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Mycotoxins in pistachios (*Pistacia vera* L.): Development and validation of a multi-mycotoxin analytical method using QuEChERS followed by Ultra High-Pressure Liquid Chromatography-Mass Spectrometry

Dissertação no âmbito do Mestrado em Segurança Alimentar orientada pela Professora Doutora Angelina Lopes Simões Pena e coorientada pela Doutora Ana Teresa Sanches Silva apresentada à Faculdade de Farmácia da Universidade de Coimbra

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RESUMO

O consumo de pistácios (*Pistacia vera* L.) tem vindo a aumentar devido ao seu reconhecido benefício na saúde humana. Apesar de constituírem uma excelente fonte nutricional, os pistácios têm riscos químicos associados, como as micotoxinas, resultantes da contaminação de fungos e do seu metabolismo secundário. Os pistácios são um dos frutos secos com maior contaminação por micotoxinas em todo o mundo, especialmente por aflatoxinas. As aflatoxinas são as mais tóxicas para os seres humanos, particularmente a aflatoxina B1 com efeito hepatotóxico e classificada como comprovadamente carcinogénica para o homem pela Agência Internacional para a Investigação do Cancro (IARC). Outras micotoxinas são relevantes como ocratoxina A (OTA), fumonisinas (FBs), zearalenone (ZEA) e trichotecenes (T2, HT2 e DON) e mais recentemente, as micotoxinas emergentes.

Este estudo desenvolveu um método rápido, fácil, barato, eficaz, robusto e seguro (QuEChERS) seguido de Cromatografia Líquida de Ultra Resolução combinada com Espectrometria de Massa de Tempo de Voo (UHPLC-ToF-MS) para a determinação das micotoxinas em pistácios. Diferentes abordagens no *clean-up* pela técnica de extração de Fase Sólida Dispersiva (d-SPE) foram avaliadas. Para isso, foram utilizados adsorventes clássicos, como C18 (octadecil sílica) e PSA (amina secundária primária) e novas classes de adsorventes, nomeadamente, EMR-Lipid (*enhanced matrix removal-lipid*) e Z-Sep (gel de sílica modificada com óxido de zircónio).

O método com 100 mg de Z-Sep proporcionou o melhor desempenho analítico, com uma boa recuperação (79 a 120%), boa repetibilidade (RSD_r<10%) e a boa precisão inter-dia (RSD_R<10%) de acordo com critérios estabelecidos pelo Regulamento N.º 401/2006 da Comissão Europeia para a análise das micotoxinas. O método foi validado para aflatoxinas (AFB1, AFB2, AFG1 e AFG2), ocratoxina A (OTA), zearalenona (ZEA), toxina T2 (T2) e toxina HT-2 (HT2). Os LODs para as AFs variaram entre 0,125 e 0,25 μg/Kg, concentrações inferiores aos níveis máximos para frutos secos regulados pela UE.

O método foi aplicado a 16 amostras de pistácios e em 6 destas foi determinada uma micotoxina (AFB1, HT2 ou FB1), mas em baixas concentrações. A concentração de AFB1 foi inferior ao valor máximo permitido de acordo com a legislação da União Europeia. Além disso, AFB2 e FB1 foram detetadas nas cascas de pistácios. Assim, os pistácios selecionados para o presente estudo e disponíveis no mercado português são considerados seguros para o consumo humano no que diz respeito à presença de micotoxinas.

Palavras-chave: Adsorventes; Aflatoxinas; Cromatografia Líquida; Espectrometria de Massa; Micotoxinas; Pistácios; *Pistacia vera* L.; QuEChERS.

ABSTRACT

The consumption of pistachios (*Pistacia vera* L.*)* has been increasing, given its important benefit in human health. In addition to an excellent nutritional source, it has associated chemical hazards, such as mycotoxins, resulting of fungal contamination and its secondary metabolism. Pistachios are one of nuts with higher mycotoxin's contamination worldwide, especially Aflatoxin B1 with a hepatotoxic effect and classified as proven carcinogenic to humans by the International Agency for Research on Cancer (IARC). More mycotoxins as ochratoxin A (OTA), fumonisins (FBs), zearalenone (ZEA) and trichothecenes (T2, HT2 and DON) and emerging mycotoxins have been concerned in nuts.

This study developed a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method followed by Ultra-High Performance Liquid Chromatography combined with Time-of-Flight Mass Spectrometry (UHPLC–ToF-MS) for the determination of multi-mycotoxins in pistachios.

Different approaches in dispersive solid phase extraction (d-SPE) as clean-up for high-lipid matrix were to evaluate. For this, classic sorbents, like C18 (octadecyl modified silica) and PSA (primary secondary amine) and new classes of sorbents, namely EMR-Lipid (enhanced matrix removal-lipid) and Z-Sep (modified silica gel with zirconium oxide) are used.

Method with 100 mg Z-Sep sorbent provided the best analytical performance, with good recovery (79 to 120%), repeatability (RSD_r<10%) and precision inter-day (RSD_R<10%) in agreement with criteria established by Commission Regulation EC No. 401/2006 for mycotoxins analysis. The method was validated for aflatoxins (AFB1, AFB2, AFG1 and AFG2), ochratoxin A (OTA), zearalenone (ZEA), toxin T2 (T2) and toxin HT-2 (HT2). The LODs for AFs ranged from 0.125 to 0.25 μg/Kg, which are lower than the maximum levels in nuts regulated by the EU.

The method was applied to 16 real pistachio samples and 6 of these presented one mycotoxin (AFB1, HT2 or FB1) but at low concentrations. The concentration of AFB1 was lower than the maximum permitted level established by the EU legislation. Also, AFB2 and FB1 are detected in pistachio shells. In this line, pistachios samples selected in the present study and available in the Portuguese market are safe for human consumption concerning mycotoxins content.

Keywords: Aflatoxins; Liquid Chromatography; Mass Spectrometry; Mycotoxins; Pistachios; *Pistacia vera* L.; QuEChERS; Sorbents.

