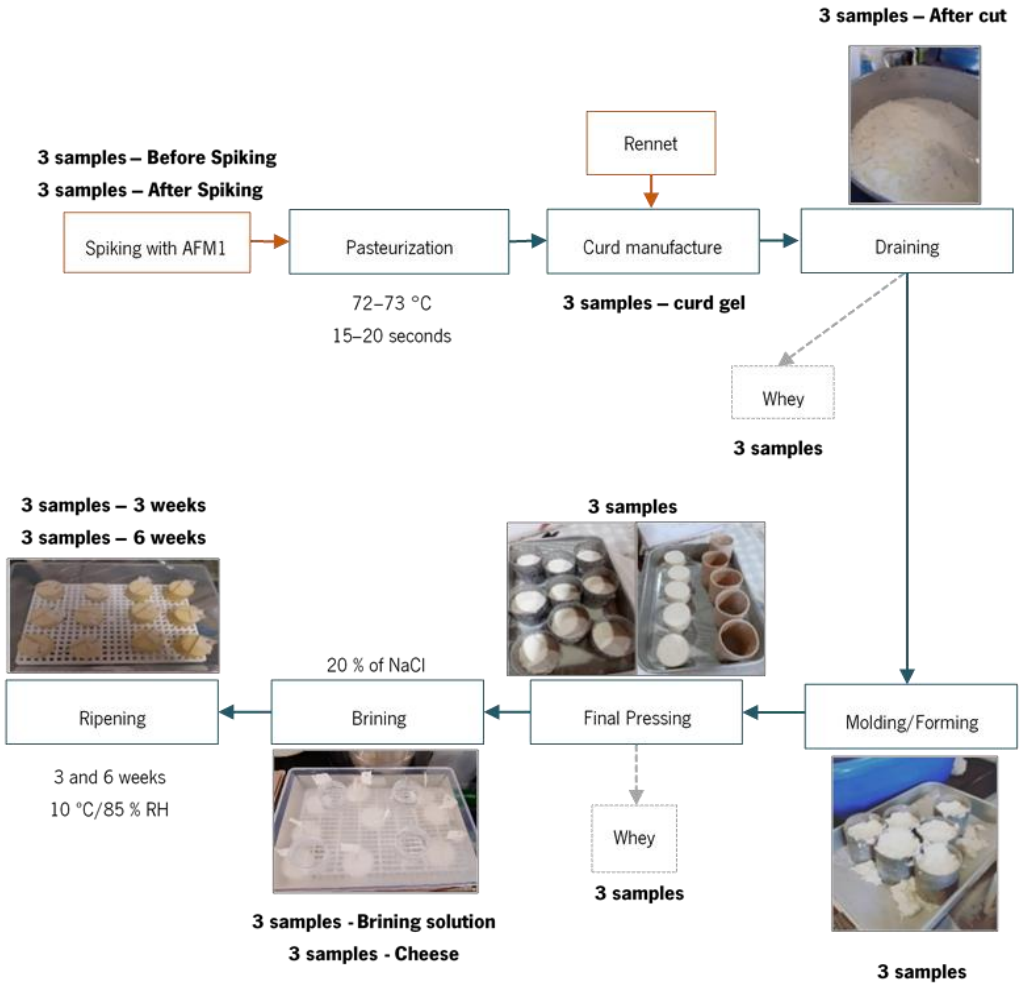


Figure 14. Scheme of sampling carried out during the cheese process.

The chemicals and supplies used in the study were: Aflatoxin M1 solution 0.5 µg/L in acetonitrile, analytical standard (34031-2mL, Sigma Aldrich Co. St. Louis, USA); Methanol and Acetonitrile, HPLC gradient grade ≥ 99.9 % (Fisher Chemical); Hexane, HPLC gradient grade ≥ 95 % (Fisher Chemical); Immunoaffinity Columns (IAC) AflaM1 HPLC (VICAM); Glass Microfiber Filters 150 mm/1.5 µm (934 AH ™, Whatman); Liquid rennet – Reniplus *(Luso Coalho);* Sulfuric acid ≥ 95 % (Fisher Chemical); Hydrogen peroxide (516813-500mL, Sigma Aldrich Co. St. Louis, USA); Sodium chloride and Sodium acetate (Analar NORMAPUR VWR Chemicals); Acetic Acid 99.7 % (Fisher Chemical); Kjeldahl TM 8400 Tecofor Line; Water bath (precisterm J.P. Selecta); Centrifuge (Sigma 3-16P); Evaporator (Reacti-Therm heating Module No, 187790); Magnetic Stirrer (Phoenix Instrument RSM – 04H); Mixer (Autovortex SA6).

### 4.2.3 Methodologies – AFM1 extraction, clean-up and determination

#### 4.2.3.1 Sample Extraction and Clean-up

Curd and cheese samples

Curd and cheese samples were analyzed for AFM1 using an adaptation from the R-biopharm method (r-biopharm, 2015). Briefly, 20 grams of sample were rigorously weighted and grounded with 60 mL of 70 % methanol in a suitable mixer. The whole sample was transferred to a tube and incubated at 50 °C for 30 min. During this incubation period, it was vigorously stirred 5 times. The suspension was then centrifuged for degreasing during 10 min, at 3000 g and 10 °C. Sixty mL of the aqueous phase (without fat) were transferred to a separating funnel and 45 mL of hexane were added. The mixture was shaken at least three times and the phases allowed to separate. Forty mL of the lower aqueous phase were collected and diluted with 160 mL of water. This solution was filtered before being added to the IAC for clean-up.

The clean-up of the extracts was performed according to the VICAM method (VICAM, 2019) with some adaptations. One hundred and sixty mL of extract were filtered through a glass microfibre filter and applied to the IAC. The column was washed with water, and AFM1 was eluted with 1.5 mL of a mixture of acetonitrile:methanol (3:2). The eluates were stored at -20 °C until analysis.

Milk, whey samples and brining solution samples

All samples were analyzed using an adaptation from the VICAM methodology (VICAM, 2019). Fifty mL were centrifuged at 1540 g for 15 minutes. The top fat layer was removed and the defatted sample was used for analysis.

For the clean-up, 50 mL of the defatted sample was applied to the IAC. The column was washed with water, and AFM1 was eluted with 1.5 mL of a mixture of acetonitrile:methanol (3:2). The eluates were stored at -20 °C until analysis.

#### 4.2.3.2 HPLC determination of AFM1

AFM1 was quantitated by high-performance liquid chromatography (HPLC). The chromatographic system coupled with fluorescence detection (FLD) (λexcitation = 360 nm, λemission = 430 nm), at 25 °C, isocratic elution with a water-acetonitrile-methanol mixture (68:24:8, v/v/v), flow rate 1 mL/min, and injection volume 100 μL.

#### 4.2.3.3 Validation of methodologies

Analytical validation data play a fundamental role in chemistry analysis, as it makes it possible to know the limitation and reliability of the method (Brito et al., 2003; Ludwing Huber, 2007; Relacre, 2000; Thompson et al., 2002).

The validation process should check that the method performs adequately for the purpose, throughout the range of the analyte concentrations and test materials to which it is applied. Furthermore, it must be considered when developing or adapting previously validated methodologies, including new techniques or using different equipment (Brito et al., 2003; Ludwing Huber, 2007; Relacre, 2000; Thompson et al., 2002). However, it is important to have in consideration that validation is always a compromise between costs, risks and technical ability.

The minimum requirements for the validation of internal methods depends on the type of method and comprise, in general, the study and knowledge of the following parameters (Huber, 2010):

– Working range/Linearity: The linearity of an analytical procedure is the parameter that determines if the method allows to obtain results directly proportional to the analyte concentrations (within a defined range of concentrations) and can be demonstrated by the correlation coefficient ($r^2$), which must be statistically equal to 1 (Moosavi & Ghassabian, 2012).
– Analytical thresholds (Ludwing Huber, 2007; Relacre, 2000):
  - LOD: corresponds to the lowest amount of analyte that can be detected and is obtained by dividing three - times the standard deviation of the regression by the slope of the calibration curve.
  - LOQ: corresponds to the lowest amount of analyte that can be quantified and is obtained by dividing ten - times the standard deviation of the regression by the slope of the calibration curve.
– Accuracy: allows to assess the agreement between the real value of the analyte in the sample and the

value obtained by the analytical process. The study of this parameter can be done through recovery tests, which allows to evaluate the efficiency of the extraction (Brito et al., 2003; Huber, 2010; Thompson et al., 2002).

– Precision evaluated through the repeatability tests (RSDr): this parameter allows to evaluate the degree of dispersion or degree of agreement between a series of measurements made on several samples of a homogeneous batch under the same conditions (same laboratory; same analyst; same equipment; same type of reagents and short time intervals) (Brito et al., 2003; Huber, 2010; Relacre, 2000). The precision is determined as percentual relative standard deviation (% RSDr), dividing the standard deviation by the mean value.

For linearity purposes, the calibration curve for AFM1 was prepared using 9 standard solutions of AFM1 (0.025 to 3.5 µg/L), in duplicate. During the experiments, whenever a change was made, such as changing the fluorescence lamp, changing the column and / or other maintenance of the equipment, a new calibration curve was prepared. LOD and LOQ were determined as described before.

The validation of the methods to determine AFM1 levels in milk and cheese were carried out taking into account the harmonized guidelines of Commission Regulation (EC) nº 401/2006 of 23 February. Three levels of spiking – 0.02, 0.05 and 0.10 µg/L and 0.05, 0.10 and 0.25 µg/kg for milk and cheese methodologies, respectively, were performed, in triplicate, in two different days. For that, 20 g of cheese were spiked with the standard toxin and left for 24 hours in the dark at room temperature to allow the incorporation of the toxin into the matrix and evaporation of the solvent. In the case of milk, the total amount of milk used for the analysis of triplicates, about 200 mL, was added with the standard toxin and left to stir for 30 minutes to ensure homogenization. After that, the toxin was extracted using the methodology described in section 4.2.3 – *Methodologies – AFM1 extraction, clean-up and determination*.

