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Optimization and internal validation of a method for extraction and detection of ochratoxin A in cheese

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Abstract

Mycotoxins are toxic secondary metabolites produced by fungi when they grow in food and feedstuff. Milk and dairy products especially cheese continue to raise concerns with regard to contamination with mycotoxins. However, for some items, such as cheese, there is a lack of precise information available about mycotoxin contents.

The objective of this study was to develop an extraction, cleanup and detection method for the determination of ochratoxin A (OTA) occurrence in "Serra da Estrela" cheese.

To achieve this goal, extraction conditions were selected by testing different OTA extraction methods. The influence of two variables on OTA recovery was evaluated: extraction efficiency of digestive enzymes (lipase and protease) and type of purification (liquid-liquid and immunoaffinity columns purification). Under optimized conditions, recoveries of OTA from spiked samples ranged from 62.7 to 70.6%. The HPLC determination by fluorescence detection allowed limits of detection and quantification of 0.45 and 1.51 μg/kg, respectively. A survey was conducted on twenty five "Serra da Estrela" cheeses obtained from 6 different certified producers using the proposed method. None of the samples was found to be contaminated with detectable levels of OTA.

Resumo

As micotoxinas são metabolitos secundários tóxicos produzidos por fungos quando crescem em alimentos e rações. O leite e os produtos lácteos, especialmente o queijo, continuam a suscitar preocupações no que diz respeito à contaminação por micotoxinas. No entanto, para alguns itens, como queijo, há uma falta de informação precisa disponível sobre o conteúdo de micotoxinas.

O objetivo do presente estudo foi otimizar um método de extração, limpeza e detecção para a determinação da ocorrência de ocratoxina A (OTA) no queijo "Serra da Estrela".

Para atingir esse objetivo, as condições de extração foram selecionadas testando diferentes métodos de extração de OTA. A influência de duas variáveis na recuperação da OTA foi avaliada: eficiência de extração de enzimas digestivas (lipase e protease) e tipo de purificação (purificação líquido-líquido e colunas de imunoafinidade). As taxas de recuperação obtidas pelo método selecionado variaram entre 62,7 e 70,6%. A determinação por HPLC por detecção de fluorescência permitiu limites de detecção e quantificação de 0,45 e 1,51 μg/kg, respectivamente. Foi realizada uma pesquisa de OTA em 25 amostras de queijo Serra da Estrela. Nenhuma das amostras apresentou níveis detectáveis de OTA.

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1 INTRODUCTION

1.1 Framework

Foodborne diseases are currently one of the biggest worldwide health problems. These diseases are caused by various agents, particularly pathogenic microorganisms and their toxins. Mycotoxins are secondary metabolites produced by filamentous fungi that cause a toxic response when ingested by humans and animals even at very low amounts.

Mycotoxins are mainly produced by different fungal species belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Several mycotoxins have been identified and reported in the last decades, but the main mycotoxins that present a major prevalence in contaminated food items are aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (FB), zearalenone (ZEA) and patulin (PAT). The same toxin can be produced by various fungal species and, conversely, the same fungal strain can produce several mycotoxins (Zain, 2011). The complexity of mycotoxin production in food products is enhanced by the fact that strains within a given species have different capacities to produce toxins, both in quantitative and qualitative terms.

Human exposure to mycotoxins ensues directly from the intake of contaminated agricultural commodities or processed food products, or indirectly through the consumption of animal-derived products (meat, milk, eggs) from animals fed with mycotoxin contaminated feed, a phenomenon called carry-over effect (Díaz Nieto et al., 2018). Mycotoxins are generally chemically stable, and some resist to high temperatures, fermentation, storage and manufacturing processes, and are regularly found in finished products (Alshannaq & Yu, 2017).

Fungal growth and mycotoxin production in foods are highly dependent on both intrinsic and extrinsic factors in the food chain and are influenced by multiple interacting factors, such as substrate composition, temperature, aw, pH and microbial interactions (Benkerroum, 2016; Magan et al., 2007; Meftah et al., 2018; Vipotnik et al., 2017). In milk, AFM_1 has been the most studied of the mycotoxins (Flores-Flores et al.,

2015). AFM_1 is the metabolized form of AFB_1 excreted in milk by animals fed with AFB_1-contaminated feed. Thus, contamination of dairy products with this toxin is a result of carry-over (Ketney et al., 2017).

Among the dairy products, ripened cheese is the only product really susceptible to fungal growth due to the physicochemical characteristics of cheese during ripening, and many types of cheese are an excellent substrate for the growth of several toxigenic *Penicillium* and *Aspergillus* species (Leggieri et al., 2017; Sengun et al., 2008), as they are particularly well adapted for growth on the cheese matrix. One of the most important toxic metabolites produced by these fungi is OTA, which may cause nephrotoxicity, hepatotoxicity, carcinogenicity and has been classified as a group 2B carcinogen by the international agency for research on cancer (IARC, 1993).

To assess the risk of contamination of cheese with OTA, it is of great importance to optimize accurate extraction, cleanup and detection methods especially since cheese is known to be a matrix difficult to manipulate due to its richness in protein and fat. Also, methods in OTA analysis should be simple, easy-to-use and, relatively fast, and should have a good recovery and precision.

1.2 Objectives

In this work, we aimed to optimize the extraction procedure of OTA from a dairy matrix, specifically a soft cheese (the PDO Portuguese cheese "Serra da Estrela"). For this purpose, to the specific objectives were:

- Optimize OTA extraction from the matrix, by testing enzymatic and chemical extraction techniques.

- Optimize the cleanup step, by testing chemical and immunochemical methods.

- Optimize the detection procedure, by using different HPLC conditions.

After the method validation, several samples of cheese were analyzed for evaluating OTA contamination.

2 LITERATURE REVIEW

2.1 General aspects of mycotoxins in food

Fungi are major spoilage agents of foods and feedstuffs. The proliferation of various fungi in agricultural products leads to reduction in yield and quality, with significant economic losses. They produce secondary metabolites, referred to as mycotoxins, which have been found to be present in most food substrates (Adeyeye, 2016). The Food and Agriculture Organization (FAO, 2004) estimates that 25% of the world's food crops, overall, are affected by mycotoxins. Considering that these food crops include cereals, nuts, fruit and vegetables, which comprise a significant part of the European consumer's diet, there is potentially a significant exposure to mycotoxins. Exposure of consumers to mycotoxins was thought to be mainly via plant foods. However, an additional potential exposure may be via foods of animal origin such as milk, cheese and meat, as a result of consumption of contaminated feed by animals, or by direct production of mycotoxins in animal-based food items as a result of fungal infection (Sengun et al., 2008).

Several kinds of mycotoxins may contaminate the food, the most toxic for human health being AFs, OTA and FB (Petzinger & Weidenbach, 2002). Mycotoxins are naturally occurring molecules produced by various fungal species and the same fungal strain can produce several mycotoxins (Zain, 2011). Most mycotoxins are very stable chemically and once formed will continue to contaminate that commodity and foods or feeds manufactured from it.

2.1.1 Conditions of mold growth

Mycotoxins are secondary metabolites produced in food as a result of certain filamentous fungi growth (Sweeney & Dobson., 1998). Mycotoxins are considered very stable, and their contamination in agricultural commodities can be pre-harvest or during the storage stages (Udomkun, 2017). Several conditions can affect molds growth

and mycotoxins formation including biological, chemical, and physiological (Afsah-Hejri et al., 2013).

Some of the highlighted factors, particularly ecological and environmental factor, can participate considerably in toxin production (Zain, 2011). Appropriate temperature and humidity are the predominant environmental factors for mycotoxigenic fungi to produce mycotoxins (Gallo et al., 2015). With regards to the mycotoxins production, most of them are produced in hot and humid climates, like those in the subtropical and African countries (Patriarca & Fernandez Pinto., 2017). In a study carried out by Wawrzyniak et al. (2018), it was observed that temperature and water activity (a_w) of barley grain significantly affected fungal growth and mycotoxins production during 40 days of storage, and indicate that a_w had the greater share of total variation in molds growth and OTA formation.

2.1.2 Health effects of mycotoxins

Mycotoxins ingestion can cause a disease named "mycotoxicosis" that can attack animals or humans (Chavarría et al., 2015). Mycotoxins are considered to be nephrotoxic, hemorrhagic, teratogenic and mutagenic to a wide range of organisms and can attack the body organs such as liver and kidney (Chhonker et al., 2018). Several factors are responsible for the toxicity of mycotoxins. These factors are related to the toxin involved, the organ affected, the age, sex, the individual nutrition, and the dose of toxin (Datsugwai et al., 2013). Multitoxin contamination of food commodities results in more severe effects when compared to single toxin contamination due to synergism effects (Mansfield & Kuldau, 2007).

2.1.3 Tolerable daily intake of the main mycotoxins encountered in foods

Humans are exposed to mycotoxins metabolites when consuming contaminated agricultural products such as cereals, fruits, etc., or that are carried over into food

products obtained from animals which were fed with contaminated material such as milk, eggs, cheese, etc. (Flores-Flores et al., 2015). Human ingestion of mycotoxins can also occur from the ingestion of processed animal-based products, namely cured cheese and cured meat products, as a result of the direct production of toxins by fungi able to grow during the ripening and curing periods (Vipotnik et al., 2017).

The Tolerated Daily Intake (TDI) refers to the theoretical maximum amount of substance that can be administered, daily and during over a lifetime, to an individual without risk of causing harmful effects to his health (EFSA, n.d). Table 2.1 illustrates the TDI of some mycotoxins according to Benkerroum (2016).

Table 2.1 Tolerable Daily Intake (TDI) of some mycotoxins (Benkerroum, 2016)

Mycotoxin	TDI µg/ kg (body weight) / day
Aflatoxin M_1	0.002
Patulin	0.4
Ochratoxin A	0.017
Fumonisin B	2.0
Zearalenone	0.25

Milk and dairy products continue to raise concerns with regard to contamination with mycotoxins. Indeed, mycotoxins have been repeatedly detected in white and blue mold-matured cheeses and, in some instances, in amounts exceeding the maximum regulatory levels (Benkerroum, 2016).