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LIST OF SIMBOLS, ACRONYMS AND ABBREVIATIONS

ACN – Acetonitrile

ADONs – Sum of 3-acetyl and 15-acetyl-deoxynivalenol

AFB1 – Aflatoxin B1

AFB2 – Aflatoxin B2

AFBO – Aflatoxin B1-exo-8,9-epoxide

AFG1 – Aflatoxin G1

AFG2 – Aflatoxin G2

AFM1 – Aflatoxin M1

AFM2 – Aflatoxin M2

AFs – Aflatoxins

ALARA – as low as reasonably achievable

ALP – Alkaline phosphatase

ARfD - Acute Reference Dose

a_w – water activity

BEA – Beauvericin

bw – body weight

C18 – Octadecyl silica

CAST – Council for Agricultural Science and Technology

CLEIA – Chemiluminescent Enzyme Immunoassay

CLIA – Chemiluminescent Immunoassay

d-SPE – Dispersive Solid Phase Extraction

Da – Daltons

DLLME – Dispersive Liquid–Liquid Microextraction

DNA – Deoxyribonucleic Acid

DON – Desoxynivalenol

EC – European Commission

EFSA – European Food Safety Authority

ELISA – Enzyme-Linked Immunosorbent Assay

EMR-Lipid – Enhanced Matrix Removal-Lipid

ENNs – Enniatins

ESI – Electrospray Ionization

EU – European Union

FA – Formic acid

FAO – Food and Agriculture Organization of the United Nations

FB1 – Fumonisin B1

FB2 – Fumonisin B2

FDA – Food and Drug Administration

FLD – Fluorescence detection

FUS – Fusaproliferin

G – Guanine

GC – Gas Chromatography

GCB – Graphitized Carbon Black

HBC – Hepatitis C Virus

HBV – Hepatitis B Virus

HCC – Hepatocellular Carcinoma

HPLC – High-Performance Liquid Chromatography

HRMS – High-Resolution Mass Spectrometry

HRP – Horseradish Peroxidase

HT2 – Toxin HT-2

IAC – Immunoaffinity columns

IARC – International Agency for Research on Cancer

INE – Instituto Nacional de Estatística

IS – Internal Standard

JECFA – Joint FAO/WHO Expert Committee on Food Additives

LC – Liquid Chromatography

LOD – Limit of detection

LOQ – Limit of quantification

ME – Matrix effect

MeOH – Metanol

MON – Moniliformin

MS – Mass Spectrometry

MS/MS – Tandem Mass Spectrometry

MSPD – Solid Phase Matrix Dispersion

NOW – Navel Orangeworm

°C – Degree Celsius

OTA – Ochratoxin A

PBS – Phosphate Buffer Saline

PCD – Post Column Derivatization

PMTDI – Provisional Maximum Tolerable Daily Intake

PortFIR – Plataforma Portuguesa de Informação Alimentar

PSA – primary secondary amine

PTWI – Provisional Tolerable Weekly Intake

Q-Orbitrap – Quadrupole-Orbital Ion Trap

QqQ – Triple Quadrupole

QqToF – Double Quadrupole-ToF

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

r^2 – Determination coefficient

RASFF – Rapid Alert System for Food and Feed

RDS – Relative standard deviation

RNA – Ribonucleic Acid

RSD_R – Reproducibility

RSDr – Repeatability

S/N – Signal-to-Noise ratio

SPE – Solid Phase Extraction

SPME – Solid Phase Micro-Extraction

SSE – Signal Suppression-Enhancement

STC – Sterigmatocystin

T – Timin

T2 – Toxin T2

TDI – Tolerable Daily Intake

TeA – Tenuazonic acid

TLC – Thin-layer Chromatography

ToF – Time-of-Flight

UHPLC – Ultra-High Pressure Liquid Chromatography

USA – United States of America

UV – Ultraviolet

WHO – World Health Organization

Z-Sep – Zirconium oxide

ZAN – Zearalanona

ZEA – Zearalenone

λem – Emission wavelength

λexc – Excitation wavelength

PART I

STATE OF THE ART

Notes:
Part I was published as a review paper: MATEUS, A.R.S.; BARROS, S.; PENA, A.; SILVA, A.S. **Development and Validation of QuEChERS Followed by UHPLC-ToF-MS Method for Determination of Multi Mycotoxins in Pistachio Nuts**. Molecules. 26 (2021), 5754. https://doi.org/10.3390/molecules2619575
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MATEUS, Ana Rita Soares.; BARROS, Sílvia; PENA, Angelina; SILVA, Ana Sanches - **Challenges of mycotoxins decontamination processes for pistachio (*Pistacia vera* L.).** In: International Scientific Conference Food & Climate Changes, Croatia. October 2021 (accepted).

1. Pistachios (*Pistacia vera* L.)

Pistachios (*Pistacia vera* L.*)* are one of the most popular nuts in the world, due to their flavour, nutritional quality and health benefits. Consumption of nuts like hazelnut, almonds, walnuts, pistachio and cashew nuts is characteristic of the Mediterranean diet (Widmer *et al.*, 2015). Worldwide, the consumption of pistachios amounted to approximately 761.71 mil tons in 2020. In five years, the consumption increased by approximately 198 mil tons. In United States of America (USA), the per capita consumption of pistachios increased substantially from 0.095 kg in 2015 for 0.245 kg in 2020 (Shahbandeh, 2020, 2021). The consumption of nuts has been increasing in Portugal, on average, one Portuguese person consumes 6.5 kg of nuts per year (INE, 2020). This is in part related with the fact that the consumption of nuts has been associated with a healthy dietary pattern and recommended by health professionals, namely nutritionists, due to pistachios being low in calories, high in mono-unsaturated fatty acids and low in saturated fatty acids. In addition they are a good source of proteins, carbohydrate and dietary fibers, vitamins (A, E, K, B1 and B6) and minerals (potassium, phosphorus, magnesium and iron) (Dreher, 2012; Kashaninejad e Tabil, 2011). About 100 g of pistachios provides 4 g of the essential amino acid tryptophan (PortFIR, 2019).

The composition of nuts is determinant for beneficial effects. From a health point of view, several studies indicate that pistachios reduce the risk of coronary heart disease, since there is a reduction in cholesterol levels and a decrease in blood pressure (Kashaninejad e Tabil, 2011). Other studies suggest a reduction in oxidative and inflammatory stress, blood glucose control, better appetite management and consequent weight control (Dreher, 2012).

Pistachio is a very versatile nut, consumed as a snack (raw, roasted, salted or flavoured) and also used in ice cream and bakery goods. Pistachios are the fourth most produced tree nut fruit in the world, with about 655 000 tons in 2019/2020 (Shahbandeh, 2021). In 2019, the global market of pistachio was dominated by Iran and USA, which produced 337,000 tons and 335,000 tons, respectively, followed by China and Turkey (Bui-Klimke *et al.*, 2014; FAO STAT, 2019). The pistachio tree grows in subtropical, warm and Mediterranean climates, with average temperatures of 30°C (Wickens, 1995).

2. Fungal contamination and mycotoxins

Similar to other nuts, pistachio contains low amounts of water after being dried, which restrict spoilage by microorganisms. However, some fungi are able to develop, since they require a smaller amount of water to multiply (Mendes, Santos e Soares, 2016). Fungal contamination can occur along the food chain, in the development of the plant in field, as well as in post-harvest, drying, transport, storage and processing. Contamination may occur in these phases following harvest or there may be an increase in previous contamination (CAST, 2003). Fungal contamination is closely related to environmental conditions, such as temperature and humidity, which must be favorable to its growth. Moreover, crop damage due to insect infestation and improper drying of crops before storage are factors to be taken into account (Sataque Ono *et al.*, 2011)(**Figure 1**).

As a result of this contamination, mycotoxins appear in nuts. The word "mycotoxin" is derived from the Greek word "*mykes*" meaning "*fungus*" and the Latin word "*toxicum*" meaning "poison" (Aiko e Mehta, 2015). Mycotoxins are secondary metabolites of filamentous fungi; low mass molecules produced by multiples genera and species of fungi and have in common toxic effects in animals and humans. Mycotoxins are a heterogeneous group due to several chemical structures, biosynthetic origins and biological effects (Bennett e Klich, 2003). Food may be contaminated with several different mycotoxins because, when conditions are favorable for fungal contamination, more than one fungal species can contaminate food, and also, a single species of fungi can produce several toxic metabolites (CAST, 2003; Yang *et al.*, 2020). It is also important to mention that the presence of fungi may not be related to the presence of mycotoxins. On the one hand, not all fungi are mycotoxin producers, and on other hand, mycotoxins are only produced under certain conditions. In fact, the occurrence of aflatoxin contamination is sporadic and, although large populations of *A. flavus* infect crops, serious outbreaks are associated with above-average temperature and below-average rainfall (CAST, 2003).