The recovery rates of each method were calculated for the three replicates of the three spiking levels, by the ratio of recovered AFM1 concentration relative to the known spiked concentration. Precision was calculated in terms of intra and inter-day repeatability from the obtained recovery rates. Based on the equation described by Horwitz & Albert (2006), the Commission Regulation (EC) nº 401/2006 of 23 February issued that the recommended values of experimental RSDr for each concentration level must be lower than, or equal to, two-thirds of the value derived by the Horwitz equation (Equation 10), which determines the value for reproducibility ($RSD_R$). The theoretical RSD % is calculated on the basis of the analyte concentration, independently of the matrix and analytical method used.

$$RSD_R = 2^{1-0.5 \log C} \quad (10)$$

where $C$ corresponds to the analyte concentration rate (e.g., $5 \times 10^{-8}$ for a spiked concentration of 0.05 µg/kg).

For the spiking levels 0.02, 0.05, 0.10 and 0.25 µg/L, the theoretical RSDr are: 19.22, 16.74, 15.08 and 13.14 %, respectively. Furthermore, an external validation was done by participating in an inter-laboratorial test organized by Progetto Trieste – Mycotoxins, Proficiency Testing Service by Test Veritas S.r.l, for cheese samples. Test Veritas S.r.l has been accredited according to the guidelines of EN ISO/IEC 17043:2010 "Conformity assessment General requirements for proficiency testing" by Accredia. Also, the quality management system has been assessed and certified by SGS Italia as meeting the requirements of UNI EN ISO 9001:2015 for the development and production of materials for agrifood analyses and proficiency supply. The laboratory performance evaluation was established taking into account the following criteria for $Z$ – score (Relacre, 2000): acceptable (satisfactory) when $|Z| < 2$; warning signal (questionable) when $2 < |Z| < 3$, and action signal (unsatisfactory) when $|Z| > 3$.

In addition to the parameters mentioned above, the global uncertainty (U) was also calculated, despite not being a minimum requirement in the validation process. According to Ratola et al. (2004), the study of this parameter allows to attribute an effective meaning to the data. In this study four individual sources of uncertainty were considered.

1) Uncertainty associated with preparation of standards (U1)

This uncertainty was calculated using equation 11:

$$U_1 = \left[\sum (\Delta v_i / vi)^2\right]^{1/2} \quad (11)$$

where $vi$ represents the reagent volume in each measurement and $\Delta vi$ is the error associated with measurement. The parameter $\Delta vi$ was evaluated as half of the smallest segment of the measuring equipment.

2) Uncertainty associated with the calibration curve (U2)

This uncertainty was calculated using equations 12 to 14:

$$U_2 = S_{x0} / x0 \quad (12)$$

$$S_{x0} = \frac{S_{y/x}}{b} \times \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(\bar{y_0} - \bar{y})^2}{b^2 \times \sum (x_i - \bar{x})^2}} \quad (13)$$

$$S_{y/x} = \left[\sum (y_i - y_{icalc})^2 / (n-2)\right]^{1/2} \quad (14)$$

where:

$S_{x0}$ – represents the standard deviation of the concentration, calculated from the calibration graph

$x0$ – concentration calculated from the calibration graph

$n$ – number of experimental points to build the calibration curve

$b$ – slope of the calibration graph

$m$ – experimental values obtained for each $x$ value

$\overline{y_0}$ – average of experimental values of $y$ for each level of concentration (in case of duplicates, it is the mean of the area obtained for both)

$\bar{y}$ – average of all the $y_i$ experimental values

$x_i$ – concentration of standards (x) used in the calibration curve

$\bar{x}$ – average of all the $x_i$ values

$S_{y/x}$ – standard error of the estimate

$y_i$ – the experimental values (area obtained for each concentration)

$y_{icalc}$ – value calculated from the calibration curve

3) Uncertainty associated with precision (U3)

This uncertainty was calculated using equation 15:

$$U_3=(s/\sqrt{n})/x_0 \quad (15)$$

where $s$ is a standard deviation of the experimental data.

This uncertainty was only evaluated for standards with higher U1 value. Each standard chosen was injected four times.

4) Uncertainty associated with accuracy (U4)

This uncertainty was calculated using equation 16:

$$U_4=s(\eta)/\sqrt{n} \quad (16)$$

where $s(\eta)$ is the relative standard deviation of the percentual recovery.

This uncertainty was evaluated for each spiking level for both methods, milk and cheese. Thus, for each method the U4 value corresponds to the mean of three values.

5) Global Uncertainty (U)

The global uncertainty (U) is then calculated using equation 17:

$$U=(U_1^2+U_2^2+U_3^2+U_4^2)^{1/2} \quad (17)$$

The results obtained from equation 17 correspond to the combined uncertainty i.e. to the sum of all uncertainties; however, these results are always expressed as an expanded global uncertainty (which corresponds to twice the combined uncertainty value) (Ratola et al., 2004).

### 4.2.4 Determination of milks composition

The total protein and casein were determined for goat and sheep milks according to the Kjeldahl method (AOAC International, 2010; ISO and IDF, 2016). Besides, the total content of fat (Folch et al., 1957) and dry weight of samples (FAO/SIDA, 1986) were also analyzed.

### 4.2.5 Calculations

#### 4.2.5.1 Determination of AFM1 distribution in whey and curd

To calculate AFM1 distribution between whey and curd, the distribution of milk between whey and curd was firstly estimated by a water balance (equations 18 and 19), and then the distribution of AFM1 was estimated based on the results from the water balance and on the AFM1 concentration determined analytically (equations 20 and 21):

$$\%\ MD_{curd} = 100 \times ((\%\ DW_{milk} - \%\ DW_{whey}) / (\%\ DW_{curd} - \%\ DW_{whey})) \quad (18)$$

$$\%\ MD_{whey} = 100 - \%\ MD_{curd} \quad (19)$$

where $\%\ MD_{curd}$ and $\%\ MD_{whey}$, represent the percentage of mass distribution between curd (curd yield) and whey (whey yield) and $\%\ DW_{milk}$, $\%\ DW_{whey}$, $\%\ DW_{curd}$, represent the dry weight in milk samples, whey samples and curd samples, respectively.

$$\%\ \text{AFM1 distribution}_{curd} = \%\ MD_{curd} \times (|AFM1|_{curd} / |AFM1|_{milk}) \quad (20)$$

$$\%\ \text{AFM1 distribution}_{whey} = \%\ MD_{whey} \times (|AFM1|_{whey} / |AFM1|_{milk}) \quad (21)$$

where $/AFM1/_{curd}$ $/AFM1/_{milk}$ and $/AFM1/_{whey}$, represent AFM1 concentration in curd samples, milk samples and whey samples, respectively.

#### 4.2.5.2 Determination of Enrichment Factor (EF)

The EF of AFM1 was calculated by using equation 22.

$$EF = |AFM1|_{curd} / |AFM1|_{milk} \quad (22)$$

#### 4.2.5.3 Determination of AFM1 concentration on dry basis

Aflatoxin M1 concentration on a wet basis was calculated from the dry weight and the AFM1 concentration in each sample (equation 23)

$$|AFM1|_{DW} = (|AFM1|_{WB}/\%\,DW)\times 100 \quad (23)$$

where, $/AFM1/_{DW}$ and $/AFM1/_{WB}$ represent AFM1 concentration in dry weight and AFM1 concentration on a wet basis, respectively.

### 4.2.6 Statistical Analysis

Data were analyzed using IBM SPSS Statistics 27.0 statistical software. The means of AFM1 concentration at different steps were analyzed and discussed using ANOVA followed by the Tukey test to evaluate differences between groups. The homogeneity of variance was verified using Levene's test ($p < 0.05$). Once this assumption was violated, the robust Brown-Forsythe's test and Welch's test were applied to check for differences between groups, followed by the Post-Hoc Games-Howell test. Regarding composition of milk, means of total protein, casein and fat content were analyzed using Test F followed by the Test T to evaluate the differences between means. Significance was established at $p < 0.05$ for all tests.

## 4.3 Results and Discussion

### 4.3.1 Validation of methodologies

The values obtained in the study of HPLC-FLD linearity and sensitivity for AFM1 detection and quantification in milk and cheese samples are summarized in Table 16. These results show the high sensitivity of the methods, which allow AFM1 detection and quantification in milk samples contaminated with levels below the European maximum legal limit (Vaz et al., 2020). There are no limits legally established for cheese.

Table 16. HPLC-FLD performance parameters for AFM1 detection and quantification in milk and cheese samples: linear range, calibration curve, correlation coefficient and limits of detection and of quantification

| **Linear range (µg/L)** | **Calibration Curve** | **$r^2$** | **Milk (µg/L)** | | **Cheese (µg/kg)** | |
|---|---|---|---|---|---|---|
| | | | LOD | LOQ | LOD | LOQ |
| 0.25 – 3.5 | Área (uV.min) = 2462.5±8.7\|AFM1\|(µg/L) + 9.9971± 17.3095 | 0.9998 | 0.002 | 0.005 | 0.008 | 0.03 |

The results obtained for the accuracy and precision determined for the milk and cheese AFM1 extration methods are summarized in Table 17. The average recoveries determined for milk and cheese samples were in the range of 62 % – 87 % and of 54 % – 78 %, respectively. Although recovery values should be as close as possible to 100 %, recoveries of 50 % or more are unlikely to affect the integrity of the method. Methods that give rise to moderate recoveries can demonstrate good precision and accuracy, as long as they present good sensitivity (Causon, 1997).

Table 17. Mean recovery, and intra- and inter-day standard deviation (% RSDr) for three levels of spiking in milk and cheese samples

| Samples | Spiking level (µg/L) | Mean recovery (%) | RSDr (%) | |
|---|---|---|---|---|
| | | | Intra-day | Inter-day |
| Milk | 0.02 | 68 ± 4 | 4.2-8.8 | 5.8 |
| | 0.05 | 82 ± 6 | 3.6-9.5 | 6.2 |
| | 0.10 | 80 ± 5 | 3.4-6.3 | 5.4 |
| Cheese | 0.05 | 69 ± 4 | 5.3 | 4.8 |
| | 0.10 | 74 ± 3 | 2.8-5.6 | 3.7 |
| | 0.25 | 61 ± 4 | 5.9-8.7 | 6.2 |

For milk, the intra and inter-day precisions (RSDr) were in the range of 3.4 % – 9.5 % and 5.4 % – 6.2 %, respectively. For cheese, the intra and inter-day precisions (RSDr) were in the range 2.8 % – 8.7 %, and 3.7 % – 6.2 %, respectively. The values obtained are in accordance with the guidelines of Commission Regulation (EC) nº 401/2006. Therefore, after analysis of the values obtained for the RSDr, it is possible to state that this method has high accuracy. However, for the spiking level of 0.05 µg/L, there were problems during the injection of samples resulting in the loss of 2 samples in one of the days of analysis. Thus, for this level, only 4 values were used for the calculation and not 6 values, as in the other levels of spiking.