2.2 Cheese

Cheese is the generic name for a group of fermented milk-based food products, produced in a wide range of flavors and forms throughout the world. There are more than 2000 varieties of cheeses, produced worldwide (Tunick, 2014).

According to the FAO (n.d), more than 6 billion people in the world consume milk and dairy products, especially cheese; the majority of these people live in developing countries. The range of dairy products consumption and production varies considerably from region to region and between countries, depending on dietary habits, available milk processing technologies, market demand and social and cultural circumstances. Cheese is one of the most traded dairy products in the world with EU production of 8.5 million tons in 2014 (Table 2.2). This generates huge revenues for leading cheese exporting economies (Ramesh, 2016).

Table 2.2 Cheese production and consumption in selected countries in 2012-2014. Values are presented in 1,000 Million Tons (Ramesh, 2016).

	2012		**2013**		**2014**	
Country	production	consumption	production	consumption	production	consumption
European Union	9.142	8.444	9.175	8.450	9.250	8.500
Brazil	700	724	722	751	736	762
United States	4.940	4.788	5.035	4.851	5.115	4.939
Australia	330	240	329	232	360	248
Canada	549	537	550	560	545	565
Japan	47	282	47	290	48	295

2.2.1 Cheese making

Cheese manufacturing includes several steps, which depend on the cheese variety. The general process of cheese making include the following steps (Enab et al., 2012; Fox et al., 2016):

1. Selection, standardization and pasteurization of the whole raw milk: The composition of cheese is strongly influenced by the composition of the milk, especially the content of fat, protein, calcium and pH. The milk composition

depends on the animal breed, feed, and stage of lactation. The three main methods for standardizing milk for cheese making are the addition of skimmed milk powder, the addition of liquid skim milk and the removal of cream. Calcium has a major role in the coagulation of milk by rennet, and it is common practice to add $CaCl_2$ to cheese milk. The use of standardized milk avoids the manufacture of cheese containing excess fat and minimizes fat and casein losses into the whey. For public health reasons, the pasteurization of cheese milk became widespread, moreover to provide a milk supply of more uniform bacteriological quality and to improve its keeping quality. There are several methods of milk pasteurization (temperature, treatment with H_2O_2 , activation of the lactoperoxidase-H_2O_2 hiocyanate system, bactofugation and microfiltration).

2. Acidification: It is usually achieved by the in situ production of lactic acid through the fermentation of the milk sugar, lactose, by lactic acid bacteria, but it can also be achieved by adding acid (usually lactic acid or HCl) or acidogen. This alternative may be used for cheese varieties for which texture is more important than flavor.
3. Milk coagulation by acidification or limited proteolysis: The mainly characteristic step in the cheese making of all varieties involves coagulation of the casein component of the milk protein system to form a gel which entraps the fat. Coagulation may be achieved by limited proteolysis by selected proteinases (rennet).
4. Pressing the cheese curd: It removes the supernatant whey from the curds with a specific value of pressure for each variety of cheese and allows the curd formation into characteristic shapes.
5. Salting: is usually made by adding sodium chloride to enhance flavors and control microbial and enzymatic activity.
6. Ripening of the curd: During ripening, the flavor, aroma and texture of the mature cheese are largely predetermined by the manufacturing process, NaCl and pH, level of residual coagulant activity and by composition, especially moisture and the type of starter.

Figure 2.1 shows an outline of the basic steps.

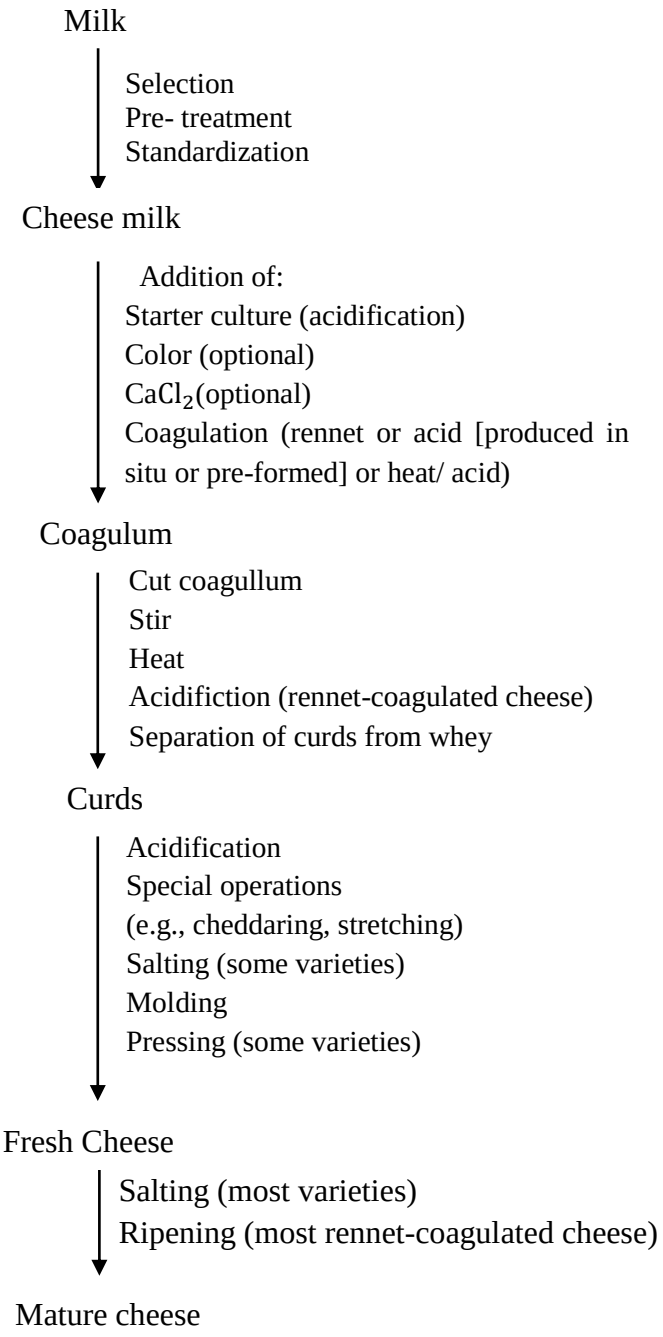

Figure 2.1 General protocol for cheese manufacturing (Fox et al., 2016)

2.2.2 "Serra da Estrela" cheese

2.2.2.1 General characterization

The Serra da Estrela PDO (Protected Denomination of Origin) cheese is a cured cheese made from raw (non-pasteurized) ewe milk, which presents a semi-soft buttery

paste and white-yellowish shade (Serra da Estrela cheese) or semi-hard to extra-hard paste and brownish orange color (Serra da Estrela Velho cheese). It is made with milk from two autochthonous Portuguese sheep breeds, the Bordaleira Serra da Estrela and the Churra Mondegueira. Serra da Estrela cheese was granted with the PDO status by the European Union; therefore, its production follows tight rules and is confined to a specific region in the center of Portugal, corresponding to the mountainous region of Serra da Estrela (Portuguese Traditional Products) (Figure 2.2).

This is the oldest Portuguese cheese being recognized internationally for its organoleptic characteristics. It was introduced in Portugal by Roman people, and it then represented a nutritious and sustainable food source being used by great explorers during their voyages.

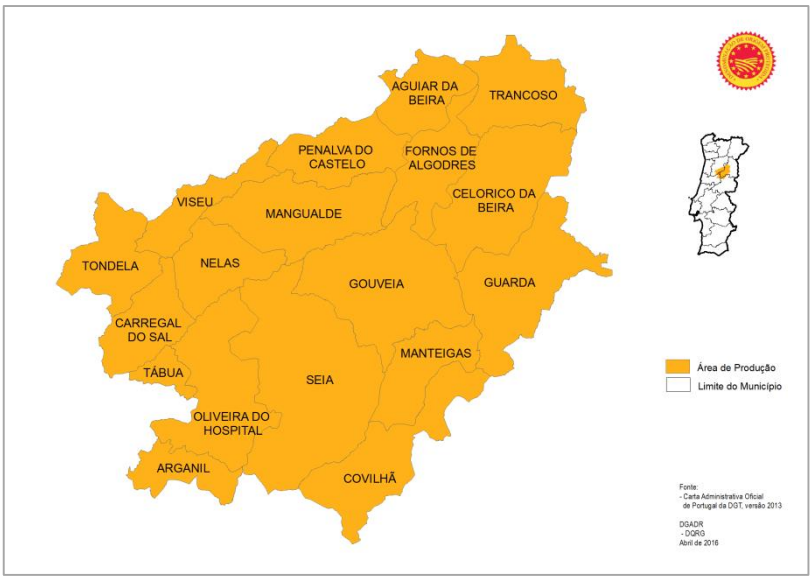

Figure 2.2 Geographic area of Serra da Estrela cheese production, in the center of Portugal, corresponding to the Denomination Region of Serra da Estrela (Portuguese Traditional Products, n.d) .

2.2.2.2 Chemical profile of Serra da Estrela cheese

The main milk components (protein, fat, and minerals) are the primary components of cheese, and they are concentrated to provide sound nutrition, a wide type of varieties, and new flavors and textures to the consumer (Ramesh, 2016). Fat is the most important component of Serra da Estrela cheese, at level of 27 g/100 g and includes solid fat and liquid oils, in the form of triglycerides. Protein in cheese composes the network in

which water and the fat globules are held. All proteins consist of amino acids linked together in a three dimensional network, and typically almost all the protein in cheese curd is casein. Several other proteins are found in whey, as is the case of albumin (Casper et al., 1998). Proteins present a high amount in Serra da Estrela cheese at level of 21g/100g (Guiné et al., 2015; Tunick., 2014). The general composition of Serra da Estrela cheese is given in Table 2.3.

Table 2.3 Nutritional composition of Serra da Estrela cheese, for 100 g of wet matter (adapted from Associação Portuguesa de Nutrição, 2018).