Mycotoxins have different adverse effects in human health, such as, carcinogenicity, mutagenicity, teratogenicity, cytotoxicity, neurotoxicity, nephrotoxicity, immunosuppression and estrogenic effects (Silva *et al.,* 2019). The severity of effects depend on amounts ingested, duration of exposure and on individual characteristics, such as age, gender, weight, diet or health status (Abrunhosa *et al.,* 2016); for example, a low variety and insufficient diet constitute a risk factor for greater severity of negative effects of mycotoxicosis (Magnussen e Parsi, 2013). In addition, the interaction between mycotoxins could result in antagonistic, additive, or synergistic effects (Šegvić Klarić, 2012; Smith *et al.*, 2016).

Mycotoxins are a concern for food safety, Food and Agriculture Organization of the United Nations (FAO) estimates that 25% of foods are contaminated by mycotoxins, with consequences on health but also leading to economic losses at all levels of the food chain (Boutrif e Canet, 1998; CAST, 2003). Mycotoxins are more common in developing countries, where less concern for food safety, insufficient quality control, hot weather, inadequate production techniques and poor crop storage conditions are suitable for the growth of fungi (Williams *et al.*, 2004). However, contamination is a global concern because is an unpredictable and inevitable problem, one of the most challenging to food safety, even when all good practices in food chain are implemented.

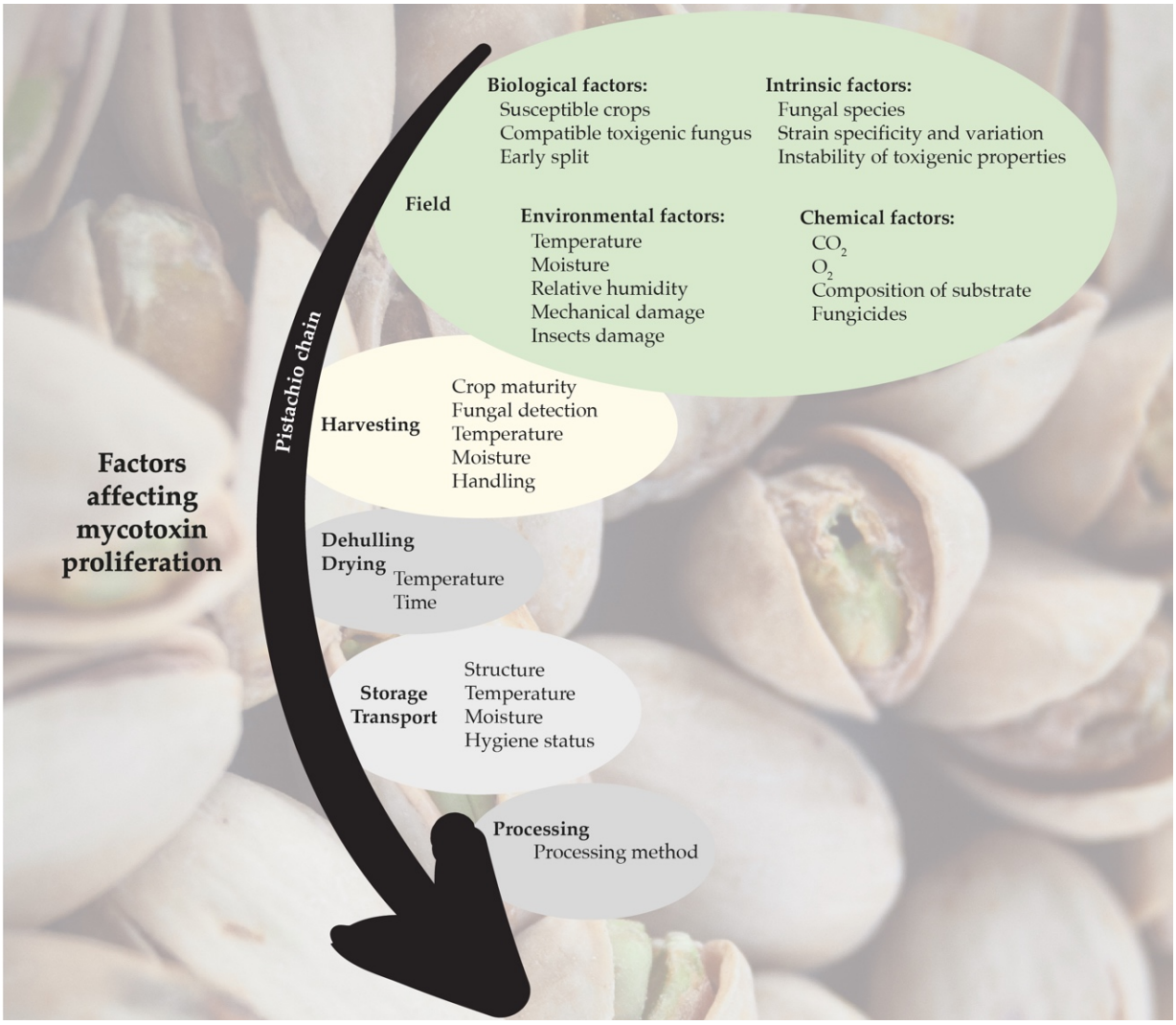


Figure 1. Major factors influencing mycotoxins' proliferation along the food chain in pistachios.

The Rapid Alert System for Food and Feed (RASFF), in 2018, reported 569 notifications for mycotoxins, predominantly in the group of dried fruits, derived from dried fruit and seeds, such as pistachios and almonds. The most prevalent reported group are aflatoxins, followed by ochratoxin A. The same trend is maintained in 2019, with 588 notifications for mycotoxins

and 90% of notifications are from countries outside EU, particularly, Turkey and Argentine (European Comission, 2020). In pistachio nuts, RASFF, between January 2020 and June 2021 reported 84 notifications, mostly from Turkey, Iran and the USA, related with aflatoxins and one notification concerning ochratoxin A (32.8 µg/kg) in pistachio from USA (European Comission, 2021; European Commission, 2020).

Thus, pistachios, despite their beneficial effects on human health, also have chemical hazards and are an important source of exposure to mycotoxins, especially aflatoxins, constituting a current public health problem. Pistachios are considered to be the ones with the highest risk of contamination by aflatoxins, largely due to shell splitting at end of maturation (Cheraghali *et al.*, 2007; Varga *et al.*, 2013). This shell protects the pistachio kernel and, as a consequence of splitting, pistachios are susceptible to molds and insect invasions. For example, navel orangeworm (NOW) (*Amyelois transitella*) is a common pest of pistachio nuts in the field. This worm causes direct physical damage in pistachio due to worm's growth, feeding on kernels and insect excrement (Siegel e Kuenen, 2011). However, it also causes indirect damage because it predisposes contamination by the aflatoxin-producing fungi. In fact, a study focused on California pistachio showed that kernel infested by NOW had substantially more infections by *Aspergillus* fungi producers of AFs and OTA, as *A. flavus* and *A. niger*, respectively, and consequently AFs are more frequently and found in higher levels (Doster, 1994).

2.1. Aflatoxins

Aflatoxins (AFs) are a class of mycotoxins produced by fungi of genus *Aspergillus*, especially the species *A. flavus* and *A. parasiticus*. Fungi *A. nomius, A. pseudotamari, A. bombycis* and *A. ochraceoroseus* are also producers of aflatoxins but found less frequently (Kumar *et al.*, 2017). *Aspergillus* are distributed worldwide, but the great predominance is in countries with subtropical climate and warm temperate. They are characteristically greenish to greyish molds, grow in hot (15 to 40°C) and humid conditions (Kumar *et al.*, 2017).