An external validation (inter-laboratory test) was performed for the cheese methodology, for which a $Z$– score of 1.02 was obtained, which indicates a satisfactory result.

Concerning global uncertainty, Table 18 presents the individual uncertainties at each concentration level for both methods. The expanded global uncertainty towards AFM1 concentration for milk and cheese methods can be seen in Figure 15. The global data is available in Table A.2. in Appendix to Chapter 4.

As shown in Figure 15, the uncertainty variation ranges between 4 – 12 %. For the studied AFM1 concentration range, the global uncertainty is higher for concentrations below 0.25 µg/L and, therefore, it will be better to work in the range between 0.25 – 3.5 µg/L, where the expanded uncertainty is lower than 10 %.

Table 18. Results obtained for individual uncertainties U1, U2, U3 and U4

| **Standard (µg/L)** | **0.25** | **0.50** | **0.75** | **1.00** | **1.50** | **2.00** | **2.50** | **3.00** | **3.50** |
|---|---|---|---|---|---|---|---|---|---|
| U1 | 0.007 | 0.007 | 0.010 | 0.009 | 0.018 | 0.015 | 0.014 | 0.015 | 0.018 |
| U2 | 0.054 | 0.026 | 0.017 | 0.013 | 0.009 | 0.006 | 0.005 | 0.004 | 0.004 |
| U3 | - | - | - | - | 0.004 | - | - | - | 0.002 |
| U4 | For milk methodology: 0.021 | | | | | | | | |
| | For cheese methodology: 0.015 | | | | | | | | |

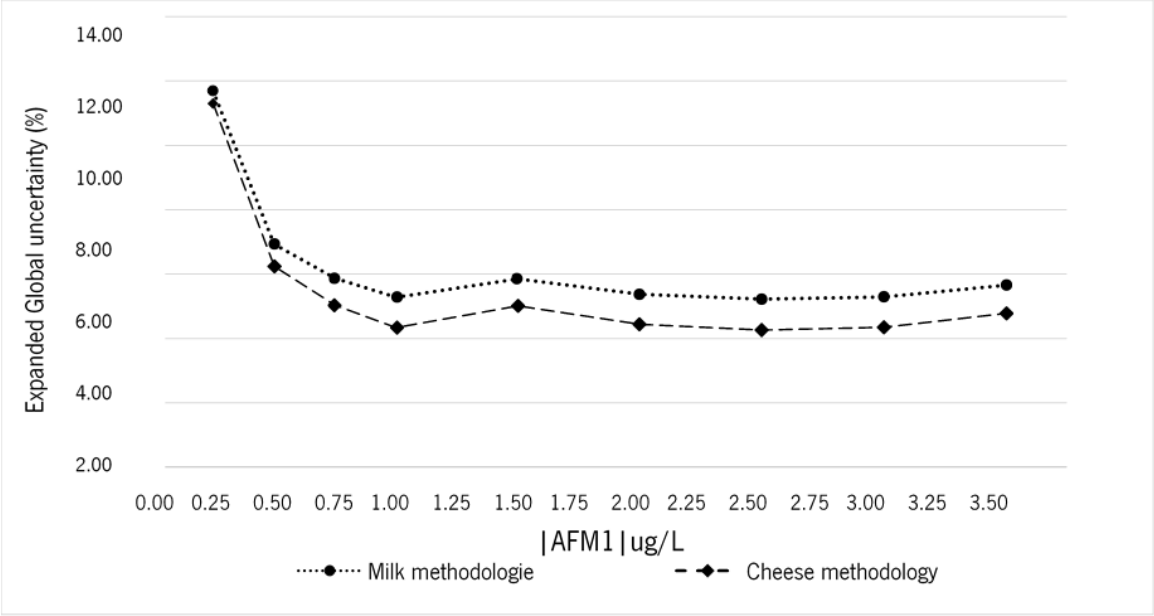


Figure 15. Global uncertainty for the two extraction methos evaluated.

The validation process indicates that the methods used for milk and cheese samples are appropriate for analysis of AFM1, allowing to obtain reliable results. The mean concentration values of AFM1 determined in this study were corrected for the recovery of the analytical method, 77 % for the milk methodology and 68 % for the cheese methodology.

### 4.3.2 Influence of milk composition in AFM1 distribution during cheese making

Table 19 shows the characterization of milks in terms of total protein, casein, total fat and dry weight. Differences were observed in milk composition in the summer (1st production) and winter (2nd production) seasons, but these differences were more pronounced in the goat milk, where the dry weight of the 2nd production was significantly higher. The composition of milk is influenced by several factors, such as animal breed, type of diet, season, stage of lactation and environment (Guo et al., 2004; Raynal-Ljutovac et al., 2008), which can explain the observed differences.

For goat milk, the percentage of total protein and casein was higher ($p < 0.05$) in the second production, whereas in sheep milk a similar percentage of casein ($p > 0.05$) was observed in both productions. Cheese is a dairy product that results from the concentration of milk. Therefore, a higher curd yield and consequent cheese production is expected when milk presents a higher percentage of casein and fat (Tetra Pak, 2018). This trend was observed for goat cheese with higher casein and fat content, which resulted in a higher curd yield ($p < 0.05$). Concerning sheep cheese, despite the similar casein content ($p > 0.05$) the curd yield was significantly lower ($p < 0.05$) for the second production, where it was observed a higher fat content of the milk ($p < 0.05$). In fact, the dry matter in curd was strongly and positively correlated with the casein plus fat contents in milk ($r^2 = 0.88$). The process of cheese production, more specifically the cutting stage, can also influence the whey loss, and therefore the curd yield. If more whey is lost, a lower wet weight can result in higher dry weight in curd (Table 19).

A higher percentage of AFM1 was observed, as well as a higher concentration, in curd than in whey for all samples (Table 19). Previous studies (Table 20) have reported percentages of AFM1 in curd from 40 to 64 %, corroborating findings from this study. Concerning sheep cheese, despite very similar ($p > 0.05$) casein in both productions, the EF obtained was quite different ($p < 0.05$), possibly due to the higher fat content or the cutting stage for whey draining. According to Pecorelli et al. (2019), the EF is independent of the amount of toxin in milk, but is significantly correlated to milk quality parameters (protein, casein and fat) and cheese yield. Comparing with previous studies (Table 20), lower EF values have been observed in the present study. However, the whey content drained in those studies was also greater, increasing the concentration of AFM1 in the curd and consequently leading to a higher EF. Besides that, the findings of this study are in accordance with study the by Alnaemi (2019), which observed a trend to lower AFM1 concentration in sheep whey, in comparison to goat whey.

Table 19. Physicochemical composition of goat and sheep milks: dry weight, total protein, casein and total content of fat. Percentage of curd obtained (Curd Yield), % AFM1 distribution in whey and curd and respective AFM1 concentration in whey and curd

| Type of milk | Production | Physicochemical characterization of the milk [1] | | | | Mass balance [1] | | | AFM1 distribution [1] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % Dry weight | Total protein (g/100g) | Casein (g/100g) | Total Fat (g/100g) | Curd Yield (%) (A) | %Dry weight of curd (B) | Dry matter (AxB) | % AFM1 in whey | $\|AFM1\|_{whey}$ (µg/kg) [2] | % AFM1 in curd | $\|AFM1\|_{curd}$ (µg/kg) | EF |
| Goat | 1st production | 11.288± 0.005[a] | 3.579± 0.036[a] | 3.235± 0.018[a] | 3.811 ± 0.027[a] | 33.35± 0.26[a] | 21.45± 0.01[a] | 7.15± 0.72[a] | 38.70± 2.87[a] | 0.050±0.004[a] | 47.57± 1.29[a] | 0.123± 0.002[a] | 1.43±0.04[a] |
| | 2nd production | 17.744± 0.001[b] | 5.471± 0.032[b] | 4.941± 0.041[b] | 6.637 ± 0.068[b] | 41.22± 0.057[b] | 30.75± 0.01[b] | 12.68± 0.09[b] | 28.13± 2.80[a] | 0.047±0.005[a] | 75.09± 1.09[b] | 0.180± 0.003[b] | 1.81±0.03[b] |
| Sheep | 1st production | 13.974± 0.007[a] | 4.960± 0.068[a] | 4.482± 0.077[a] | 5.366 ± 0.075[a] | 36.47± 1.22[a] | 26.15± 0.01[a] | 9.54± 0.53[a] | 29.39± 0.48[a] | 0.042±0.001[a] | 54.82 ±3.00[a] | 0.137± 0.012[a] | 1.50±0.08[a] |
| | 2nd production | 15.450± 0.002[a] | 5.008± 0.090[a] | 4.587± 0.081[a] | 5.596 ± 0.084[b] | 26.35± 1.55[b] | 35.37± 0.01[b] | 9.32± 0.49[a] | 32.12± 5.27[a] | 0.043±0.007[a] | 66.95± 3.76[b] | 0.248± 0.014[b] | 2.54±0.14[b] |

[1]Results are expressed as mean ± standard deviation for n=3, where the symbol n, represents the number of tests performed. Different letters indicate significant differences between the samples of the same animal species determined by T-test ($p < 0.05$); [2]Results are expressed as mean ± standard deviation for n=3 for sheep whey and n=2 for goat whey. The symbol n represents the number of tests performed.