Energy	**333 kcal**	
Lipids	27 g	
Saturated fatty acids	14.2 g	
Carbohydrates	0.2 g	
Proteins	21 g	
Salt	1.78 g	
Vitamin A	240 μg	800 μg RDD
Vitamin D	0.2 μg	25 μg RDD
Vitamin B2	0.6 mg	1.6 mg RDD
Vitamin B3	5.6 mg	15 mg RDD
Vitamin B6	0.09 mg	1.4 mg RDD
Vitamin B12	1.4 mg	2.5 μg RDD
Folate	31mg	
Potassium	80 mg	
Calcium	700 mg	
Phosphorus	480 mg	
Magnesium	53 mg	
Iron	0.7 mg	
Zinc	2.8 mg	

RDD: Recommended daily portion.

2.2.2.3 Production process

The manufacturing process of Serra da Estrela cheese starts with the milk filtration through muslin clothes. The milk is then heated to 28-32 ºC and salted, and the thistle flower *Cynara cardunculus* is added to clot the casein. After 45 to 60 minutes the curd is manually cut, and a new filtration is performed to remove the remaining whey. This is followed by molding, pressing and new salting processes. The following maturing step consists of two phases. The first maturation step takes 15-20 days at 6 to 12 ºC and relative humidity of 85 to 90%. Cheeses are turned and washed on a daily basis. The second phase takes 20 more days at 6-14 ºC and relative humidity of 90 to 95%, with occasional turning and washing, depending of the rind aspect.

To produce Serra da Estrela Velho cheese the same temperatures and relative humidity are used, but the maturation time-period is of 120 days.

2.3 Mycotoxins in cheese

Among the dairy products, cheese seems to be the main product susceptible to mold growth (Van Egmond, 1983). Fungal growth in or on cheese causes undesirable changes which can affect the quality of the final products (Beresford et al., 2001).

The first studies related to mycotoxins in cheeses led to the discovery of AFs (Hymery et al., 2014). Aflatoxins are toxic fungal metabolites, which can be found in feed. Upon ingestion by ruminants, aflatoxin B_1 is partially destroyed in the rumen, whereas the absorbed AFB_1 undergoes hepatic metabolism to generate several metabolites which include AFM_1. Diverse factors facilitate the passage of AFM_1 to milk in lactating animals (Chavarría et al., 2015), a phenomenon called carry-over effect (Díaz Nieto et al., 2018), an indirect contamination of cheese with mycotoxins (Sengun et al., 2008). Many research works have considered AFM_1 in cheese (Chavarría et al., 2015; Erdogan et al., 2003; Oliveira et al., 2011). AFM_1 concentration in cheese has even been shown to be generally greater than that of the original milk due to the fact that an important proportion of AFM_1 binds to the milk casein and is concentrated in curd after draining (Benkerroum, 2016).

The direct contamination of cheese with mycotoxin can be exogenous via an accidental growth of molds during cheese making, and some of these mycotoxins may be highly toxigenic (Sengun et al., 2008).

The most common mycotoxins that were found to be stable in cheese are citrinin, cyclopiazonic acid, penitrem A, roquefortine C, sterigmatocystin and AFs. Others including PAT, penicillic acid and roquefortins do not persist in cheese (Taniwaki et al., 2001). It is reported that these toxins are inhibited in foods rich in proteins by compounds that contain sulfhydryl, such as cysteine and glutathione (Sengun et al., 2008). Table 2.4 shows the mycotoxin occurrence in several types of cheese.

Table 2.4 Mycotoxin occurrence in several types of cheese**.**

Cheese	Mycotoxin	Range	Reference
Gouda and Edam	Sterigmatocystin	5 to 600 µg/kg	Northolt et al. (1980)
Italian white cheeses	Cyclopiazonic acid	20 to 80 µg/kg	Zambonin et al. (2001)
Blue-molded tulum cheese	Patulin, Penicillic acid PR toxin Roquefortine	ND ND ND 2.1 to 3.8 mg/kg	Erdogan et al. (2003)
Tulum cheese	Aflatoxin M1	0.011 to 0.202 µg/kg	Gurses (2004)
Blue-white mold cheese	Roquefortine C	0.8 to 12 mg/kg	Kokkonen et al. (2005)
Blue cheeses: **Gorgonzola** **Bleau** **Roquefort** **Bergader**	Ochratoxin A	 0.2-3.0 µg/kg ND 0.1-1.4 µg/kg ND	Dall'Asta et al. (2008)
Turkish cheeses	Aflatoxin M1	0.058 to 0.85 µg/kg	Aydemir et al. (2010)
Roomy cheese: **small scale** **large scale**	Ochratoxin A	 3.67± 0.22 µg/kg 3.92 ± 0.30 µg/kg	Awad et al. (2012)
hard cheese	Ochratoxin A	1.62 to 54.07 µg/kg	Biancardi et al. (2013)
Semi hard cheese	Patulin Ochratoxin A	15 to 460 µg/kg 1 to 262.2 µg/kg	Pattono et al. (2013)
Blue veined cheese	Aflatoxin M1 Roquefortine C, Mycophenolic acid	ND 1 mg/kg to 14.125 mg/kg 3 mg/kg to 6190 mg/kg	Evin Fontaine et al. (2015)
Grana cheese	Ochratoxin A	1 to 1439 µg/kg	Decontardi et al. (2017)
Surk cheese	Aflatoxin M1 Ochratoxin A	0.058 to 5.04 µg/kg 0.615±0.228 µg/kg	Sakin et al. (2018)

2.3.1 Factors modulating mycotoxins in cheese

The growth of fungi and the production of mycotoxins in food is influenced by multiple factors including abiotic and biotic factors (Filtenborg et al., 1996). In cheese,

concerning biotic factors, we can consider three that modulate the production of mycotoxins. The first factor is related to the capacity of a given mold species to produce mycotoxins. The second factor represents the physiological state, and the third factor is about the interactions that can occur between the organism and other cheese microbiota members (Figure 2.3). The principal abiotic factors that can modulate the production of mycotoxin in cheese could be divided into three factors; manufacturing factors, environmental factors, and physicochemical factors. The environmental factors include temperature and humidity. The manufacturing factors include ripening time, and storage atmosphere used during cheese making. While the physicochemical factors involve chemical composition (carbon and nitrogen sources, C/N ratio, NaCl content, pH, a_w, and redox potential (E°) (Hymery et al., 2014).

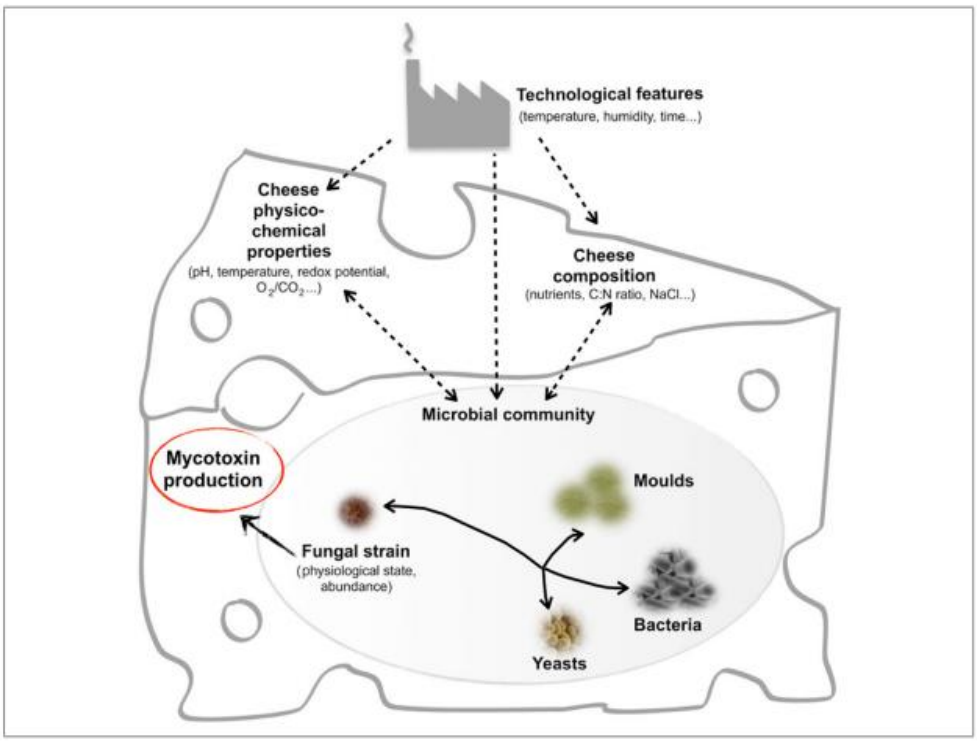

Figure 2.3 Main biotic and abiotic factors modulating mycotoxin production in cheese (Hymery et al., 2014).

2.3.1.1 Effect of nutritional factors on mycotoxin production

It is assumed that the metabolism of fungi is more suitable to substrates rich in carbohydrates (Pitt & Hocking, 2009). Despite cheese is considered as a poor matrix on

carbohydrates, presenting a low C/N ratio, and having a low ripening temperature, it cannot be assumed that it is a poor substrate in terms of its nutritional composition for mycotoxin production. Probably, a mold has the ability to produce mycotoxins in cheese is rather species-or strain-specific. The ability of *Penicillium crustosum* to produce penitrem A on the cheese analogue could be connected to the high protein content of this substrate, as amino acids are required in the synthesis of tremorgenic mycotoxins (Kokkonen., 2005).

Sodium chloride is added to cheese to enhance flavor and to control microbial growth and enzymatic activity through a_w reduction, by drawing some of the water out of a cell, it also enables the growth of salt-tolerant microorganisms. However, NaCl may also affect mycotoxin production. Indeed, Geisen et al. (2018) reported that *Penicillium citrinum* can be found in salt-rich foods like cheeses and that high amounts of OTA were produced by *Penicillium nordicum* over a large concentration of NaCl.

2.3.1.2 Effects of temperature, aw, pH, and atmosphere composition on mycotoxin production

A wide number of research works (Erdogan et al., 2003; Le Bars, 1979; Taniwaki et al., 2001) highlighted that mycotoxins produced by fungi in cheese are directly correlated with temperature, with low or no production at refrigerated temperatures, intermediate production at 12 °C, and optimal production at 20 to 25 °C. Furthermore, Leggieri et al. (2017) confirmed that during grana cheese ripening, elaborated between 15 and 22 °C, OTA, penitrem A, roquefortine C and mycophenolic acid are apparently at the highest production risk.