2.1.1. Physical and chemical characteristics

Aflatoxins are low molecular weight molecules, among 312-346 Da (Kumar, 2018), composed of carbon, oxygen and hydrogen atoms (**Figure 2**). They are highly oxygenated heterocyclic compounds derived from difuranocumarinic, where difuran group is attached to one side of the cumarin nucleus and the pentatone ring is connected to the other side, in the case of AF-B series, or the hexagonal lactone ring, in the case of AF-G series (Kumar *et al.*, 2017). The designation of series is related to fluorescence of molecules under UV light: B

series has a blue color and G series has a green color, while associated numbers are related to the mobility of molecules in chromatography (CAST, 2003).

More than 20 aflatoxins are known, but the four main ones are: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), as well as the metabolites of AFB1 and AFB2, aflatoxin M1 (AFM1) and M2 (AFM2), respectively, as they were primarily found in animal milk (Bennett e Klich, 2003). It should be noted that *A. flavus*, more common in dried fruits (Kumar *et al.*, 2017), mainly produces B-series aflatoxins, while *A. parasiticus* produces both aflatoxins B and G (Fletcher e Blaney, 2016). In terms of toxicity, the most toxic aflatoxin is AFB1, followed by AFG1, AFB2 and, the least toxic, AFG2 (Nazhand *et al.*, 2020), while AFM1 has similar toxicity to AFG1 (Benkerroum, 2020).

These mycotoxins are characterized by being crystals that are colorless to light yellow. They present fluorescence under UV light, but UV light is instable in the presence of extreme oxygen and pH (<3 or >10). The melting points of these molecules are between 240 and 280°C. AFs are soluble in organic solvents, such as chloroform and methanol; moderately soluble in water and insoluble in non-polar solvents (Nazhand *et al.*, 2020).

2.1.2. Toxicokinetics

AFB1 is the best studied aflatoxin due to its relevance in human health and to its being the one that most frequently occurs in food, reflecting metabolism of other AFs. AFB1 is rapidly absorbed by the gastrointestinal tract, reaching maximum concentrations in the bloodstream after 1 hour (IARC, 2012). About 95% of AFB1 and metabolites are excreted in urine in the first 24 hours after exposure (IARC, 2012). AFB1 is metabolized in the liver by cytochrome P450 system, by epoxidation, to an electrophilic and very reactive molecule, aflatoxin B1-exo-8,9-epoxide (AFBO), capable of covalently binding to DNA, RNA and proteins (Bennett e Klich, 2003). Conjugation of AFBO with glutathione by glutathione-S-transferase is a detoxification route since it inhibits the ability of AFBO to bind to DNA, forming an inert metabolite, followed by biotransformation with mercapturic acid, and then excreted in urine (Rushing e Selim, 2019). In addition to epoxidation, AFB1 can be metabolized by hydroxylation reaction and also by cytochrome P450 system enzymes, resulting in several metabolites: aflatoxin M1 (AFM1), aflatoxin Q1 (AFQ1), aflatoxin P1 (AFP1), aflatoxicol (AFL), aflatoxicol H1 (AFH1) and aflatoxin B2a (AFB2a). AFM1 is the predominant metabolite, most commonly found as a consequence of AFB1 exposure, and the most carcinogenic, by similar mechanism concerning AFB1. Moreover, these metabolites have toxic effects on humans (Rushing e Selim, 2019). AFM1 and AFQ1, although toxic, are less reactive than other molecules and are eliminated directly in urine (Wacoo *et al.*, 2014).

2.1.3. Toxicity

Aflatoxins are the leading cause of non-infectious diseases of food origin. It is estimated that 4.5 to 5.5 billion people are exposed to these mycotoxins (Kew, 2013). AFs are genotoxic, carcinogenic and hepatotoxic; therefore, there is no threshold level for their toxicity and a Tolerable Daily Intake (TDI) is not established. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), in 1997, through epidemiological data, estimated that intake of 1 ng AFB1/kg bw/day increases the incidence of liver cancer by 0.013 cancer cases /year per 100 000 subjects, for HBsAg-negative individuals, concerning risk assessment (JECFA, 2017). In 2016, JECFA recalculated the cancer risk associated with aflatoxin exposure and conclude that European people and those other developed countries had a lower risk cancer, ranging from <0.01 to 0.1 aflatoxin-induced cancers per year and per 100,000 subjects (JECFA, 2017; Schrenk *et al.*, 2020).

2.1.3.1. Acute toxicity

Exposure to high concentrations of aflatoxins in a short period of time leads to hepatotoxic effect, manifesting early as anorexia, malaise and low fever, and maybe progressing to vomiting, abdominal pain and jaundice, as well as pulmonary and cerebral edema, coma and convulsions (Alshannaq e Yu, 2017). In addition, acute exposure to a high AF content can lead to death by hepatitis (Kumar *et al.*, 2017). Estimated total aflatoxin intake that causes a mortality risk is >1 mg/day, i.e., >20 μg/kg body weight/day in adults (IARC, 2012). Children are a more vulnerable population group since consumption of food by body weight is higher compared to adults; immune and neurological systems are immature and diet is more restricted, so there is greater susceptibility to develop complications (Magnussen e Parsi, 2013). AFB1 may cause weight loss, growth delay or even malnutrition states in children (Bhat, Rai e Karim, 2010). Acute exposure to AFs is associated with Kwashiorkor Syndrome, identified through epidemiological studies and outbreaks that have occurred throughout history. Kwashiorkor syndrome is intermediate malnutrition associated with high carbohydrate intake due to lack of proteins and vitamins and occurs mainly in children. Studies indicate that children with this syndrome are more exposed to AFB1 by cereals consumed and have higher frequency and higher concentration of aflatoxicol in serum, indicating a change in AFB1 metabolism and interference in micronutrient absorption (Rushing e Selim, 2019). Some studies also indicate a relationship with Reye Syndrome, an acute encephalopathy with visceral fat degeneration, more common in adolescents; however, the cause-effect relationship of aflatoxins with this syndrome has not yet been fully established (CAST, 2003).

2.1.3.2. Chronic toxicity

Aflatoxin B1 is considered the most potent hepatic carcinogenic of aflatoxins, and the International Agency for Research on Cancer (IARC) since 1987 classified in group 1, proven carcinogenic to humans, related to hepatocellular carcinomas, since there is sufficient scientific evidence in both studies conducted in animals with human studies (International Agency for Research on Cancer, 1993; Ostry *et al.*, 2017). Toxicity mechanisms are related to the metabolite of AFB1. AFBO is capable of linking to DNA, by nucleophilic addition, to nitrogen 7 (N7) of guanine base, forming AFB1-N7-guanine adduct. The formation of this adduct in DNA leads to G-to-T transversion during cell replication. One consequence is the AGG→AGT (Arginine→Serina) transversion, resulting in the inactivation of p53 tumor suppressor gene in codon 249, responsible for cell cycle control, DNA repair and apoptosis (CAST, 2003; Wacoo *et al.*, 2014). In addition, AFBO can bind to primary amine groups of amino acids (such as lysine) and proteins (namely albumin), forming adducts found in the bloodstream (Bennett e Klich, 2003; Wacoo *et al.*, 2014).