Table 20. Compilation of literature data. Milk origin, percentage of whey obtained during production (Whey yield), concentrations and percentage distribution of AFM1 in milk, whey and curd and respective curd enrichment factor

| Milk origin | Whey Yield (%) | Milk | Whey | | Curd | | EF | Reference |
|---|---|---|---|---|---|---|---|---|
| | | \|AFM1\| (µg/kg) | \|AFM1\| (µg/kg) | % of AFM1 retained | \|AFM1\| (µg/kg) | % of AFM1 retained | | |
| Cow | 86 | 0.045 | 0.023 | 44 | 0.175 | 54 | 3.9 | (Govaris et al., 2001) |
| Cow | 86 | 0.088 | 0.041 | 40 | 0.386 | 60 | 4.4 | (Govaris et al., 2001) |
| Cow | 86 | 0.245 | 0.159 | 56 | 0.782 | 42 | 3.2 | (Oruc et al., 2006) |
| Cow | 86 | 0.049 | 0.033 | 58 | 0.144 | 40 | 2.9 | (Oruc et al., 2006) |
| Cow | 86 | 0.730 | 0.448 | 53 | 2.465 | 46 | 3.4 | (Oruc et al., 2006) |
| Cow | 85 | 0.049 | 0.027 | 47 | 0.175 | 53 | 3.6 | (Oruc et al., 2007) |
| Cow | 86 | 0.244 | 0.125 | 44 | 0.926 | 55 | 3.8 | (Oruc et al., 2007) |
| Cow | 86 | 0.732 | 0.342 | 40 | 2.943 | 58 | 4.0 | (Oruc et al., 2007) |
| Cow | 79 | 1.230 | 0.560 | 32 | 3.72 | 56 | 3.0 | (O. Deveci, 2007) |
| Cow | 79 | 3.100 | 1.370 | 32 | 9.81 | 59 | 3.2 | (O. Deveci, 2007) |
| Cow | 78 | 0.950 | 0.370 | 29 | 2.950 | 64 | 3.1 | (Motawee & McMahon, 2009) |
| Cow | 80 | 1.830 | 0.690 | 28 | 6.000 | 64 | 3.3 | (Motawee & McMahon, 2009) |
| Cow | N/C[1] | 0.030 | 0.017 | 59 | 0.143 | 41 | 4.6 | (Pietri et al., 2016a) |

[1]N/C – not possible to calculate

### 4.3.3 Evolution of AFM1 concentration in the production of goat and sheep cheeses

The distribution of AFM1 throughout the stages of goat and sheep cheese production was studied and AFM1 concentration on a wet basis and on a dry basis is presented in Figure 16 and Figure 17, respectively. The global data for each step in both bases can be seen in Appendix to Chapter 4.

In the first production stage – curd formation, an increase in AFM1 was observed. Along the other processing stages, it would be expected that the AFM1 concentration would keep increasing, due to the loss of moisture and to the preferential affinity to the milk casein fraction through non-covalent interactions during curd formation (Alnaemi, 2019; Cavallarin et al., 2014). Pressing can lead to toxin losses in whey; however, since AFM1 concentration in whey is lower, the result is an increased AFM1 concentration in the pressed curd (mean of 48.05 ± 0.30 %). After pressing, the brine step follows. This is the most controversial step, since decreases of up to 30 % have been reported (Table 21), mainly attributed to the diffusion of AFM1 into the brine solution.

Table 21. Literature data on changes in AFM1 concentration in cheeses after the brining step

| $\lvert AFM1 \rvert_{Cheese}$ (µg/kg) | $\lvert AFM1 \rvert_{After\ Brining}$ (µg/kg) | % Decrease | Brining time | Reference |
|---|---|---|---|---|
| 6.00 | 5.50 | 8.3 | – | (Brackett & Marth, 1982) |
| 0.175 | 0.168 | 3.6 | 7 days | (Govaris et al., 2001) |
| 3.72 | 3.42 | 8.1 | 3 months | (O. Deveci, 2007) |
| 2.95 | 2.07 | 30 | 10 hours | (Motawee & McMahon, 2009) |

After brining, a decrease in AFM1 content is observed in Figure 16 (mean of 13.54 ± 0.07 %) and Figure 17, although statistically not significant. In this study, brining took place for just 2 hours, while in the studies mentioned in Table 21 the brining time was higher. Thus, time seems not to be the determinant factor for AFM1 reduction during this stage.

During ripening, due to humidity loss, an increase in AFM1 concentration is observed. In all processes, milk's AFM1 content (on a wet basis) was statistically different from all other samples and was the sample with the lowest toxin content. On the other hand, ripening cheeses were the samples with the highest toxin content. The highest AFM1 levels observed in ripening cheeses can be directly linked to the loss of moisture that occurred during the process, which leads to the increase of the matrix dry weight. Nevertheless, no statistical changes were observed between ripening cheese for three and six weeks. The same was reported by Oruc et al. (2006), Oruc et al. (2007) Deveci et al. (2006), Deveci

(2007), Fernandes et al. (2012), Iha et al. (2013) and Pecorelli et al.(2019).

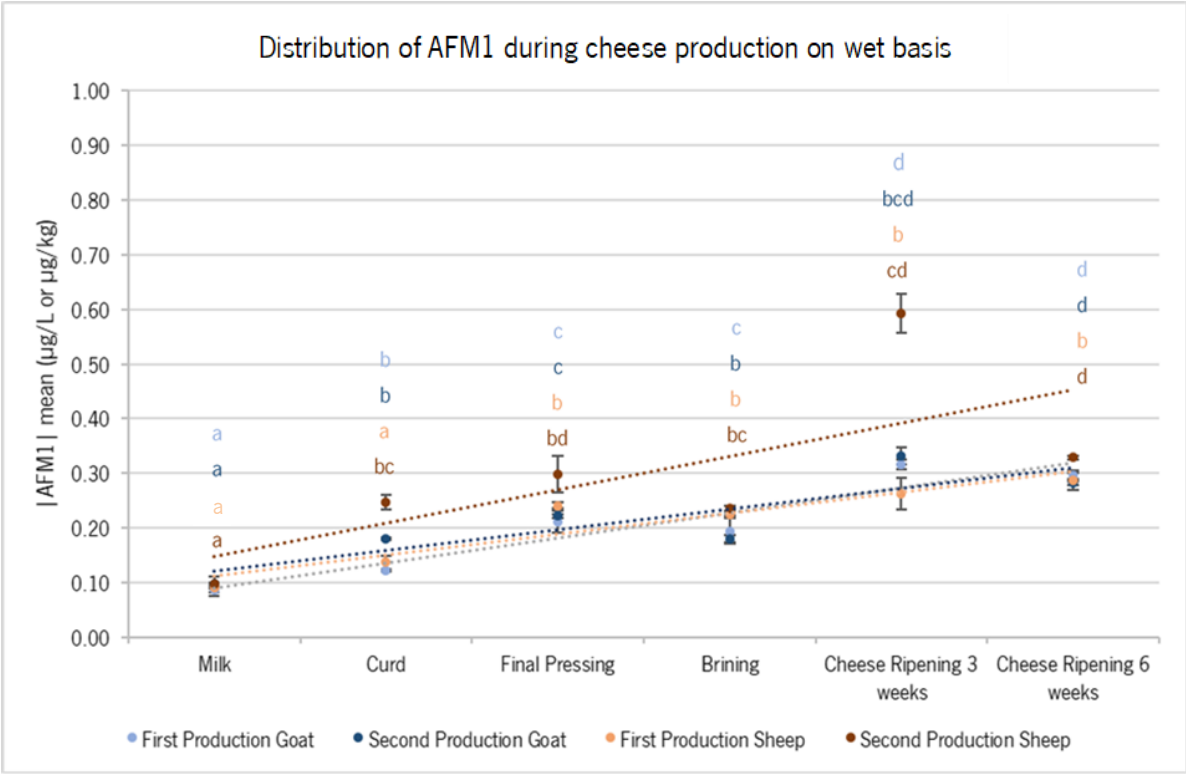


Figure 16. AFM1 distribution throughout the goat and sheep cheese production stages. Different letters indicate significant differences between samples in each production as determined by the Tukey test ($p < 0.05$) for the first production of goat cheese and by the Games-Howell test for the rest of the productions.

On a dry basis (Figure 17), the toxin concentration is expected to remain constant unless the toxin is removed in whey at the initial stages or degraded by the action of the rennet during the maturation stage. However, at the initial stages, the reduction of toxin compared to milk was significant only for the first production of both species. Regarding the final stages of the production process (brining and ripening stages), the toxin concentration remained constant, except for the second process of sheep. These results indicate that there was no degradation. To confirm this result, a complementary test was done to evaluate the rennet's ability to degrade the toxin Appendix to Chapter 4, and it was observed that no toxin degradation occurred. Therefore, the increase of AFM1 concentration on final stages in wet basis can be related to the loss of moisture.

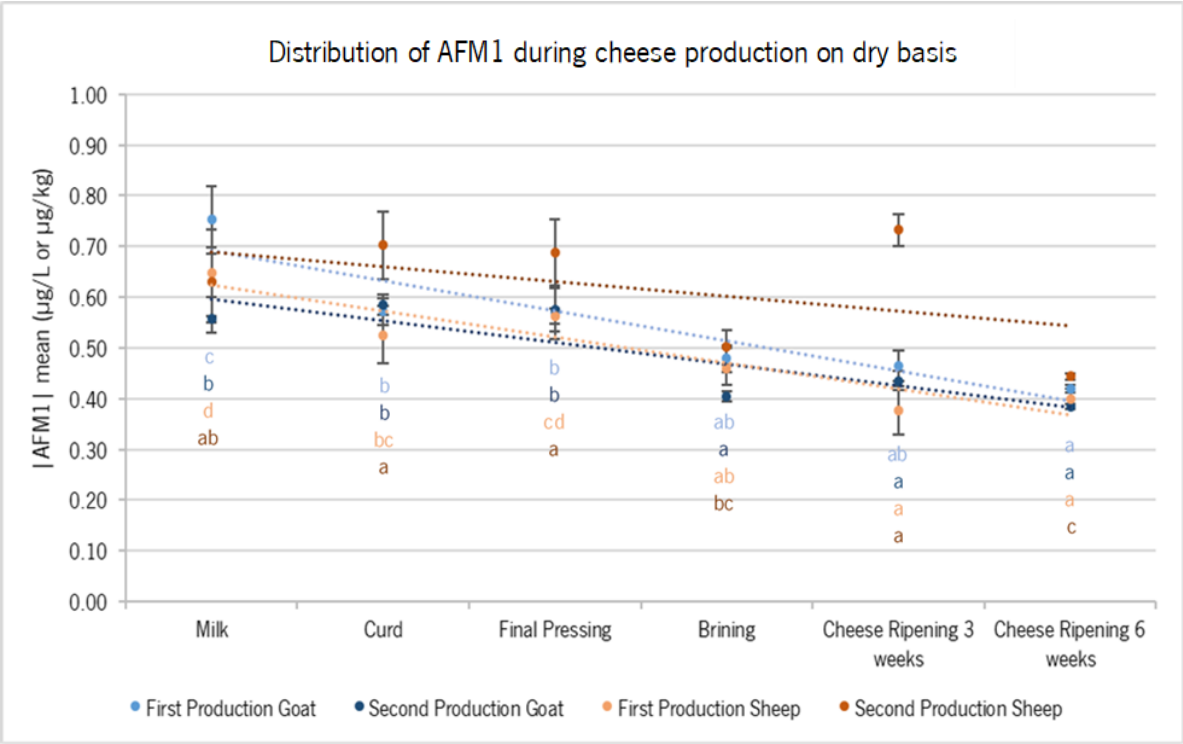


Figure 17. AFM1 distribution throughout the goat and sheep cheese production stages. Different letters indicate significant differences between samples in each production as determined by the Tukey test ($p < 0.05$).