The majority of fungi having the ability to grow in cheese are considered as salt-tolerant and can grow at relatively low a_w conditions (Hymery et al., 2014). According to Marín et al. (2014) *Penicillium* spp. as the dominant species on hard cheese, *Penicillium* and *Aspergillus* spp. were highly tolerant to water restriction, making them more competitive with other fungi during cheese ripening.

Atmospheric gas composition, such as CO_2 and O_2 levels, exert an important impact on mycotoxin production by cheese related fungi. Taniwaki et al. (2001) compared the production of mycotoxins by strains of *Aspergillus flavus*, *Penicillium roqueforti* and

Penicillium commune on sliced cheddar cheese under modified and not modified atmosphere conditions, which combines the inhibitory effect of low oxygen and elevated carbon dioxide levels. The production of mycotoxins significantly decreased with modified atmosphere conditions compared to production in the air.

2.4 Ochratoxin A

Ochratoxins are a group of mycotoxins mainly produced as secondary metabolites by several fungal species such as *Aspergillus* and *Penicillium*, there are three different chemical structures in ochratoxin family which are OTA, OTB and OTC (Selvaraj et al., 2015).

Among the ochratoxin group, OTA seems to be the most important toxic metabolite due to its prevalence in food and toxicity, produced by several molds primarily *Aspergillus carbonarius*, *Aspergillus niger*, *Aspergillus ochraceus*, *P. nordicum* and *Penicillium verrucosum*. It is a chlorinated polyketide mycotoxin containing the amino acid phenylalanine (Dall'Asta et al., 2008).

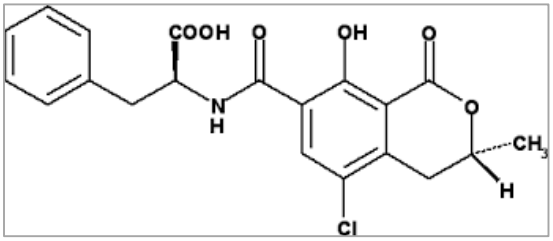

Figure 2.4 Chemical structure of ochratoxin A (Dall'Asta et al., 2008).

OTA is a moderately stable molecule of molecular weight 403.81 g/mol, heat-stable, crystalline solid agent with melting point at 168–173 °C) (Kőszegi & Póor, 2016). OTA is considered to be involved in severe pathological response from humans and animals due to its nephrotoxic, immunosuppressive, carcinogenic and teratogen effects (Selvaraj et al., 2015), and has classified as a potential carcinogen (group B2) for humans by the International Agency for Research on Cancer (IARC, 1993).

Due to the risk of food contamination by OTA known to cause losses of quality in food products and adverse effects to human health, legislation has been created to minimize this risk. Thus, European Commission has established the maximum tolerable limits for OTA in several types of food products or ingredients from 0.5 to 80 µg/kg (EC, 2006). No Maximum Tolerable Limits have been established for OTA in cheese.

2.4.1 OTA occurrence in cheese

OTA contamination is found in a wide variety of food items such as cereals, coffee, spices, wine, grape juices dried fruits and in protein–rich food like fermented meat, milk, egg and cheeses (Bogs et al., 2006; Lund & Frisvad., 2003). Many studies have been focused on OTA occurrence in cheese (see Table 2.4).

The most hazardous mycotoxins found in cheese are OTA and AFM_1. OTA is produced by toxigenic fungi, via direct cheese contamination (an environmental cross contamination), which grow on cheese during ripening and AFM_1 through an indirect contamination, a carry-over effect (Hymery et al., 2014). Dall'Asta et al. (2008) reported that OTA may be present in different commercial samples of blue-mold ripened cheeses at very low levels: 0.2 to 3.0 µg/kg in Gorgonzola and at level of 0.1 to 1.4 µg/kg in Roquefort cheese and that OTA level increased during the shelf life in three Gorgonzola and in two Roquefort cheese samples. Sakin (2018) considered that the concentration range of OTA in surk cheese (0.615±0.228 µg/kg) was much lower than those reported for grana cheese in Italy ranging from 1 to 1439 µg/kg which detected in all grana cheese samples (Biancardi., 2013; Decontardi et al., 2017).

2.5 Methods of extraction and analysis of OTA

Analytical methods for quantitative determination of OTA follow the same steps for quantification of mycotoxins: sampling, extraction, purification and detection.

2.5.1 Extraction and purification of OTA from cheese

Pattono et al. (2013) demonstrated the feasibility of a chemical extraction for OTA in traditional semi-hard cheese using acidified acetonitrile solvent and hexane (liquid-liquid extraction, LLE), which allowed removing fat from the extract, a key factor considering the high concentration of fat in cheese. Sakin et al. (2018) focused on the occurrence of OTA in surk cheese, being the extraction performed through a chemical extraction using methanol/water solvent and immunoaffinity columns (IAC) for the cleanup step. Another study carried out by Zhang et al. (2009), developed an in situ solid phase extraction method (SPME) for direct extraction of OTA from lipid-rich, semi-solid heterogeneous matrix such as cheese, using acidified SPME fibers. However, traditional sample preparation approaches, such as liquid extraction and solid-phase extraction (SPE), have proven to be time-consuming and labor-intensive. The cost and the fragility of the non-reusable column (antibody-based IAC and active protein based affinity) prevent it from direct application to cheese sample complex matrix (Zhang et al., 2009). Therefore, several attempts to develop other alternatives for OTA extraction from a complex matrix were done, such as the study conducted by Giacomo et al. (2016) which demonstrate the benefits of using an enzymatic digestion (ED) method for OTA extraction in pig tissue. Giacomo et al. (2016) compared the ED procedure and LLE protocol from animal tissues followed by IAC, to evaluate their performance regarding OTA quantitative extraction and they confirmed that the use of the ED extraction significantly reduced matrix interference with the samples. The recovery obtained with the ED extraction method was higher than the recovery obtained with the conventional LLE. The limit of detection (LOD) and limit of quantification (LOQ) of the ED extraction method were also lower than the LOD and LOQ of the LLE method.

2.5.2 Detection of OTA in cheese

For the detection and quantification of OTA in cheese, most of studies used high performance liquid chromatography (HPLC) with fluorescence detector (FD) or liquid chromatography with mass spectrometry (LC-MS). Table 2.5 summarizes different

techniques developed for the extraction and the detection of OTA in different type of cheese, including details on method performance.

Table 2.5 Different techniques developed for the extraction and the detection of ochratoxin A, and corresponding limit of detection (LOD), limit of quantification (LOQ) and recovery rates.

Targeted food material	Method of extraction and purification	Detection techniques involved	LOD	LOQ	Recovery	Reference
Blue cheeses	Chemical extraction	HPLC-FD	0.02 µg/kg	0.1 µg/kg	97±1%	Dall'Asta et al. (2008)
Cheddar cheese	Solid phase microextraction	LC-ESI-MS/MS	1.5 ng/ml	3.5 ng/ml	39%	Zhang et al. (2009)
Roomy cheese	Immunoaffinity column	HPLC-FD	0.02 µg/kg	0.07 µg/kg	97±3%	Awad et al. (2012)
Hard cheese	Solid-liquid extraction	LC-MS/MS	1 µg/kg	----	93.75±1.36%	Biancardi et al. (2013)
Semi-hard cheese	Chemical extraction	HPLC-FD	0.17 µg/kg	0.54 µg/kg	93.9± 1.8%	Pattono et al. (2013)
Grana cheese	Chemical extraction Immunoaffinity column	HPLC-FD	0.1 µg/kg	0.3 µg/kg	-----	Decontardi et al. (2017)
Surk cheese	Chemical extraction Immunoaffinity column	HPLC-FD	0.04 µg/kg	0.11 µg/kg	98.8%	Sakin et al. (2018)

HPLC-FD: high performance liquid chromatography with fluorescence detector
LC-MS/MS: liquid chromatography with tandem mass spectrometry

3 MATERIALS AND METHODS

3.1 Chemicals and reagents

The standard stock solution of OTA (Sigma, St. Luis, USA) was dissolved in methanol (MeOH) at a concentration of 1 mg/mL. This stock solution was diluted with MeOH to obtain working solutions as needed.

HPLC grade solvents (MeOH and acetonitrile - ACN) were used in the preparation of OTA standards, in sample extraction, and in the preparation of the mobile phase (ACN). OchraRhone wide immunoaffinity columns (IACs) were obtained from R-Biopharm Rhône LTD.

Protease solution was prepared by diluting 0.005 g of protease (from *Streptomyces griseus* type XIV, ≥ 3.5 units/mg solid powder, Sigma) in 0.5 mL of sodium acetate solution 10 mM, pH 7.5, 0.5 mL of calcium carbonate solution 5 mM, pH 7.5 and 4 mL of distilled water. Lipase solution was prepared by diluting 0.005 g of lipase (porcine pancreas type II lipase, 100-500 units/mg protein, Sigma) in 0.5 mL potassium phosphate buffer 200 mM pH 7.5, 0.4 mL of magnesium chloride, 0.5 mL EDTA, 3.6 mL of distilled water.

Phosphate buffer saline (PBS) was prepared by diluting one tablet of PBS (Life Science) in 100 mL distilled water. 2% sodium bicarbonate solution was prepared by diluting 2 g of sodium bicarbonate (Biochem, Chemopharma) in 100 mL of distilled water.

3.2 Safety and decontamination procedures

Due to the toxicity of OTA, all the necessary safety considerations were taken. Solutions were handled with nitrile gloves under the chemical hood; all disposable materials, including falcons with potential OTA contamination, were decontaminated by autoclaving before being disposed; reusable materials were decontaminated by immersion in 10% bleach over-night and washed with distilled water several times.

3.3 Preliminary optimization of the analytical procedures

Before testing the extraction procedures, four UPLC columns and two OTA dissolution solvents were tested, to determine the best analytical results.

3.3.1 HPLC columns tested

To ensure a fast separation of OTA with high efficiency and sufficient resolution, four different HPLC columns were tested. A high concentration of OTA solution (50 ng/mL) diluted in ACN was injected using each column.