Through epidemiological studies, it was concluded that exposure to AFs constitutes a risk factor for the development of hepatocellular carcinoma (HCC) (Bennett e Klich, 2003). HCC is the fourth most common cause of cancer-related death worldwide. In addition to exposure to AFs, alcohol, hepatitis B and C and other metabolic liver diseases are considered risk factors for HCC (Yang *et al.*, 2019). Epidemiological studies conducted in Asia and Africa have indicated combination of AFB1 exposure and hepatitis B virus (HBV) infection increases the risk of HCC; that is, there is a synergistic effect between AFB1 and HBV. The first clinical evidence of this synergism occurred in China where it was found that HCC occurred in individuals infected with HBV living in villages with high consumption of aflatoxins, with a mortality rate 10 times higher than in individuals living in villages with lower consumption (Kew, 2013). HBV infection can sensitize hepatocytes to carcinogenic effects of AFB1, explained by different mechanisms related to mutation in codon 249. One hypothesis state that HBV genome is inserted in HBV X gene, translated into HBV X protein that inhibits DNA repair and also contributes to uncontrolled cell proliferation. Another hypothesis states that necrosis of hepatocytes and proliferation results in an increase of cells with mutation. Moreover, chronic inflammatory liver disease, resulting from the HBV virus, causes production of reactive oxygen and nitrogen species that increase oxidative stress and can induce mutation (Kew, 2013). In addition, these studies have shown that exposure to AFB1 alone was sufficient to significantly increase the risk of developing cancer (Rushing e Selim, 2019). Hepatitis C virus

(HCV) has also shown a correlation with incidence of the HCC, in synergy with exposure to AFB1, but is not yet fully established (Rushing e Selim, 2019).

Children are chronically exposed to high levels of aflatoxins in areas where food contamination is endemic, and this exposure begins in the uterine phase, in the fetal development, through mother's milk, and continues throughout life (IARC, 2012). AFs are considered as a risk factor for compromising children's growth (Rushing e Selim, 2019). Furthermore, studies show that AFB1 has the ability to decrease immune system functions, with changes in immunological parameters in populations chronically exposed to aflatoxins (IARC, 2012; Rushing e Selim, 2019).

2.2. Ochratoxin A (OTA)

Ochratoxin A (OTA) is the second most important mycotoxin produced by fungi *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum*. This occurs predominantly in cereals and derivatives, namely flours, bread, rice, breakfast cereals and infant feed (CAST, 2003). OTA have nephrotoxic effects associated with oxidative stress. In humans, epidemiological studies demonstrate a possible association with Balkan Endemic Nephropathy an endemic chronic interstitial nephropathy, but causal link is not yet been established (Alshannaq e Yu, 2017; Schrenk *et al.*, 2020). It is classified by the IARC as possibly carcinogenic to humans and belongs to group 2B since there is sufficient scientific evidence of carcinogenicity in animals, but human studies are still insufficient (IARC, 2012; Ostry *et al.*, 2017). Moreover, OTA is considered immunotoxic, neurotoxic, mutagenic, teratogenic, hepatotoxic and affects developmental (Bhat, Rai e Karim, 2010; Schrenk *et al.*, 2020). In 2008, JECFA reconfirmed a provisional tolerable weekly intake (PTWI) of 100 ng OTA/kg bw from 1995, and estimates that dietary exposure, mainly in Europe, ranging from 8 to 17 ng/kg bw per week are below the PTWI (Schrenk *et al.*, 2020).

OTA is a polypeptide derivative of dihydro-isocomarina, bound by 7-carboxylic group to 1-b-phenylalanine by an amide bond (**Figure 2**). Characterized by being a white crystal with a melting point of 90°C, when recrystallized with benzene, it is very soluble in polar organic solvents, moderately soluble in water and soluble in sodium hydrogencarbonate solutions. It presents absorption in ultraviolet to $\lambda_{MeOHmax}$ (ε) = 333 nm (6400) and intense native fluorescence, with a maximum emission at 467 nm in 96% ethanol (Duarte, Pena e Lino, 2010; Ringot *et al.*, 2006).

2.3. Fumonisins (FB1 and FB2)

Fumonisins are produced by the fungi *Fusarium proliferatum* and *F. verticillioides*, predominantly found in corn and derived products. Fumonisin B1 (FB1) is the most toxic fumonisin, followed by fumonisin B2 (FB2) (Ruyck *et al.*, 2015). However, it has recently been discovered that *Aspergillus niger* also produces FB2 (Abrunhosa *et al.*, 2016).

Fumonisins are characterized by a long chain hydroxylated hydrocarbon, hydroxyl groups in C14 and C15 esterified with terminal carboxylic group of tricarboxylic acid (**Figure 2**) (Abrunhosa *et al.*, 2016). They are different molecules from other mycotoxins because they are hydrophilic, dissolve completely in organic solvents such as methanol and acetonitrile:water (1:1) and do not present fluorescence (Bennett e Klich, 2003; IARC, 2012). FB1 and FB2 are structurally similar to sphingosine and sphinganin bases. They interfere with metabolism of sphingolipids, competitively inhibiting ceramide synthase enzyme, causing dysregulation in cell cycle (Abrunhosa *et al.*, 2016; Al-Jaal *et al.*, 2019; Alshannaq e Yu, 2017). These mycotoxins are considered to be possibly carcinogenic to humans, belonging to IARC Group 2B. They are associated with esophageal cancer (Bansal *et al.*, 2011). The largest target organs of these mycotoxins are the liver and the kidneys, and FB1 is carcinogenic, hepatotoxic and nephrotoxic (Alshannaq e Yu, 2017; Bansal *et al.*, 2011). In 2011, JECFA established a provisional maximum tolerable daily intake (PMTDI) for FB1, FB2 and FB3 of 0.002 mg/kg bw, alone or in combination (FAO/WHO, 2018).

2.4. Zearalenone (ZEA)

Zearalenone (ZEA) is a secondary metabolite of fungi of the genus *Fusarium*, mainly of the species *F. graminearum* and *F. culmorum* (Al-Jaal *et al.*, 2019), very common in cereals such as corn, wheat, barley, rye and their derivatives (Alshannaq e Yu, 2017).

This mycotoxin is a macrocyclic-resorcyclic acid lactone (**Figure 2**) (Alshannaq e Yu, 2017), with a similar structure to 17-β-estradiol, a human sex hormone, so ZEA is considered a non-steroidal estrogenic mycotoxin (Al-Jaal *et al.*, 2019; Alshannaq e Yu, 2017; IARC, 2012). Given this structural similarity, they have affinity for estrogen receptors and, as a consequence, leads to negative effects on the reproductive system, such as fertility problems, precocious puberty, change in serum levels of estradiol and progesterone (Abrunhosa *et al.*, 2016; Ruyck *et al.*, 2015). IARC categorized ZEA in group 3, not classified as carcinogenic to humans, since studies are limited (Ostry *et al.*, 2017). In 2000, JECFA established a provisional maximum tolerable daily intake (PMTDI) for ZEA of 0.5 μg/kg/bw (JECFA, 2017).

ZEA presents in the form of white crystals, is soluble in benzene, acetonitrile, methanol, ethanol and acetone, is very stable for degradation up to 120°C and is stable to hydrolysis in neutral or acid buffer solutions (IARC, 2012).