## 4.4 Conclusion

This study evaluated the fate of AFM1 during goat and sheep cheese-making. The obtained data revealed that AFM1 concentration in curd was higher than in whey in all experiments and, on average, it was approximately two-fold higher than in the contaminated milk. Furthermore, a trend to a lower percentage of AFM1 retained in sheep whey was also observed. Different findings on the content of AFM1 have been reported during brining and ripening stages. This study allowed to verify these contrasting informations, demonstrating that during brining no statistical changes were detected. Also, time seems not to be the determining factor for AFM1 reduction during this stage. Regarding the ripening stage, in general, no statistical changes were observed on a dry basis. Thus, the changes observed on wet basis can be related to the moisture content.

This work also allowed to conclude that AFM1 is a rather stable compound, under the experimental conditions used, and that its distribution is affected by milk composition. Therefore, to prevent this contaminant from being present in cheese, the control should occur at the beginning (dairy feed in good conditions) or middle of the dairy chain, using enzymes or microorganisms that are able to detoxify the toxin. However, the action of enzymes or microorganisms can be challenging due to the effect of food

matrix. Organoleptic properties can also change after this treatment. Thus, more investigation on mitigating strategies concerning the intrinsic diversity of the processing steps is crucial.

The work developed and in agreement with some studies reported in the literature demonstrate that the cheese manufacturing processes do not reduce or eliminate AFM1, but, on the contrary, they tend to concentrate the levels of toxins. This evidence and the fact that cheese is a widely consumed food matrix, lead to questions about the need to adopt legislative limits on cheese.

# **CHAPTER 5 –** CONCLUSIONS AND FINAL REMARKS

Despite the growing increase of plant origin sources that replace dairy products, such as soy drink and vegan cheese, among others, milk and its derivatives continue to be widely consumed worldwide. This market has been trying to keep up with the development of society and the growing concern with health. At present, there are already countless dairy products without lactose, skim and with appealing flavors to reach the largest possible niches of the population.

Milk and dairy products are a very important source of macro – and micronutrients including calcium, fatty acids, proteins, vitamins, and minerals essential for the growth, development and human health in general. However, despite other concerns with this matrix, such as the fact that it is of animal origin and is a source of saturated fat, milk is also the greatest source of AFM1 in human diet. The presence of this toxin in milk and dairy products, even in small quantities, represents a concern to human health, mainly because this toxin is considered as a possible carcinogen.

Several international studies have reported the occurrence of AFM1 in milk and dairy products, and the main purpose of this work was to verify the contrasting information in literature about AFM1 occurrence and its distribution during cheese-making. Also, the exposure to this toxin via dairy products consumption (by the EDI) and the risk to human health (through the HI and the HCC) were estimated.

To understand AFM1 occurrence trend and behaviour, a systematic literature search was performed using data from 329 articles about AFM1 occurrence in dairy products. Based on this data base, the relation between AFM1 concentration and sampling decade, continent, type of dairy product, and animal species was evaluated. This study revealed that there is a trend towards the increase in AFM1 concentration between 1990 – 2019, at a worldwide level. The Asian continent apparently deviated from this trend, which can be related to the outbreak of aflatoxins in Asian maize that occurred between 2005 – 2009. Comparing the continents, Africa presented the highest AFM1 levels, indicating inadequate conditions and concerns in food safety and food security in the region. However, the consumption of dairy products in Africa is the lowest, being the EDI and HI values also low. On the other hand, the African population has a higher prevalence of individuals with HBsAg+, leading to a higher impact of AFM1 on cases of HCC. Regarding Europe, it was the continent with the lowest AFM1 levels, possibly related to different factors: (i) the modern feed processing practices; (ii) adoption of strict food safety systems; (iii) highly stringent regulations applied to mycotoxins in food and feed, and (iv) advanced analytical techniques that allow continuous monitoring of compounds like aflatoxins. Nevertheless, the dairy consumption in Europe is high, so the human health risk (HI) to AFM1 is also high. On the other hand, the risk to develope cancer (HCC) is lower due to the lower prevalence of HBsAg+ individuals. Concerning

the type of dairy product, milk presented the lowest AFM1 concentration. Contrarily, butter and cheese samples presented the highest AFM1 concentration. Despite the fact that cheese represents a small slice in terms of dairy products consumption (about 13 % worldwide), the high AFM1 levels observed increase the risk to human health, representing this group 22 % of the estimated AFM1 daily intake (EDI). Also, in the particular case of the European and Latin American continents, cheese represents 13 and 25 % of the dairy products consumption, and 28 and 34 % for the EDI, respectively. From this, it is evident that the contamination with AFM1 in dairy products is an issue of concern, and the importance of these products in the human diet further drives the need for the quantification of AFM1 levels in all types of dairy products.

The levels of carry–over of AFM1 to dairy products are controversial. To verify the contrasting information, the fate of AFM1 in goat and sheep cheeses productions were evaluated. Results showed that a higher percentage and a higher concentration of AFM1 were always observed in curd (compared with whey). Correlation was established between curd yield and milk composition (sum of casein and fat). AFM1 is a rather stable compound, under the experimental conditions applied, and its distribution is affected by milk composition. Thus, interactions at molecular level might be a promising approach to demystify the true interaction of AFM1 with different proteins allowing to develop and adopt specific strategies for its reduction. This study also allowed to demonstrate that during the brining stage no statistical changes were detected. A significant increase of AFM1 concentration throughout the process in wet basis was observed and this was mainly attributed to a concentration effect due to cheese humidity losses, since an additional study proved that rennet is not able to degrade AFM1 during ripening.

Therefore, as demonstrated in this work and in most of the studies reported in the literature, AFM1 is a stable compound and its elimination or reduction is still a challenge to be overcome. The combination of several factors: (i) cheese is widely consumed; (ii) its consumption tends to increase; (iii) this matrix tends to concentrate AFM1 levels, and (iv) the AFM1 levels in dairy products tend to increase over the years, as a result of climate changes and of inadequate post-harvest storage and handling processes of feed (which increase the risk of contamination by AFB1), lead to questions about the necessity of adoption of legislative limits in cheese. The definition of legal limits is an issue that entails several difficulties, due to the existence of a huge variety of cheeses. There are several cheese production processes that have a direct impact on the distribution of the toxin through the different stages. Soft cheeses tend to concentrate less toxin than hard cheeses. On the other hand, cheeses belonging to the same hardness category (same moisture content) may have different Enrichment Factor since this parameter is significantly correlated

to the quality parameters of the milk used (protein, casein and fat) that directly influence cheese yield. Therefore, the distribution of the toxin throughout the process and, consequently, its concentration in cheese is directly related to the production process and the origin of the milk. Furthermore, since cheese is a food matrix with a higher dry extract content compared to other dairy products, all compounds are naturally concentrated. Thus, there are several issues that arise, but they must be considered so that it is possible to define reasonable and achievable limits. A wide – ranging study of controlled and systematic conditions that would allow evaluating the behavior of the toxin (starting from a high amount of milk and making different productions from this mother sample) could minimize the variability of results and better observe possible trends/correlations.

An extensive study that would allow the establishment of Enrichment Factor in the different cheese categories based on their hardness and on the milk average composition would be essential. The systematization of analytical methodologies would also allow a greater standardization of results in different studies. In this way, knowledge in this area would increase and converge in a certain direction, allowing the competent authorities to take a decision in a supported and adequate manner, always with the objective of guaranteeing food quality and safety. On the other hand, setting a minimum threshold for soft cheeses and adopting a correction factor for harder cheeses could be a strategy to be adopted. Regardless of the path to be chosen, considerable research is still needed on this topic and systematic studies can promote the establishment of limits that will certainly minimize the risk of the population against AFM1.

Additionally, special measures should be taken to protect feed from contamination with AFB1 or to reduce the exposure of dairy animals to this toxin, since the final levels of AFM1 will depend on the initial levels at the primary production. Mitigation actions should take place either upstream (feed) or downstream (in contaminated milk), by selecting rennet enzymes or fermentation organisms able to transform and detoxify the toxin.