The HPLC columns tested were:

Column #1 C18 reverse-phase column RP-18e (100 × 4.6 mm, 2 µm, Merck Chromolith Performance, Darmstadt, Germany).

Column #2 C18 reverse-phase column PLRP-S 300 Å (250 × 4.6 mm, 8 µm, Polymer laboratories).

*Column #3 C*18 reverse-phase column PLRP-S 300 Å (150× 4.6 mm, 8 µm, Polymer laboratories).

*Column #4 C*18 reverse-phase column 100-S (150 × 4.6 mm, 5µm, Nucleosil, Mancherey-Nagel).

3.3.2 OTA dissolution solvents

For the optimization and validation of OTA analysis by UPLC, two different dissolution solvents were tested: MeOH and ACN. Calibration curves for OTA eluted in MeOH and in ACN were established using 8 standard solutions with concentrations ranging from 0.19 ng/mL to 50 ng/mL. This assay was justified by the fact that in the literature different extraction methods reported the use of either MeOH or ACN as OTA final eluents.

For the selection of OTA dissolution solvents, the chromatographic separation was achieved using an isocratic elution with the HPLC column #2.

3.3.3 UPLC system and conditions

The detection of OTA was achieved by UPLC instrument (Dionex Ultimate 3000 UPLC Thermo Scientific, USA). The chromatographic system consisted of a quaternary pump, an autosampler maintained at 5 °C, a degasser, a fluorescence detector (FD) set to 330 nm excitation and 465 nm emission and an automatic thermostatic column compartment. The mobile phase consisted of acetonitrile/water/acetic acid (70:29.5:0.5; v/v/v) which was previously degassed and filtered. Two flow rates (0.8 and 1 mL/min) and four injection volumes (10, 20, 50 and 100 µL) were tested for each column. UPLC chromatograms were analyzed using Xcalibur software.

3.4 Optimization of OTA extraction procedure

In any mycotoxin analysis, the critical step is the extraction and clean-up procedure, especially when the concentration of the analytes is around ng levels and complex matrices such as cheese are used. This section describes the establishment of a sensitive and accurate method for determination of OTA in "Serra da Estrela" cheese, to achieve the best recovery for the OTA contained in the cheese while eliminating most of the interfering matrix components. To achieve this goal, extraction conditions were selected by testing different OTA extraction methods. The influence of two variables on OTA recovery was evaluated: extraction efficiency of digestive enzymes (lipase and protease) and type of purification (liquid-liquid and IAC purification).

The protocols of OTA extraction and clean-up from cheese samples described below were tested after the establishment of the best HPLC column and OTA dissolution solvent described in section 3.3.

3.4.1 Preparation of OTA standard solutions

The standard working solution used for spiking purposes was prepared by diluting the stock standard solution in MeOH to a concentration of 2000 ng/mL. The prepared solution was stored at 4 ºC.

3.4.2 Preparation of cheese samples

For the extraction tests, a Serra da Estrela cheese was bought from a local market. The cheese rind was taken and the internal mass was homogenized, divided into 5 g aliquots and stored at −20 ºC until being used for extraction tests.

For each optimization procedure, cheese aliquots were contaminated (spiked) with 10 ng of OTA per gram of cheese (10 ng/g), and left over night in the dark, so that OTA would fully adhere to the matrix, to replicate a real case scenario. All tests were made in triplicate. To ensure that the cheese was not preliminarily contaminated with OTA, a blank cheese sample was tested for each method.

3.4.3 OTA extraction methods

3.4.3.1 Method #1 Chemical extraction followed by Liquid-liquid purification as described by Pattono et al. (2013)

Method #1.1 Extraction without enzymes

Five grams of spiked cheese were homogenized with 10 mL of ACN added of 40 μL of 18% sulphuric acid to obtain a pH of 2.0 ± 0.5. The homogenized sample was then shaken horizontally for 5 min. An initial centrifugation step at 4677 RCF was performed, and 5 mL of ACN was collected in a separate vial. The extract was shaken horizontally at 600 strokes/min for 30 min with 5 mL of hexane. This step was repeated twice to remove any fat from the extract. The organic phase (ACN) was filtered through 0.2 μm PTFE syringe filter and stored at 4 °C until the chromatographic analysis.

Method #1.2 Enzyme-assisted extraction with some modifications proposed by Chavarría et al. (2015)

Five grams of cheese spiked with OTA were homogenized for 5 minutes with 1.8 mL of 10 mM sodium acetate buffer at pH 7.5, 0.1 mL of a 1 mg/mL protease solution and 0.1 mL of 1 mg/mL lipase solution. The mixture was homogenized for a second time and was incubated at 35 °C for 24 h. The mixture was then homogenized with 10 mL of ACN added of 40 μL of 18% sulphuric acid to obtain a pH of 2.0 ± 0.5. From this step forward, the procedure was the same as described in Method #1.1. The homogenized

sample was shaken horizontally for 5 min. An initial centrifugation step at 4677 RCF was performed, and 5 mL of ACN was collected in a separate vial. The extract was shaken horizontally at 600 strokes/min for 30 min with 5 mL of hexane. This step was repeated twice to remove any fat from the extract. The organic phase (ACN) was filtered through 0.2 µm PTFE syringe filter and stored at 4 °C until the chromatographic analysis.

3.4.3.2 Method #2 Chemical extraction followed by IAC purification, carried out as described by R-Biopharm P119.V8 application method

Method #2.1 Extraction without enzymes

Cheese aliquots spiked with OTA were homogenized with 20 mL of 100% MeOH and mixed at high speed in vortex for 7 minutes. 20 mL of 2% sodium bicarbonate was added and mixed again for 5 minutes. The mixture was centrifuged at 5000 rpm for 5 minutes. The upper phase was filtered through glass microfiber filters (Figure 3.1), and an aliquot of 4 mL was taken and mixed with 12 mL of PBS. The extract was purified through the IAC containing immobilized antibodies against OTA. The IAC was adapted to a 20 mL syringe set on a hose clamp. Sixteen mL of the extract were poured into the syringe and passed through the IAC by gravity, at a rate of about 1-2 drops/sec. As soon as air came through the column, OTA was eluted from the IAC by passing 1.5 mL of MeOH through the column at a rate of 1-2 drops/sec (Figure 3.2). The sample eluate was collected into an amber vial, filtered through a 0.2 µm PTFE syringe filter and stored at 4 ºC until the chromatographic analysis.

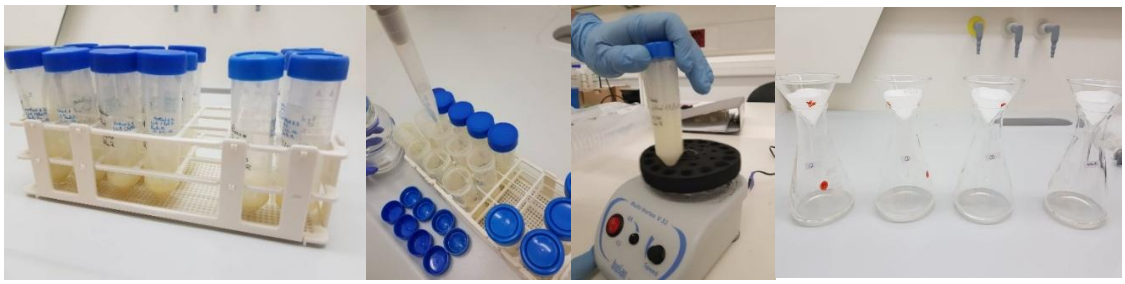

Figure 3.1 OTA chemical extraction from Serra da Estrela cheese.

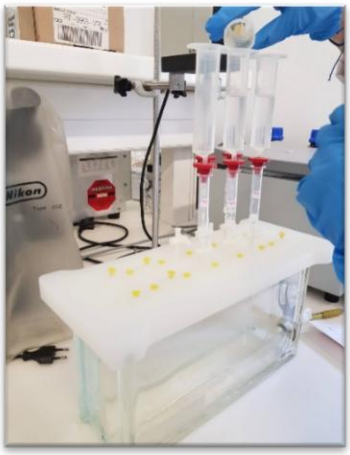

Figure 3.2 Purification step using IAC.

Method #2.2 Enzyme-assisted chemical extraction proposed by Chavarría et al. (2015) with some modifications

Cheese aliquots spiked with OTA were homogenized for 5 minutes with 1.8 mL of 10 mM sodium acetate buffer at pH 7.5, 0.1 mL of a 1 mg/mL protease solution and 0.1 mL of 1 mg/ mL lipase solution. The mixture was incubated at 37 ºC for 24 hours.

From this step forward, the procedure was the same as described in Method #2.1. To the resulting paste, 20 mL of 100% methanol were added and mixed at high speed in vortex for 7 minutes. 20 mL of 2% sodium bicarbonate were added and mixed again for 5 minutes. The samples were centrifuged at 5000 rpm for 5 minutes. The upper phase was filtered through glass microfiber filters Whatman n°4, and an aliquot of 4 mL was taken and mixed with 12 mL of PBS. The samples were purified through the IAC containing immobilized antibodies against OTA. The IAC was adapted to a 20 mL syringe set on a hose clamp. 16 mL of the extract were poured into the syringe and passed through the IAC by gravity, at a rate of about 1-2 drops/sec. As soon as air came through the column, OTA was eluted from the IAC by passing 1.5 mL of HPLC grade MeOH through the column at a rate of 1-2 drops/sec. The eluate was filtered through 0.2 µm PTFE syringe filter and stored at 4 ºC until the chromatographic analysis.

3.4.3.3 *Method #3 Chemical extraction followed by IAC purification as described by Anelli et al. (2019*)

Five grams of spiked cheese slurry (2.5 g of cheese and 2.5 g of distilled water) was extracted with 50 mL of dichloromethane plus 0.75 mL of 85% phosphoric acid (pH=3)

and 1 mL of NaCl saturated solution on an orbital shaker for 120 minutes. The extract was filtered through filter paper Whatman n°1 and extracted two times with 10 mL of NaCl saturated solution. A volume of the aqueous extract (10 mL) was cleaned up through an IAC. OTA was eluted from the IAC with 1.5 mL of 100% MeOH. The eluate was filtered through a 0.2 µm PTFE syringe filter and stored at 4 °C until the chromatographic analysis.