2.5. Trichothecenes

Trichothecenes are a group of structurally related mycotoxins produced mainly by fungi of the genus *Fusarium*. These molecules consist of a 12,13- epoxytrichothene skeleton and a double bond with several substitutions in the side chain (**Figure 2**). This group includes non-macrocyclic mycotoxins: desoxynivalenol (DON), T2 toxin and HT-2 toxin (Bennett e Klich, 2003), all classified in group 3 of IARC, due to inadequate scientific evidence in animals and lack of human studies (Ostry *et al.*, 2017). These mycotoxins are cytotoxic, interfering in synthesis of nucleotide acids and proteins and cell division (Abrunhosa *et al.*, 2016).

2.5.1. Desoxynivalenol (DON)

DON is a B-type trichothecene with carbonyl group in carbon 8 (**Figure 2**) (Bennett e Klich, 2003). Mainly produced by the species *Fusarium graminearum* and *F. culmorum*, it is very common in cereals such as wheat and corn (Ruyck *et al.*, 2015). DON is known as vomitoxin, due to its acute exposure and is linked to gastroenteritis in humans with nausea, vomiting, abdominal pain, headache, fever and also with immunosuppressive effects, mostly reported in Asia (Knutsen *et al.*, 2017). They deregulate the normal functioning of cells, by inhibiting protein synthesis, influence on signaling, differentiation and cell proliferation (Vidal *et al.*, 2013). In 2011, JECFA established PMTDI for DON and its acetylated derivatives (3-Ac-DON and 15-Ac-DON) of 1 mg/kg bw/ day, and also established an acute reference dose (ARfD) of 8 mg/kg bw (JECFA, 2017; Knutsen *et al.*, 2017). DON was later recognized as responsible for an epidemic in Japan called "red mold poisoning" due to consumption of maize and moldy wheat, whose symptoms were nausea, vomiting, diarrhea and seizures (Fletcher e Blaney, 2016). DON is characterized by white needle-shaped crystals. It is soluble in chloroform, ethanol, methanol and ethyl acetate and stable at pH 4 even at high temperatures (IARC, 2012).

2.5.2. HT-2 Toxin and T-2 toxin

HT-2 and T-2 toxins are A-type trichothecene, with a hydrogen or an ester group in lateral chain in carbon-8; the difference between these two molecules is the carbon-4-bound group: in the case of HT-2 it is a hydroxyl group, and in the case of T2 it is an acetate group (**Figure 2**) (Bennett e Klich, 2003). These mycotoxins are produced by species *Fusarium sporotrichioides* and *Fusarium poae*, found especially in oats and also in corn and wheat (Ruyck *et al.*, 2015).

HT-2 toxin (HT2) is a metabolite of T-2 toxin (T2). T-2 toxin has a haematotoxicity effect and is linked to food toxic aleukia (ATA), a condition that involves irritation of gastrointestinal tract, vomiting, diarrhea and, in the most severe cases, leukemia, anemia and even death (Abrunhosa *et al.*, 2016; Bennett e Klich, 2003). Some in vivo studies show that T2 and HT2 have anorectic effects upon short-term exposure (Knutsen *et al.*, 2017). In 2016, EFSA established a tolerable daily intake (TDI) for T2 and HT2 of 0.02 mg/kg bw/ day based on immune- and haematotoxicity of T2 (Arcella *et al.*, 2017). The EFSA Scientific report (Arcella *et al.*, 2017) shows a high chronic exposure in lower age groups.

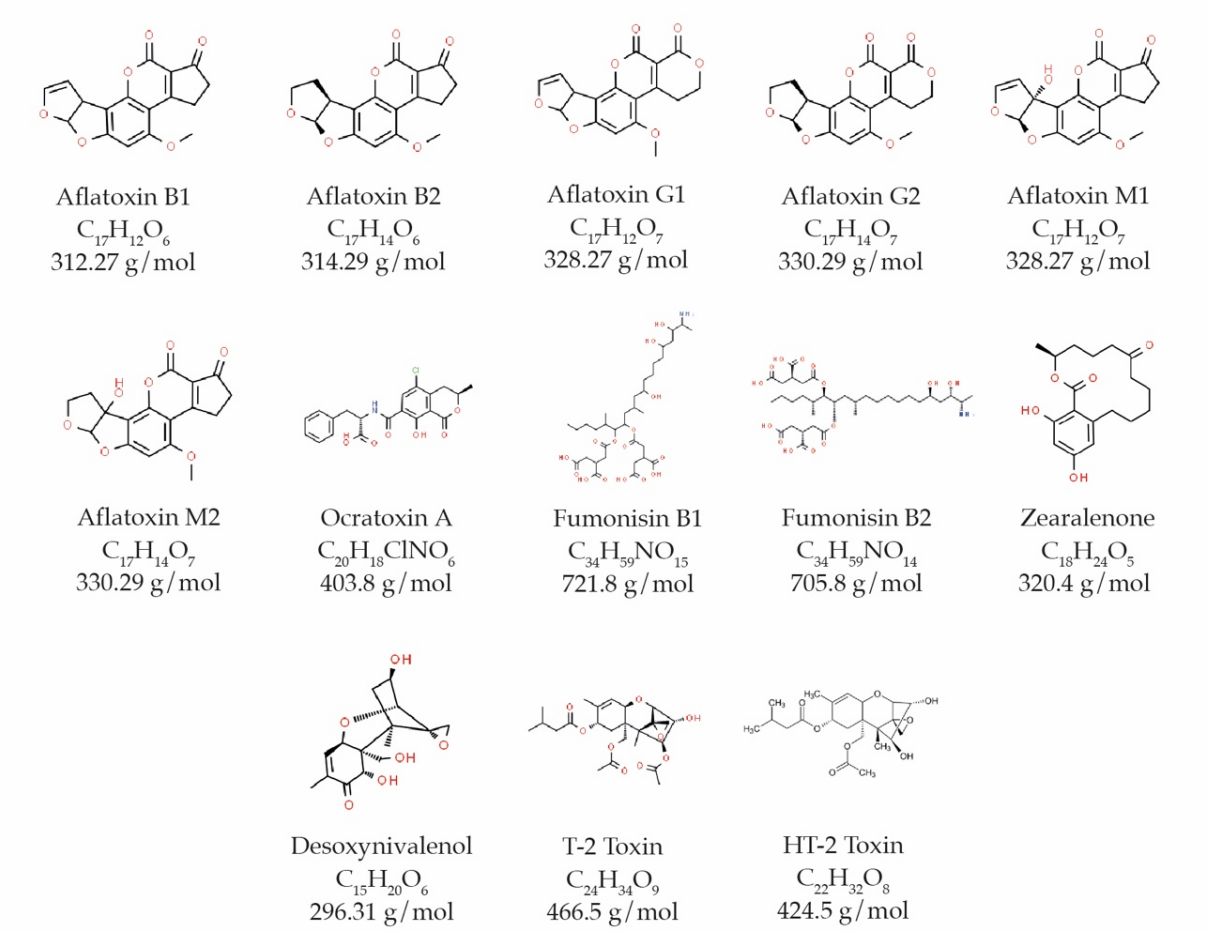


Figure 2. Examples of main mycotoxins most common determinate in foods (structures from www.chemspider.com)

2.6. Emerging mycotoxins

Besides common mycotoxins, there is also a group of emerging mycotoxins, defined as *"mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing"* (Vaclavikova *et al.*, 2013). These new mycotoxins are more usually found in cereals like wheat, maize and barley, and Mediterranean crops; determination on pistachio and other nuts are rare.