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# APPENDICES

# Appendix to Chapter 2

Table A.1. Determination of AFM1 in milk and dairy products using different methods

| Product | Sample Preparation | Extraction | Clean-up | Quantification Method | References |
|---|---|---|---|---|---|
| Milk | Centrifugation<br>Filtration | Magnetic Solid Phase Extraction (MSPE) | NA[2] | HPLC-FLD | (Hashemi et al., 2014) |
| Milk | Centrifugation<br>Filtration | AOAC 2000.08<br>Pepsin-HCl (P-HCl) method | IAC | HPLC-FLD | (Pietri et al., 2016) |
| Milk | Homogenization<br>Centrifugation | C18-SPE columns | NA[2] | HPLC-FLD | (Manetta et al., 2005) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | Chemiluminescent Enzyme-linked immunosorbent Assay (CL-ELISA) | (Vdovenko et al., 2014) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | Fluorometric ELISA | (Kanungo & Bhand, 2013) |
| Milk | Centrifugation | NA[2] | NA[2] | Potentiometric aflatoxin M1-immunosensor | (Rameil et al., 2010) |
| Milk | Homogenization<br>Centrifugation<br>Filtration | QuEChERS | QuEChERS<br>C18 sorbent; PSA | UHPLC–MS/MS | (Michlig et al., 2016) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | Intramolecular electro-chemiluminescence resonance energy transfer (ECL-RET) | (Liu et al., 2017) |
| Milk | NI[1] | Captiva ND lipids extraction (lipids and proteins removal)-<br>Organic solvents: methanol | NA[2] | Impedimetric aptasensor | (Istamboulié et al., 2016) |
| Milk | NI[1] | Addition to Apt GMAPs-GO-LAgNPs;<br>Magnetic separation | NA[2] | Electrochemiluminescence (ECL) aptasensor | (Khoshfetrat et al., 2018) |
| Milk<br>Milk powder | Homogenization | Organic Solvents: Acetonitrile<br>SPE: Oasis HLB cartridge | NA[2] | UHPLC–ESI–MS/MS | (Huang et al., 2014) |
| Milk | Centrifugation | NA[2] | IAC | HPLC-FLD | (Shuib et al., 2017) |

Table A.1. (Continued)

| Product | Sample Preparation | Extraction | Clean-up | Quantification Method | References |
|---|---|---|---|---|---|
| Milk | Centrifugation | NA[2] | IAC | HPLC-FLD | (Shuib et al., 2017) |
| Milk powder | NI[1] | Organic Solvents: chloroform<br>Magnetic molecularly imprinted polymer I (MMIP-SB) | NA[2] | HPLC–MS/MS | (Díaz-Bao et al., 2016) |
| Milk powder | NI[1] | Organic Solvents: methanol/water | NA[2] | RT-qPCR based aptasensor | (Guo et al., 2016) |
| Milk | Homogenization<br>Centrifugation<br>Filtration | NA[2] | IAC | HPLC-FLD | (Yoon et al., 2016) |
| Milk | Homogenization<br>Centrifugation<br>Filtration | NA[2] | IAC | HPLC-FLD, TLC, ELISA | (Al-Mossawei et al., 2016) |
| Milk | Centrifugation<br>Filtration | NA[2] | IAC | HPLC-FLD | (Fernandes et al., 2012) |
| Milk | Centrifugation<br>Filtration | SPE column | NA[2] | UHPLC–QqQ–MS/MS | (Aguilera-Luiz et al., 2011) |
| Milk | NI[1] | Organic Solvents: acetonitrile<br>Organic Solvents: acetone<br>QuEChERS | NA[2] | UHPLC–QqQ–MS/MS | (Aguilera-Luiz et al., 2011) |
| Milk | NI[1] | MIP | NA[2] | Scanning electron microscopy, energy dispersive spectroscopy, and Fourier-transform infrared spectroscopy | (Bodbodak et al., 2018) |
| Milk | NA[2] | QuEChERS | QuEChERS - C18 sorbent and PSA | UHPLC-Q-Orbitrap HRMS | (Rodríguez-Carrasco et al., 2018) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | ELISA, HPLC-FLD | (Norian et al., 2015) |

Table A.1. (Continued)

| Product | Sample Preparation | Extraction | Clean-up | Quantification Method | References |
|---|---|---|---|---|---|
| Milk | Homogenization<br>Centrifugation<br>Filtration | NA[2] | IAC | HPLC-FLD | (Öztürk Yilmaz & Altinci, 2019) |
| Milk | NI[1] | liquid–liquid extraction -<br>Homogenization<br>Organic Solvents: ethyl acetate, acetic acid | NA[2] | HPLC-UV | (Curticapean & Curticapean, 2013) |
| Milk | NA[2] | Organic Solvents: chloroform | silica gel column chromatography | TLC | (Fallah, 2010; Kamkar, 2005) |
| Milk | Homogenization<br>Centrifugation<br>Filtration | NA[2] | IAC | UHPLC–ESI–MS/MS | (Camaj et al., 2018) |
| Milk | NA[2] | Salt-induced liquid–liquid extraction (SI-LLE) | NA[2] | UHPLC–MS/MS | (Campone et al., 2016) |
| Milk | Centrifugation | Organic Solvents: acetonitrile<br>SPE columns | NA[2] | UHPLC–MS/MS | (Campone et al., 2018) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | ELISA | (Mohamadi Sani et al., 2012; Motawee & McMahon, 2009) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | electrochemical immunosensor | (Micheli et al., 2005) |
| Milk | Centrifugation<br>Filtration | NA[2] | NA[2] | Flexible Dispense-Printed Electrochemical Immunosensor | (Abera et al., 2019) |
| Milk | Homogenization | Organic Solvents: methanol | IAC | LC-FLD | (Iha et al., 2011) |
| Cheese | Centrifugation<br>Filtration | Organic Solvents: chloroform, hexane | IAC | HPLC-FLD | (Fernandes et al., 2012) |
| Cheese | Grated | Pepsin-HCl (P-HCl) method<br>Pepsin-pancreatin (PP) method<br>Cloroform (CH) method | IAC | HPLC-FLD | (Pietri et al., 2016) |

Table A.1. (Continued)

| Product | Sample Preparation | Extraction | Clean-up | Quantification Method | References |
|---|---|---|---|---|---|
| Cheese | Homogenization<br>Centrifugation | C18-SPE | NA[2] | HPLC-FLD | (Manetta et al., 2005) |
| Cheese | NI[1] | Organic Solvents: chloroform<br>SPE | NA[2] | TLC | (Filazi et al., 2010) |
| Cheese | Homogenization | Organic Solvents: dichloromethane/acetone<br>Organic Solvents: acetonitrile/water<br>C18-SPE columns | NA[2] | UHPLC-MS/MS | (Škrbić et al., 2015) |
| Cheese | Homogenization | Organic Solvents: dichloromethane, methanol-water-hexane | IAC | HPLC-FLD, TLC, ELISA | (Al-Mossawei et al., 2016) |
| Cheese | NI[1] | Organic Solvents: acetonitrile/water | IAC | HPLC-FLD | (Yoon et al., 2016) |
| Yoghurt<br>Cheese | NI[1] | Organic solvents: dichloromethane, methanol, heptane | NA[2] | ELISA | (Motawee & McMahon, 2009; Sarimehmetoglu et al., 2004) |
| Cheese | NI[1] | Organic Solvents: chloroform, hexane | IAC | HPLC-FLD | (Öztürk Yilmaz & Altinci, 2019) |
| Cheese | NI[1] | Organic solvents: dichloromethane | NA[2] | ELISA | (Tavakoli et al., 2012) |
| Cheese | Homogenization | Organic Solvents: methanol | IAC | LC-FLD | (Iha et al., 2011) |
| Yoghurt | NI[1] | Organic Solvents: chloroform | IAC | HPLC-FLD, TLC, ELISA | (Al-Mossawei et al., 2016) |
| Yoghurt<br>Ice Cream<br>Cheese | NI[1] | Organic Solvents: chloroform | silica gel column chromatography | TLC | (Fallah, 2010) |

Table A.1. (Continued)

| Product | Sample Preparation | Extraction | Clean-up | Quantification Method | References |
|---|---|---|---|---|---|
| Yoghurt | Homogenization | Organic Solvents: methanol | IAC | LC-FLD | (Iha et al., 2011) |
| Butter | NI[1] | Organic Solvents: chloroform, hexane | IAC | HPLC-FLD | (Öztürk Yilmaz & Altinci, 2019) |