3.4.3.4 Method #4 Chemical extraction followed by IAC purification as described in the R-Biopharm P119.V8 application method with some modifications

Five grams of cheese slurry (2.5 g cheese and 2.5 g distilled water) spiked with OTA were homogenized with 20 mL of 100% MeOH and mixed at high speed in vortex for 7 minutes. 17.5 mL of 2% sodium bicarbonate were added and homogenized for 120 minutes. The extract was centrifuged at 5000 rpm for 5 minutes. The upper phase was filtered through glass microfiber filters Whatman n°4, an aliquot of 4 mL was taken and mixed with 12 mL of PBS. The filtered extract was passed through the IAC and OTA was eluted from the column with 1.5 mL of 100% MeOH. The eluate was filtered through a 0.2 µm PTFE syringe filter and stored at 4 °C until the chromatographic analysis.

3.5 OTA detection and quantification

The detection of OTA was achieved in a HPLC system equipment with: Smartline pump (1000, Knauer, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057, Jasco, Easton, MD, USA), and a fluorescence detector (FP-2020, Jasco) set to 330 nm excitation and 465 nm emission. The chromatographic separation was achieved using an isocratic elution with the HPLC column #2 (C18 reverse-phase column PLRP-S 300Å; 4.6×250 mm, 8 µm), which gave the best results in the preliminary tests (as described in section 3.3.1). The mobile phase consisted of acetonitrile/water/acetic acid (70:29.5:0.5; v/v/v). Oven temperature was set to 35 °C (7971 R Grace oven). The flow rate was set at 0.8 mL min^{-1} and the injection volume was 50 µL. Under these conditions, the retention time of OTA was 5.6 min. Calibration curves were prepared using standard solution of OTA at concentrations of 0.19, 0.39, 0.78, 1.56, 3.125, 6.25 ng mL^{-1} in pure MeOH. Quantification of OTA was performed by measuring area's peak at OTA retention time and plotting against the calibration

curve. OTA was quantified by gram of cheese, taking into consideration the dilution factors resulting from the different extraction methods.

3.6 Method internal validation

3.6.1 Linearity

The linearity of an analytical procedure can be defined as the capability (within a given range) to obtain test results of variable data which are directly proportional to the concentration of the analyte in the sample. The calibration parameters evaluated for OTA were the determination coefficient (R^2), the limit of detection (LOD) and the limit of quantification (LOQ), which reflect the linearity of the equipment. LOD can be defined as the lowest amount of an analyte in a sample which can be detected but not necessarily quantified as an exact value; LOQ corresponds to the analyte concentration which is measurable within a certain level of confidence (Taverniers et al., 2004). Below LOD and LOQ, determination and quantification are possible, but become unreliable as the uncertainty associated with it at these lower levels is higher than the measurement value itself (Taverniers et al., 2004).

Linearity, LOD and LOQ were determined by using 6 standard solutions in MeOH with concentrations ranging from 0.19 ng mL^{-1} to 12.5 ng mL^{-1}. LOD and LOQ were calculated according to the following equations (Taverniers et al., 2004):

$$LOD = 3 \times (s_a/b)$$

$$LOQ = 10 \times (s_a/b)$$

where s_a is the standard deviation of the intercept of the regression line (i.e, calibration curve) and b is the slope of the line.

3.6.2 Accuracy (recovery) and precision

Method validation, expressed by the recovery rate (%), is the closeness of agreement between the values that are accepted either as conventional true values or an accepted

reference value and the value found (Chan, 2004). Accuracy is usually reported as percent recovery by assay, using the proposed analytical procedure, of a known amount of analyte added to the matrix (spiking). Recovery rate (%) was calculated by the ratio of recovered OTA concentration relative to the known spiked concentration.

Method precision was determined in terms of repeatability and intermediate reproducibility at two standard concentrations (10 ng/g and 2 ng/g) on the same day with three replicates (% RSD_r, intra-day precision) and on two different days (% RSD_R, inter-day precision) with three-replicates.

Definitions for the performance criteria are as follows (Commission Regulation (EC) NO 401/2006; Taverniers et al., 2004):

- SD_R= Standard deviation, calculated from results generated under repeatability conditions.
- RSD_R (%) = Relative standard deviation, calculated from results generated under repeatability conditions [(SDr/mean) × 100].
- SD_R = Standard deviation, calculated from results under intermediate precision conditions.
- RSD_R (%) = Relative standard deviation calculated from results generated under intermediate precision conditions [(SD_R /mean) × 100].

In this study, since there are only two values for mean recovery to calculate Intermediate Precision, the Mean Deviation (MD) and Relative Mean Deviation (RMD) substitute the commonly used Standard Deviation (SD) and Relative Standard Deviation (RSD). The data variable used for quantitation of the analyte was the peak area.

3.7 Analysis of cheese samples

3.7.1 Sampling

A total of 25 Serra da Estrela cheese samples produced between February 2019 and April 2019 were purchased from 6 different certified producers located in 5 municipalities (Oliveira do Hospital, Celorico da Beira, Penalva do Castelo, Gouveia and Nelas) within the PDO geographical region (Table 3.1). Samples were identified and kept frozen until OTA extraction and analysis.

Table 3.1 Serra da Estrela cheese samples.

Producer	Geographical origin	Production date	Sample code
Producer #1	Oliveira do Hospital	February 2019	122752B
		March 2019	123031B
		April 2019	220710B
Producer #2	Celorico da Beira	January 2019	070363B
		February 2019	071862B
		March 2019	071867B
		April 2019	072914B
Producer #3	Penalva do Castelo	February 2019	197978B
		March 2019	202760B
			202761B
		April 2019	236929B
			236934B
Producer #4	Penalva do Castelo	February 2019	161740B
		March 2019	162114B
			162112B
Producer #5	Gouveia	February 2019	188191B
			188192B
		March 2019	205338B
			205339B
		April 2019	229988B
Producer #6	Nelas	February 2019	060993B
			060991B
		March 2019	062712B
			062668B
		April 2019	063713B

3.7.2 OTA detection and quantification

OTA was analyzed in cheese samples using method #4, as described in section 3.4.3.4. The HPLC conditions were those described in section 3.5.

4 RESULTS AND DISCUSSION

4.1 Establishment of preliminary conditions of the analytical procedure

4.1.1 Selection of the best HPLC column

In the analysis of the best column to be used, three parameters were taken into account: i) retention time - the shortest the retention time the fastest the analysis, with reduced costs in time and eluents; ii) area of the peak – for a given analyte concentration, the biggest the area of the peak the highest the sensitivity; and iii) peak shape and co-elution with other matrix components.

The results obtained for the different HPLC columns tested using a standard solution of OTA (at 50 ng/mL in ACN) are shown in Table 4.1. Figure 4.1 shows the corresponding HPLC chromatograms. Even though several injection volumes and flow rates were tested, only the best result achieved with each column is shown in Table 4.1.

Table 4.1 Best results obtained for each HPLC column tested.

Column	Injected volume (µL)	Flow rate (mL/min)	Retention time (min)	Area (a.u.)	Height (a.u.)
Column #1 C18 reverse-phase column (100 × 4.6 mm, 2µm, Merck Chromolith Performance)	10	0.8	2.33	1.14E+07	1.88E+06
Column #2 C18 reverse-phase column PLRP-S 300Å (250 × 4.6 mm, 8µm, Polymer laboratories)	100	1	4.28	6.65E+07	4.42E+06
Column #3 *C*18 reverse-phase column PLRP-S 300Å (150× 4.6mm, 8µm, Polymer laboratories)	100	1	2.60	4.70E+07	2.84E+06
Column #4 *C*18 reverse-phase column 100-S (150 × 4.6 mm, 5µm, Nucleosil, Mancherey-Nagel).	100	0.8	2.53	6.34E+07	4.82E+06

(a.u.) : arbitrary unit.

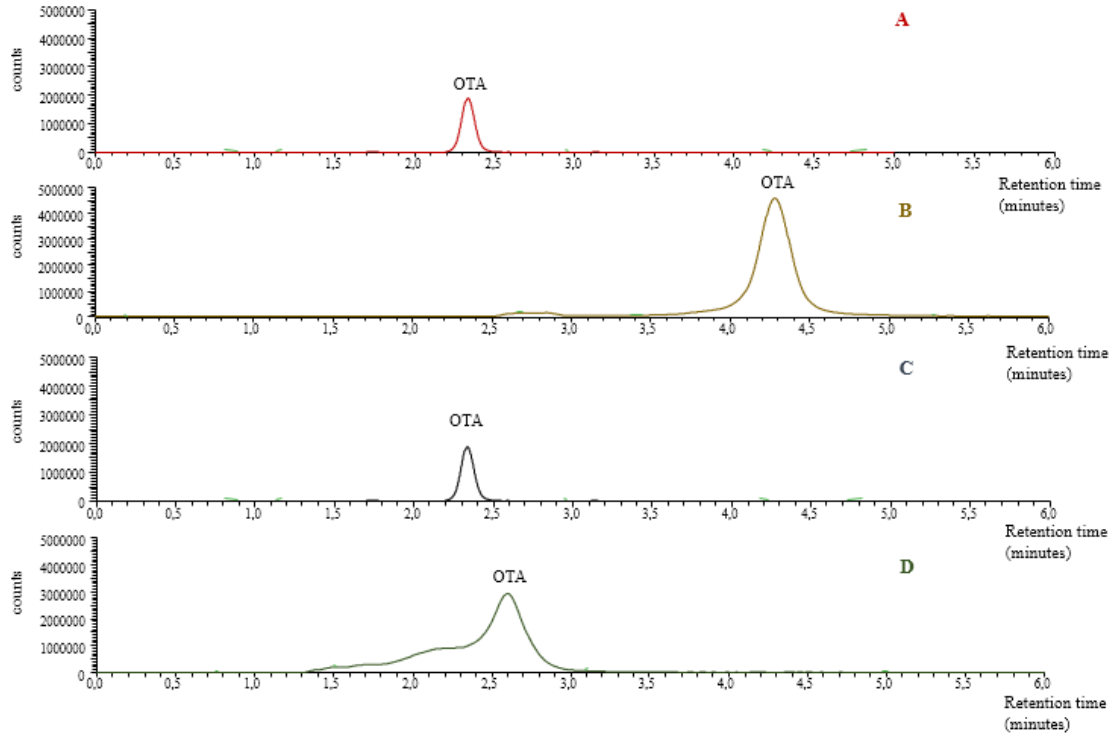

Figure 4.1 HPLC chromatograms for a standard solution of OTA at 50 ng/mL (in ACN) using different chromatographic columns. A) column #1: C18 reverse-phase column (100 × 4.6 mm, 2 µm); B) column #2: C18 reverse-phase column PLRP-S 300 Å (250 × 4.6 mm, 8 µm), C) column #3: C18 reverse-phase column PLRP-S 300Å (150× 4.6 mm, 8 µm), D) column #4: C18 reverse-phase column 100-S (150 × 4.6 mm, 5µm).