Fusarium second metabolites like fusaproliferin (FUS), beauvericin (BEA), enniatins (ENNs), and moniliformin (MON) are included in the group of emerging mycotoxins. Moreover, fusaric acid, culmorin, butanolide (Gruber-Dorninger *et al.*, 2017) and, more recently, NX-2 (Agriopoulou, Stamatelopoulou e Varzakas, 2020) are *Fusarium* emerging mycotoxins. Moniliformin (MON) was first described by Cole *et al.* (1973) isolated from the *Fusarium* strain, initially called *F. moniliforme*, which contaminated cereals like maize. MON is a small, water-soluble and very acidic molecule that occurs in nature typically as sodium or potassium salt (Jestoi, 2008). The toxicity of MON is due to inhibition of thiamine enzymes, compromising the tricarboxylic acid cycle and resulting in cytotoxic effects for lymphocytes and cardiomyocytes. Muscle weakness, breathing difficulties and myocardial lesions are reported symptoms resulting from MON exposure, based on animal studies, and the heart is the main target organ (Fraeyman *et al.*, 2017). However, MON is suspected to be associated with development of Keshan's disease, an endemic disease reported in China characterized by myocardial insufficiency (Gruber-Dorninger *et al.*, 2017; Jestoi, 2008).

Beauvericin (BEA) and Enniatins (ENNs) are structurally very similar mycotoxins found in grains and cereal based food. *Fusarium* species like *F. proliferatum*, *F. subglutinans* or *F. verticillioides* produces BEA, primarily found in 1969, and *F. avenaceum*, *F. poae*, or *F. tricinctum* produced ENNs, and ENN A, A1, B and B1 are the most commonly detected in food. *F. oxysporum* produces both mycotoxins. The toxicity of BEA and ENNs are based on their ionophore proprieties; they act as transporters for mono- or divalent cations, for example, K^+ or Ca^{2+}, resulting in disrupting of normal physiological concentrations, inducing DNA fragmentation and apoptosis. BEA and ENNs have also been demonstrated to inhibit acyl-CoA:cholesterol acyltransferase (ACAT) which causes the accumulation of cholesteryl ester in atherogenesis. BEA and ENNs have no cytotoxic *in vitro* studies and no mutagenicity in Ames test. Moreover, they show pharmacological properties, such as anticonvulsant, antineoplastic and lower cholesterol levels of blood (Jestoi, 2008). EFSA (2014) conclude that acute exposure to BEA and ENNs is not a concern to human health and since there is a lack of toxicity in *in*

vivo data, there are no conclusions concerning chronic exposure (Gruber-Dorninger *et al.*, 2017; Jestoi, 2008).

Fusaproliferin (FUS) is one of the most recent mycotoxins, discovered in 1993 by Randazzo *et al.*, so, very little is known about it yet. FUS is produced by *Fusarium proliferatum*, *F. subglutinans*, and *F. verticillioides*, occurring in grains and grain-based foodstuff (Gruber-Dorninger *et al.*, 2017). Most of the studies are in plants, insects and cells cultures. These studies indicate that FUS have phytotoxic properties, are moderately cytotoxic to human B lymphocyte, interact with DNA and show teratogenic effect (Gruber-Dorninger *et al.*, 2017; Jestoi, 2008). However, toxicity and mode of action have not been comprehensively investigated and there is still an insufficient amount of toxicity data to assess the impact on human health.

In addition *Aspergillus, Alternaria* and *Penicillium* are also fungi that produced emerging mycotoxins. Sterigmatocystin (STC) is an *Aspergillus* mycotoxin, mainly produced by *A. nidulans* and *A. versicolor*, and structurally closely related and toxic precursor to aflatoxins (Gruber-Dorninger *et al.*, 2017). Studies show mutagenicity and cytotoxic effects, with formation of DNA adducts. In 1987, IARC classified STC in group 2B (possibly carcinogenic to humans) (Battilani *et al.*, 2008). *Alternaria* mycotoxins are mostly produced by *Alternaria alternata* and included alternariol, alternariol monomethyl ether, tenuazonic acid (TeA) and altertoxins with some effects in animals (Gruber-Dorninger *et al.*, 2017; Mujahid *et al.*, 2020).

2.7. European Legislation

To ensure consumer health, the occurrence of mycotoxins is monitored, and maximum levels are regulated worldwide. In the European Union, the European Food Safety Authority (EFSA) is responsible for scientific opinions concerning risks associated with mycotoxins and advice to the European Commission (EC), which established Regulation No. 1881/2006 concerning the maximum levels of certain contaminants, including certain mycotoxins (**Table 1**). The levels of aflatoxins in foodstuffs not for direct human consumption are higher as they will still be processed. Based on the toxicity of different aflatoxins, a limit is provided for the total aflatoxins in food, corresponding to the sum AFB1, AFB2, AFG1 and AFG2), as well as the individual content of AFB1 since this is aflatoxin with the greatest concern given its carcinogenicity. Peanuts and nuts available on the market for the consumer must have a content of AFB1 less than 2 μg/kg and total aflatoxin content of less than 4 μg/kg. As aflatoxins (AFs) are carcinogenic substances, maximum levels should be imposed at a level that is as low as reasonably achievable (ALARA), defined as "*the concentration of a substance that cannot be eliminated without seriously compromising the availability of main food nutrients*" (CAST, 2003).

The maximum levels of mycotoxins are not equivalent worldwide. For example, in Canada and Australia there is a maximum limit for dried fruits and derived of 15 μg/kg; the US FDA (Food and Drug Administration) only sets a maximum level for total AFs of 20 μg/kg; in India, for all foodstuffs, the maximum limit set is 30 μg/kg. In Israel, for dried fruits, peanuts, dehydrated fruits there is a distinction at the maximum levels for AFB1 (5 μg/kg) and AFs (15 μg/kg) (Ismail *et al.*, 2018). All these limits are higher than those established in the European Union.

In Codex Alimentarius, maximum levels for total aflatoxins in treenuts, including almonds, hazelnuts, pistachios and shelled Brazil nuts, for human direct consumption are 10 μg/kg and for treenuts still to undergo further processing are 15 μg/kg. However, maximum levels of DON, FB1, FB2 and OTA in nuts are not established (FAO/WHO Codex Alimentarius Comission, 1995).

EFSA (2007) publish a scientific opinion concluding that increasing the maximum level of AFs in pistachio, almonds and hazelnuts to 8 or 10 μg/kg would increase aflatoxin exposure in 1%, with more impact in groups with a high level of nut consumption, and, despite the minor effects on cancer risk, EFSA strengthens that exposure to AFs should be low as reasonably achievable.

European legislation covers other mycotoxins, for example, OTA, but in dried fruit other than raisins, the maximum levels are not defined yet, and for DON, ZEA, FB1, FB2, T2 and HT2 there is no references of the maximum levels in nuts because, so far, there is no significant reported occurrence.

Table 1. Aflatoxins content in foodstuffs, according to Annex in Regulation No. 1881/2006 and its amendments

Foodstuffs	Maximum levels (μg/kg)	
	AFB1	**AFs**
Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs.	8.0*	15.0*
Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs.	5.0*	10.0*
Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs.	2.0*	4.0*
Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs.	5.0	10.0
Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs.	2.0	4.0

* The maximum levels shall apply to the edible part of peanuts and nuts. If whole peanuts and nuts are analyzed, when calculating the aflatoxin content, it should be assumed that all contamination is in the edible part.