NI [1]- Not Indicated; NA[2] - Not Applicable

# Appendix to Chapter 3

## A.3.1 Data transformation using the *log(x+1)* function

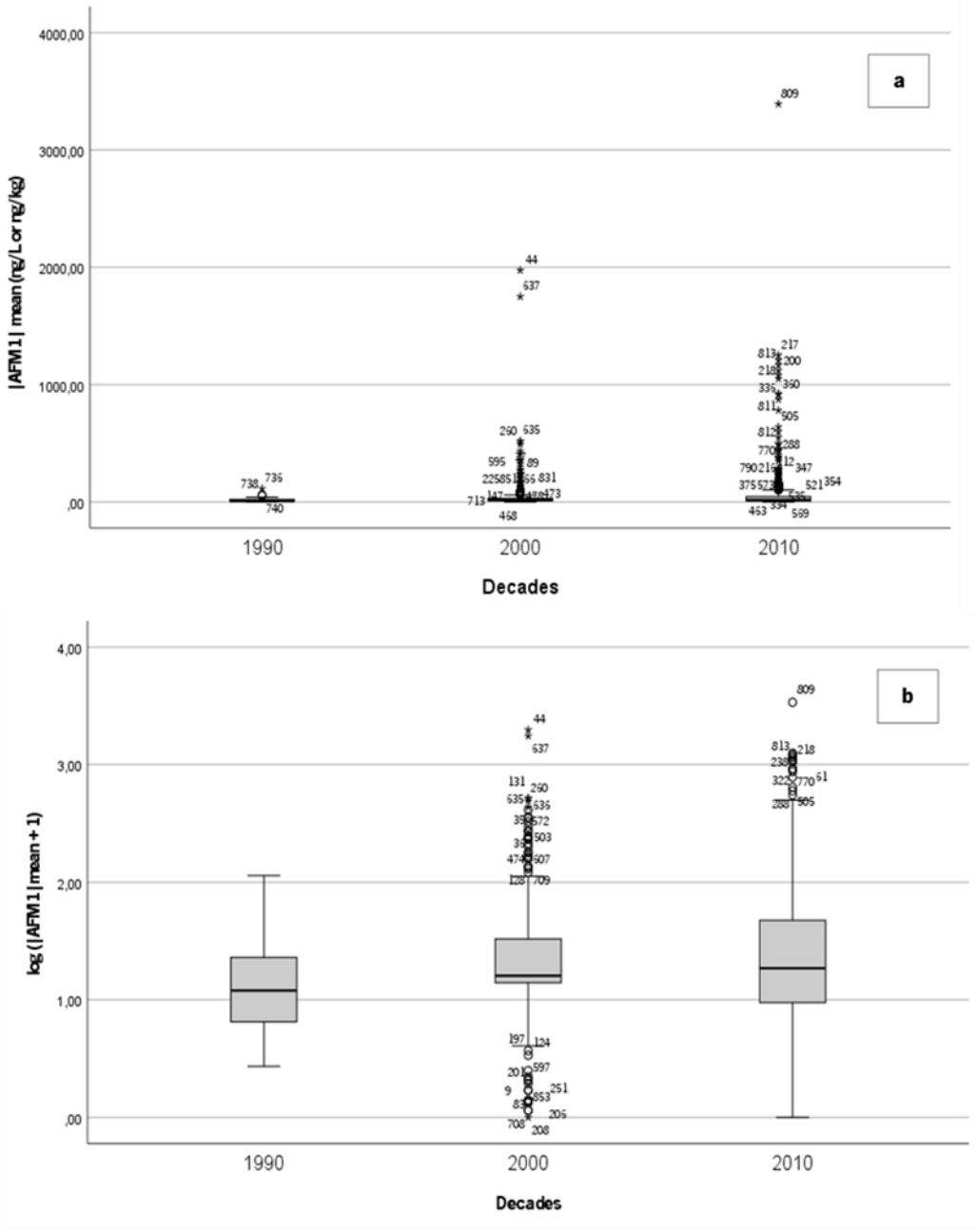


Figure A.1. AFM1 mean concentration of all samples by decades (a) expressed in concentration units, and (b) after transformation with log(x+1) function.

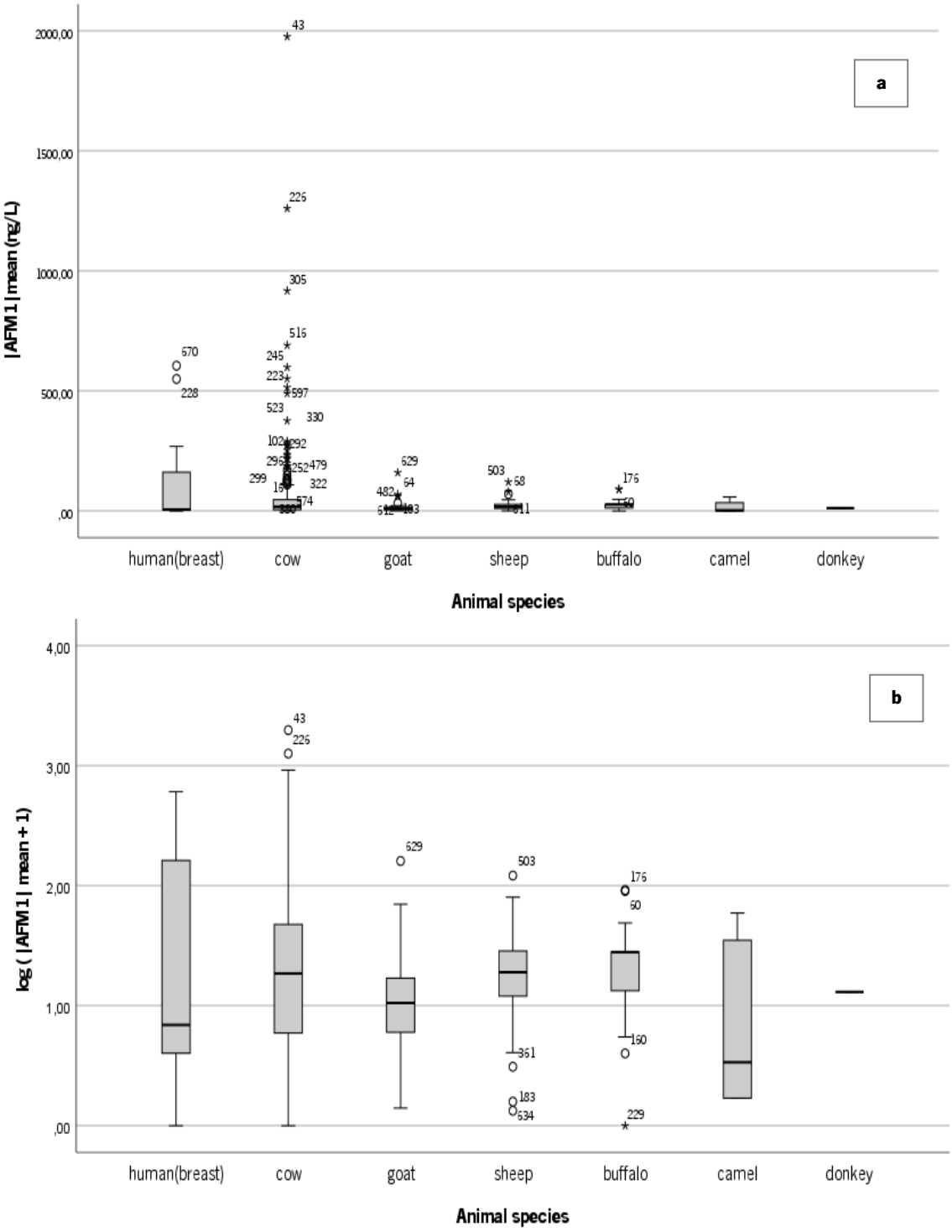


Figure A.2. AFM1 mean concentration of all samples by animal species (a) expressed in concentration units, and (b) after transformation with log(x+1) function.

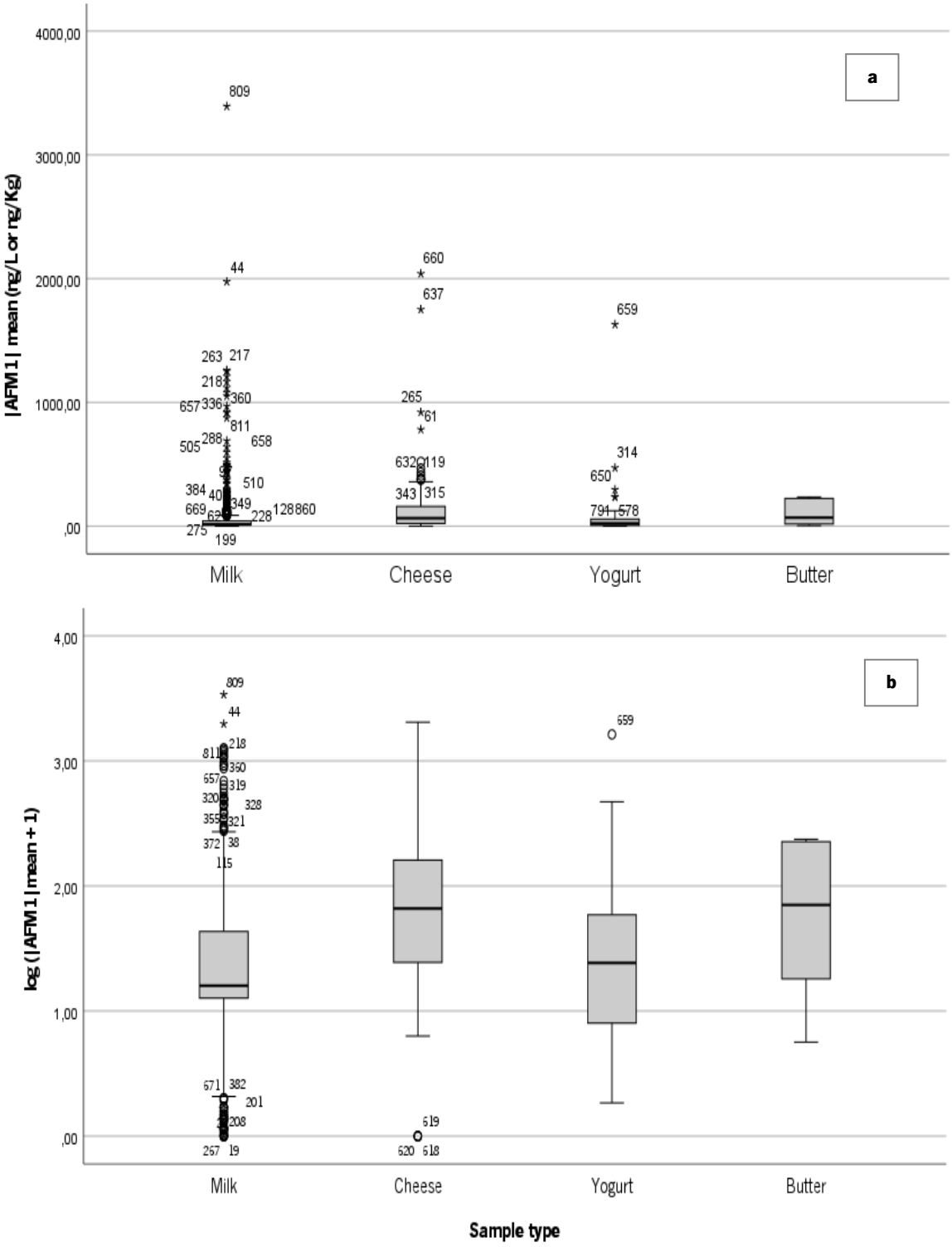


Figure A.3. AFM1 mean concentration of all samples by sample type (a) expressed in concentration units, and (b) after transformation with log(x+1) function.