For the selection of the best HPLC columns, different injection volumes (10, 20, 50 100 µL) and flow rates (0.8, 1 mL/min) were tested for the 4 columns. Injection volumes, as well as optimal flow rates, are limited by the size of the column (cross sectional area and the length) which explain the use of 10 µL injection volume instead of 100 µL and 0.8 mL/min flow rate instead of 1 mL/min for the column #1.

According to Figure 4.1 and Table 4.1, we were able to determine the best column based on the three parameters. Column #1 and column #3 had low retention times, but the peak areas were also the lowest, thus resulting in low sensitivity. The best results in terms of OTA detection (peak area) were achieved using column #2 and column #4. However Figure 4.1 shows that the peak shape was better defined using column #2. For these reasons column #2 was selected for further OTA chromatographic analysis.

4.1.2 Selection of OTA dissolution solvents

For the selection of OTA dissolution solvents, calibration curves were performed for OTA standards dissolved in MeOH and ACN. The obtained calibration parameters of instrumentation are presented in Table 4.2. Calibration curves are shown in Figures 4.2 and 4.3. As revealed in Figure 4.3, a plot of OTA standards dissolved in ACN revealed that the data were unsatisfactory for the purposes of calibration: although there were 6 data points, the distribution of the points indicated that this was in effect a two-point calibration, which made it necessary to create two calibration curves. Table 4.2 shows that the LOQ and LOD values for the calibration curves of OTA standards eluted in ACN for a tested range from 12.5 to 50 ng/mL were higher than those obtained using MeOH dissolution solvent. For this reason, MeOH dissolution solvent was selected for further tests.

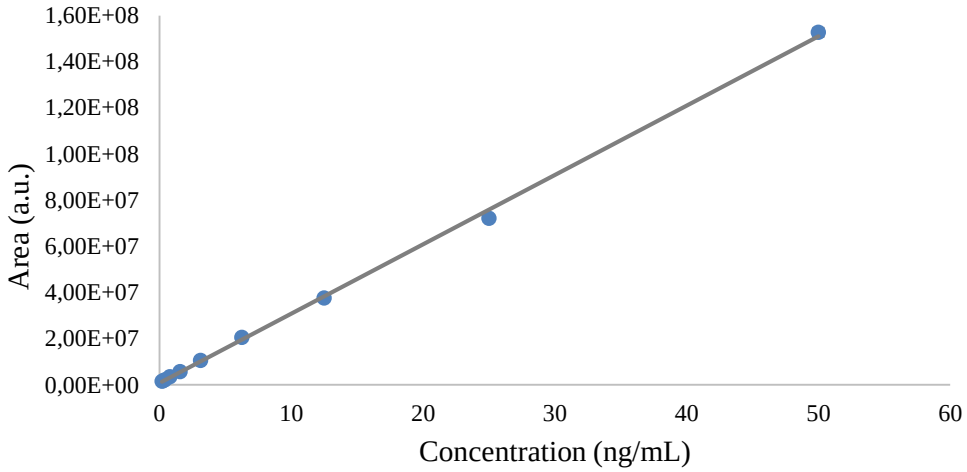

Figure 4.2 Calibration curve obtained for OTA standards using MeOH solution from 0.19 to 50 ng/mL.

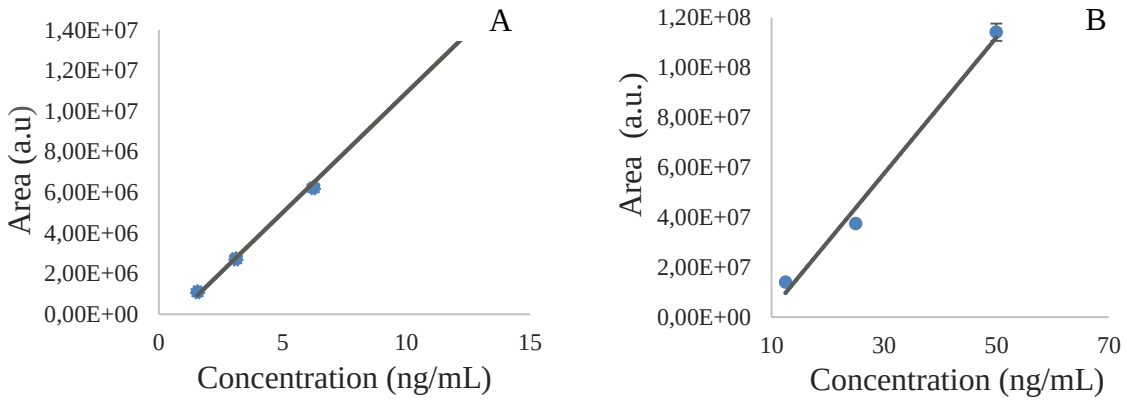

Figure 4.3 Calibration curves obtained for OTA standards using ACN solution: A) from 1.56 to 12.5 ng/mL, B) from 12.5 to 50 ng/mL.

Table 4.2 Calibration parameters of instrumentation using different dissolution solvents of OTA.

Dissolution solvent	**Concentration dynamic range (ng/mL)**	**Calibration curve**	**r^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**
Methanol	[0.19, 50]	Area (a.u.) = 3140291 (±12776) × OTA (ng/mL) + 460786 (±250484)	0.9995	0.26	0.80
Acetonitrile	[1.56, 12.5]	Area (a.u.) = 1210499 (±18073) × OTA (ng/mL) - 1007252 (±18073)	0.9984	0.34	1.02
	[12.5, 50]	Area (a.u.) = 2725196 (±294951) × OTA (ng/mL) - 24342188 (±9754605)	0.9884	---	---

4.2 Selection and validation of OTA extraction methods

The results regarding the extraction methods evaluated in this work for OTA extraction from cheese are presented and discussed in this section. Also, the internal validation study of the selected method is described.

The use of a different HPLC equipment was necessary due to some problems in the detector of the HPLC system used for the preliminary tests described in section 3.3 (high background noise: low analyte signal) For this reason, the assessment of the instrumental precision had to be performed for the new HPLC system.

4.2.1 Instrument precision

The HPLC conditions allowed the determination of OTA with retention time of approximately 5.6 minutes (Figure 4.4). A clean chromatogram was obtained with spiked cheese, and is shown in Figure 4.5. These analyses were used to obtain a calibration curve and to determine LOD and LOQ (Table 4.3).

The calibration parameters (linearity) that determine the accuracy of the equipment were satisfactory. Linearity is generally considered to be good when coefficient of determination $r^2 \geq 0.997$ (Chan, 2005), being in the present study a r^2 equal to 0.9995 obtained.

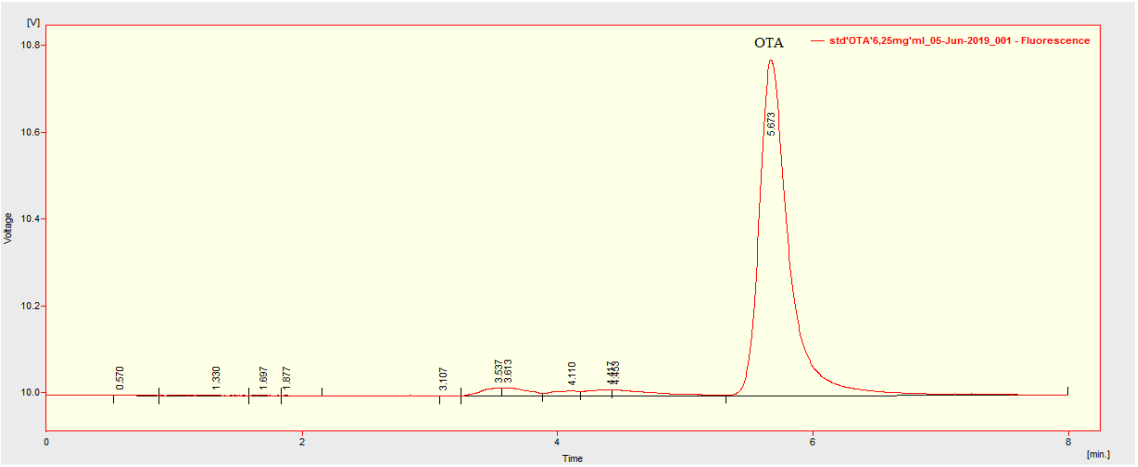

Figure 4.4 HPLC chromatogram for a standard solution of OTA at a concentration of 6.25 ng/mL.