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### A.3.3 Link of global data about AFM1 occurrence

https://docs.google.com/spreadsheets/d/1d8cmzU8o4om8kkAIq65ihy8UeOzFPq1Y/edit?usp=sharing&ouid=103472162345661507944&rtpof=true&sd=true

Password: Andreia-AFM1-010593

# Appendix to Chapter 4

## A.4.1 Expanded global uncertainty

Table A.2. Compilation of results obtained for global uncertainties at each concentration level for milk and cheese methodologies

| Standard (µg/kg) | | | 0.25 | 0.50 | 0.75 | 1.00 | 1.50 | 2.00 | 2.50 | 3.00 | 3.50 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cheese method** | U3=0.004 | Uc(%) | 5.65 | 3.12 | 2.51 | 2.17 | 2.51 | 2.22 | 2.13 | 2.17 | 2.39 |
| | U4=0.015 | Ux (%) | 11.29 | 6.24 | 5.03 | 4.33 | 5.01 | 4.44 | 4.26 | 4.34 | 4.78 |
| | U3=0.002 | Uc(%) | 5.64 | 3.10 | 2.49 | 2.14 | 2.48 | 2.19 | 2.10 | 2.15 | 2.37 |
| | U4=0.015 | Ux (%) | 11.27 | 6.20 | 4.99 | 4.28 | 4.97 | 4.39 | 4.21 | 4.29 | 4.73 |
| **Milk method** | U3=0.004 | Uc(%) | 5.85 | 3.47 | 2.93 | 2.64 | 2.93 | 2.68 | 2.61 | 2.64 | 2.83 |
| | U4=0.021 | Ux (%) | 11.69 | 6.93 | 5.87 | 5.28 | 5.85 | 5.37 | 5.22 | 5.29 | 5.65 |
| | U3=0.002 | Uc(%) | 5.84 | 3.45 | 2.91 | 2.62 | 2.91 | 2.66 | 2.59 | 2.62 | 2.81 |
| | U4=0.021 | Ux (%) | 11.67 | 6.90 | 5.83 | 5.24 | 5.82 | 5.33 | 5.18 | 5.25 | 5.62 |

[1]Uc: Ucombined; [2]Ux: Uexpanded

## A.4.2 Evolution of AFM1 concentration in the production of goat and sheep cheeses

Table A.3. AFM1 mean concentration obtained in each step of sheep and goat cheese production processes on wet basis.

| Production | Samples | Mean concentration of AFM1 on wet basis (µg/L or µg/Kg )[1] | |
|---|---|---|---|
| | | Goat | Sheep |
| 1st | Milk | 0.08588 ± 0.00921$^{a}$ | 0.09113 ± 0.00310$^{a}$ |
| | Curd | 0.12251 ± 0.00247$^{b}$ | 0.13723 ± 0.01182$^{a}$ |
| | Final Pressing | 0.21130 ± 0.02044$^{c}$ | 0.24133 ± 0.00597$^{b}$ |
| | Brining | 0.19473 ± 0.02225$^{c}$ | 0.22547 ± 0.00366$^{b}$ |
| | Cheese - Ripening 3 weeks | 0.31567 ± 0.00933$^{d}$ | 0.26297 ± 0.02887$^{b}$ |
| | Cheese - Ripening 6 weeks | 0.29550 ± 0.00494$^{d}$ | 0.28733 ± 0.01680$^{b}$ |
| 2nd | Milk | 0.09876 ± 0.00279$^{a}$ | 0.09747 ± 0.01467$^{a}$ |
| | Curd | 0.17989 ± 0.00261$^{b}$ | 0.24770 ± 0.01390$^{bc}$ |
| | Final Pressing | 0.22173 ± 0.00359$^{c}$ | 0.29867 ± 0.03338$^{bd}$ |
| | Brining | 0.17970 ± 0.00645$^{b}$ | 0.23667 ± 0.00496$^{bc}$ |
| | Cheese - Ripening 3 weeks | 0.33254 ± 0.01587$^{bcd}$ | 0.59190 ± 0.03550$^{cd}$ |
| | Cheese - Ripening 6 weeks | 0.28227 ± 0.00315$^{d}$ | 0.32927 ± 0.00346$^{d}$ |

[1]Results are expressed as mean ± standard deviation for n=3. The symbol n, represents the number of tests performed for each step. Different letters indicate significant differences between samples in each production as determined by the Tukey test ($p < 0.05$) for the first production of goat cheese and by the Games-Howell test for the other productions.

Table A.4. AFM1 mean concentration obtained in each step of sheep and goat cheese production processes on dry basis.

| Production | Samples | Mean concentration of AFM1 on dry basis (µg/L or µg/Kg )[1] | |
|---|---|---|---|
| | | Goat | Sheep |
| 1st | Milk | 0.75262 ± 0.06576$^{c}$ | 0.64869 ± 0.04933$^{d}$ |
| | Curd | 0.57161 ± 0.02578$^{b}$ | 0.52571 ± 0.05669$^{bc}$ |
| | Final Pressing | 0.57112 ± 0.05301$^{b}$ | 0.56275 ± 0.01586$^{cd}$ |
| | Brining | 0.48093 ± 0.05488$^{ab}$ | 0.46009 ± 0.00751$^{ab}$ |
| | Cheese - Ripening 3 weeks | 0.46532 ± 0.02976$^{ab}$ | 0.37606 ± 0.04792$^{a}$ |
| | Cheese - Ripening 6 weeks | 0.41916± 0.00809$^{a}$ | 0.39932 ± 0.01937$^{a}$ |
| 2nd | Milk | 0.55660 ± 0.01403$^{b}$ | 0.63161± 0.10126$^{ab}$ |
| | Curd | 0.58560 ± 0.02057$^{b}$ | 0.70201 ± 0.06775$^{a}$ |
| | Final Pressing | 0.57482 ± 0.04191$^{b}$ | 0.68800 ± 0.06535$^{a}$ |
| | Brining | 0.40495 ± 0.01048$^{a}$ | 0.50238 ± 0.00353$^{bc}$ |
| | Cheese - Ripening 3 weeks | 0.43513 ± 0.01859$^{a}$ | 0.73255 ± 0.03162$^{a}$ |
| | Cheese - Ripening 6 weeks | 0.38417$^{a}$ ± 0.00469$^{a}$ | 0.44341 ± 0.00561$^{c}$ |

[1]Results are expressed as mean ± standard deviation for n=3. The symbol n, represents the number of tests performed for each step. Different letters in the mean value indicate significant differences between samples in each production as determined by Tukey test ($p < 0.05$).

### A.4.3 The rennet's ability to degrade the toxin

#### A.4.3.1 Materials and Methods

#### A.4.3.2 Sampling

To evaluate the ability of rennet to degrade AFM1 an assay was simulated using contaminated milk with ten times more AFM1 and rennet used in cheese process productions. For assays, 0.6 L of skim milk was added to the "test" bottle. The milk was spiked with 0.5 µg/L of AFM1 and with 2 mL of the same rennet used in the production process. For blanks, 0.3 L of skim milk was added to the "blank" bottle. The mik was spiked with AFM1 at 0.5 µg/L, but no rennet was added. Both solutions were mixed during 30 minutes in a magnetic stirrer to ensure a good homogenization. After that, they were placed in a dark room at 37 °C during 9 weeks. For AFM1 analysis over the days, three samples and one control samples were taken as demonstrated in Figure A.1. during 9 weeks.

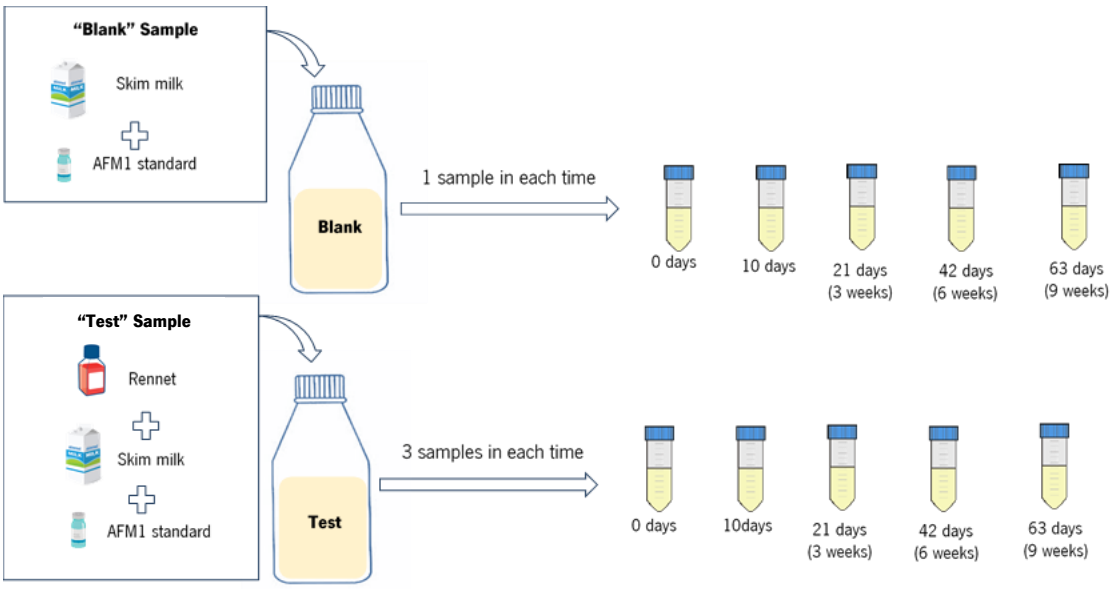


Figure A.1. Representative scheme of the rennet assay.

### 4.1.1.2 Chemicals and apparatus

The chemicals and apparatus used in the study were according the methodologies describe in section 4.2.2.

#### 4.1.1.3 Sample Extraction, Clean-up and determination

Samples were analyzed for AFM1 as curd samples according the methodologies described in section 4.2.3.

### 4.1.2 Results and discussion

The results obtained are summarized in Table A.. The ratio obtained for the first sampling time (0 days) and for the last sampling time (63 days) was around 1, which proves that no degradation occured. Considering that, the samples of intermediate times were not analyzed.

Table A.5. Results obtained for rennet test. Mean AFM1 concentration for test and blank samples in times 0 and 63 days and ratio obtained in each situation.

| Sample | Mean \|AFM1\| µg/kg | Ratio[2] |
|---|---|---|
| Blank (t = 0 days) | 0.8441 | 1.0924 |
| Test (t = 0 days)[1] | 0.9221 ± 0.0394 | |
| Blank (t = 63 days) | 0.5389 | 1.0878 |
| Test (t = 63 days)[1] | 0.5862 ± 0.0460 | |

[1]Results are expressed as mean ± standard deviation for n=3, where the symbol n represents the number of tests performed; [2]Ratio calculated as: |AFM1| Test / |AFM1| Blank