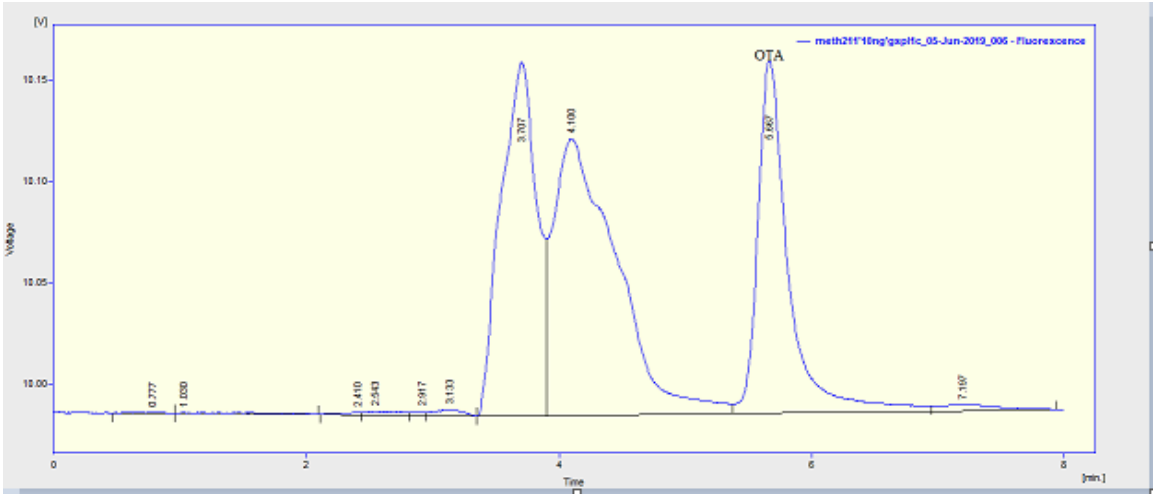

Figure 4.5 Chromatogram of OTA extracted from "Serra da Estrela" cheese spiked with 10 ng/g.

Table 4.3 Calibration parameters of instrumentation used for method validation and cheese analysis.

Mycotoxin	Calibration curve	r^2	LOD (ng/mL)	LOQ (ng/mL)
OTA	Area (a.u.) = 1920.5 (±16) × OTA (ng/mL) - 152 (±48)	0.999	0.075	0.251

4.2.2 Selection of the best OTA extraction method

Table 4.4 shows the recovery rates (RR) for each of the three replicates for each method, average RR, standard deviation (SD) and the Relative Standard Deviation (RSDr).

Table 4.4 Results obtained for the various methods tested for OTA extraction, in terms of recovery rate, mean, standard deviation (SD) and the relative standard deviation (RSDr).

Method	**Method #1**		**Method #2**		**Method #3**	**Method #4**
	Method #1.1	Method #1.2	Method #2.1	Method #2.2		
Spiking level	10 µg/kg					
Recovery (%)						
Replicate 1	27.1	34.9	47.5	41.9		61.9
Replicate 2	22.4	35.0	39.5	41.8		84.6
Replicate 3	25.1	35.1	42.6	40.5	ND	60.4
Mean (%)	24.9	35.0	43.2	41.4		69.0
SD	2.3	0.1	4.0	0.8		13.6
RSDr (%)	9.2	0.3	9.2	0.1		19.6

Considering the results of RR presented in Table 4.4, it can be seen that the RR varied according to the methods. With regards to method #1, the RR obtained was very low, independently of using enzymes (method #1.2) or not (method #1.1). Since ACN is a very toxic and expensive solvent, this method was discarded based on these two factors.

These results are lower than those obtained in a previous study carried out by Pattono et al. (2013) using a similar extraction method (method #1.1), which presented 93.9% as RR at a spiking level of 1 µg/kg.

For the method #2, the RR were also low for method #2.1 and method #2.2, which explain that the use of enzymes assisted chemical extraction (lipase and protease) do not affect the recovery of OTA extraction in "Serra da Estrela" cheese. However several studies have reported that the use of digestive enzymes had a significant influence on the RR, such as the study conducted by Giacomo et al. (2016) which demonstrate the use of enzymatic digestion (ED) method for OTA extraction in pig tissue. Giacomo et al. (2016) compared the ED procedure and LLE protocol from animal tissues followed by IAC, to evaluate their performance regarding OTA quantitative extraction and they confirmed that the use of the ED extraction significantly reduced matrix interference with the

samples. For spiked muscle samples, the recovery obtained with the ED extraction method (90.32± 0.02%) was higher than the recovery obtained with the conventional LLE (79.90± 1.80%). The increase of OTA extraction in pork products by using protease might be due to the fact that meat has serum albumin. OTA is reported to have high affinity to albumin (Kőszegi and Póor, 2016). Probably a treatment with a protease would increase the release of OTA from albumin and increase its detection. Chavarría et al. (2015) also indicated that an improvement on the RR was achieved when cheese homogenates were incubated for 24 h in 50 µg/mL protease solution for AFM_1using LLE from soft fresh cheese.

Using the method #3, OTA could not be detected. Also, the dichloromethane is known to be a toxic solvent such as ACN, so this method was not further considered.

In method #4, the RR obtained were acceptable (mean recovery 69%), which led us to choose this method for further analysis of OTA in the samples. This was also faster and more practical when compared to the method #1.

Results obtained indicated that LLE (MeOH/sodium bicarbonate) followed by IAC purification lead to the most efficient extraction method comparing to the LLE (ACN) followed by a liquid purification (hexane). Moreover, it has been reported that the solubility of OTA in methanol is high, and methanol is one of the most potent adsorbents (Jalili & Jinap, 2015). Also, IAC have been widely used as a clean-up tool and their use, although expensive, is highly recommended, allowing the isolation of the analyte from most matrix interferences, due to its specificity (Serra et al., 2004).

4.2.3 Validation of the selected method: Method #4 Chemical extraction followed by IAC purification as described in the R-Biopharm P119.V8 application method with some modifications

In order to evaluate the accuracy of the method applied, blank cheese samples were spiked at two different OTA concentrations then, recovery and standard deviation (SD), intraday and inter-day repeatability (RSD_r and RSD_R respectively) were calculated (Table 4.5).

Table 4.5 Performance and precision of OTA extraction method.

Mycotoxin	**OTA**	
Spiking level	2 µg/kg	10 µg/kg
Day 1		
Recovery (%)		
Replicate 1	48.53	74.79
Replicate 2	50.37	68.50
Replicate 3	67.92	62.01
Mean	55.60	68.43
SD	10.71	6.39
RSDr (%)	19.26	9.34
Day 2		
Recovery (%)		
Replicate 1	68.92	74.97
Replicate 2	71.99	68.56
Replicate 3	68.67	75.00
Mean	69.86	72.84
SD	1.85	3.71
RSDr (%)	2.64	5.09
Mean Recovery (%)	62.73	70.63
MD*	10.08	3.12
RMD* (%)	16.06	4.42
LOD (µg/kg) **	0.45	
LOQ (µg/kg) **	1.51	
Recommended		
Recovery (%)	70-110	
RSDr (%)	≤ 20	
RSD_R(%)	≤ 30	

* Because there are only two values for mean recovery to calculate Intermediate Precision, mean deviation (MD) and relative mean deviation (RMD) substitute the commonly used standard deviation (SD) and relative standard deviation (RSD).

** Calculated from the LOD and LOQ presented in section 0.

*** As recommended by the Regulation No. 401/2006 of the European Commission (EC, 2006).

The LOQ obtained was 1.51 µg/kg. This is a high value compared to those obtained from other reports using methodologies similar to ours vary widely. Sakin et al. (2018) for example reported a LOQ value of 0.11 µg/kg for OTA. The average RR obtained in our study was 70.63 and 62.73 % for spiking levels of 2 µg/kg and 10 µg/kg, respectively. The average RR obtained in the current study are slightly below the recommended interval according to the Commission Regulation No 401/2006 of the European Commission (EC, 2006) which is 70-110% for OTA. Recent report by Sakin et al. (2018) showed higher recoveries: 105.4 and 92.3 % within a spiking range of 1-3 µg/kg for Surk cheese in Turkey. Dall'Asta et al. (2008) reported also a mean recovery for OTA 97% within a spiking range of 0.5-2 µg/kg in different commercial samples of blue-mould ripened cheeses. The RSDr and RSD_R values were between 2.64 – 19.26 % and 4.42 - 16.06 % respectively. Comparing these values with those recommended (EC, 2006) we verify that the RSDr and RSD_R are below the recommended value.

4.3 Detection and quantification of OTA in "Serra da Estrela" cheese samples

Among the 25 "Serra da Estrela" cheese samples analyzed, none of the samples was found to be contaminated with detectable levels of OTA, since none of them reached the LOD value of 0.45 µg/kg. In Italy, a study on the subject indicated also that no OTA contamination was found in the Bleau and Bergarder cheeses examined, but 50% of Roquefort samples and 42.6% of Gorgonzola samples showed OTA at levels ranging from 0.25 to 3µg/kg) (Dall'Asta et al., 2008). Higher levels were reported by other authors for hand-made semi-hard cheeses in Italy with OTA concentration ranged from 1 to 262 µg/kg in the rind and from 18 to 146 in the cheese interior (Pattono et al., 2013). Regulation (EC) No 1881/2006 of the European Commission (EC, 2006) which lays down the levels of certain contaminants in foodstuffs has only established limits for products of plant origin and there is no limit for OTA for cheese.

5 CONCLUSIONS

The current study aimed to provide an optimized procedure of extraction, purification and detection by HPLC-FD of OTA for "Serra da Estrela" cheese. The study has shown that:

- The complexity and the variety of the cheese matrix pose a question about what kind of HPLC calibration is more suitable for OTA determination.
- MeOH is a better dissolution solvent of OTA than ACN.
- Enzymatic treatment (lipase and protease) showed no effect in the affinity of fats and proteins for OTA in "Serra da Estrela" cheese.
- Compared to the liquid-liquid extraction (LLE) and purification with ACN and hexane, the LLE with MeOH/sodium bicarbonate followed by IAC purification showed the highest OTA recovery, achieving a better clean up and an enrichment of the analyte concentration. Also, this approach avoids additional solvent extraction for sample defatting.

The optimized analytical methodology provides an acceptable result in terms of accuracy, repeatability, intermediate precision and sensitivity, and has shown to be reliable for the determination of OTA in "Serra da Estrela" cheese.

The application of the procedure to twenty-five cheese samples demonstrated that none was contaminated with detectable levels of OTA**.** In conclusion, "Serra da Estrela" cheese seems to be a product with no or low susceptibility for OTA contamination.

Further investigations of the occurrence of other mycotoxin (e.g., AFM_1, PAT) must be performed on "Serra da Estrela" cheese and as well as the possible isolation of mycotoxigenic strains from the cheese samples. This information will be essential for developing scientific tools capable of matching consumer expectations regarding the complete safety of "Serra da Estrela" cheese.

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