3. Analytical methods for the determination of mycotoxins

Mycotoxins are present in low concentrations, in the order of μg/kg, and pistachio represent a complex food matrix, mainly due to the lipid content (53%) (Cunha, Sá e Fernandes, 2018; PortFIR, 2019). Therefore, sensitive analytical methods with low limits of detection and quantification and good specificity, precision and accuracy are needed (Mbundi *et al.*, 2014). Analysis of mycotoxins, regardless analytical method, follows a common protocol: sampling, sample preparation, extraction, with or without purification and detection/quantification (Sinha, 1999). Sample preparation is very important because this step is responsible for eliminating matrix interferents and pre-concentrate mycotoxins and transferring them to an adequate solvent for the next analytical technique (Vargas Medina *et al.*, 2021).

High-performance Liquid Chromatography (HPLC) or Ultra-High Pressure Liquid Chromatography (UHPLC) with Fluorescence detection (FLD), Mass Spectrometry (MS) or tandem Mass Spectrometry (MS/MS) are the main analytical techniques reported in the scientific literature. Other researchers have more recently used immunoassays, like Enzyme-Linked Immunosorbent Assay (ELISA) and sensor methodology. Analytical methods used for mycotoxins determination in pistachio and other related food matrices are summarized in **Table 2**. The most used analytical techniques for screening and confirmatory determination

of mycotoxins in pistachio are represented in **Figure 3**. In screening analysis, immunoassays are the most applied techniques, due to simple and rapid performance; and for confirmatory analysis, liquid chromatography is the gold standard, with distinct detectors. The validation of the analytical methodology, whether it is for screening or confirmatory, is of utmost importance in order to assure reliable data.

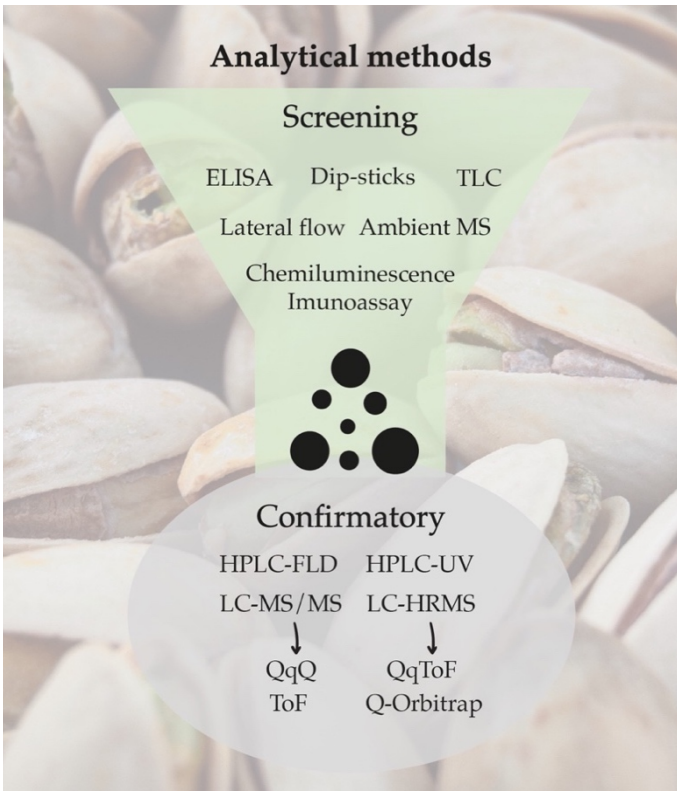


Figure 3. Main analytical methods for screening and confirmatory determination of mycotoxins in pistachios.

(ELISA - Enzyme-Linked Immunosorbent Assay; FLD - Fluorescence detector; HPLC - High-Performance Liquid Chromatography; HRMS - High-Resolution Mass Spectrometry; LC - Liquid Chromatography; MS - Mass Spectrometry; MS/MS - Tandem Mass Spectrometry; Q-Orbitrap - Quadrupole-orbital ion trap; QqQ - Triple Quadrupole; QqTOF - Double Quadrupole -TOF; TLC - Thin-layer chromatography; TOF - Time-of-flight).

Table 2. Summary of analytical methodologies used for mycotoxins determination in pistachio.

Type of sample	Analytes	Clean-up methods	Procedure of extraction	Detector	Conditions	Analytical Column	Internal Standard	LOD (μg/kg)	LOQ (μg/kg)	Ref.
Pistachio	AFB1; AFB2; AFG1; AFG2	IAC	Sample quantity: 125g Sample extraction: 475ml MeOH/H_2O/Hexane (63:16:21 v/v/v); filtration, dilution with water; IAC:10ml PBS; 75ml filtrate; wash 15ml H_2O, vacuum; elution with 0.5ml MeOH	HPLC - FLD with PCD	Mobile phase: H_2O/MeOH/ACN (42:29:17, v/v/v) Flow-rate: 1ml/min Temperature column: Injection volume: 100μl λexcitation: 365nm λemission:450mm	C18 250mmx4.6mm 5μm	-	0.1-0.4	-	Cheraghali *et al.*, 2007
Peanuts, pistachio, wheat, maize, cornflakes, raisins and figs	AFB1, AFB2, AFG1, AFG2, OTA, DON, FB1, FB2, T2, HT2, ZEA, CIT, etc.	-	Sample quantity: 25g Sample extration: 100ml ACN/H_2O (80:20 v/v), shaken 2h, diluted 1ml extract with 3ml H_2O, filtration	LC-MS/MS	Mobile phase:(A) H_2O with 0.1% FA (B) ACN with 0.1%FA Gradient program: 90%A at 0 min, 30%A at 12min, 10%A at 17.5min, 90% at 21min (t=25min) Flow-rate: 0.3ml/min. Ionization: ESI source in the positive mode Temperature column: 30°C Injection volume: 20μl Ionization: ESI source in the positive mode Capillary voltage: 2.5kV Collision gas pressure: 0.8bar Vaporizer temperature: 450°C Sheath gas pressure: Auxiliary gas flow: 600 l/h	Alltima C18 150mmx3.2mm 5μm	-	0.5-200	1-200	Spanjer, Rensen e Scholten, 2008

Dried fruits (peanuts, walnut, cashews, pistachio, almond, pecan walnut), cereals, dehydrated fruits and spices	AFB1, AFB2, AFG1, AFG2	SPME	Sample quantity: 0.5g Sample extraction: 1ml MeOH:H_2O (80:20 v/v), centrifugation, filtration of supernatant and added to in-tube SPME	HPLC – MS	Mobile phase: MeOH/ACN (60:40, v/v):5mM ammonium formate (45:55) Gradient program: After 8 min, washed with MeOH/ACN (60/40, v/v) for 2min and returned to the initial conditions in 2min Flow-rate: 1ml/min Temperature column: 40°C Injection volume: 10µl Ionization: ESI source in the positive mode Capillary voltage: 2.5kV Collision gas pressure: Vaporizer temperature: 350°C Sheath gas pressure: 30psi Auxiliary gas flow: 13L/min	Zorbax Eclipse XD8-C8 150mmx4.6mm 5µm	AFM1	0.02	0.05	Nonaka *et al.*, 2009
Pistachios	AFB1, AFB2, AFG1, AFG2, OTA	IAC	Sample quantity: 5g for AFs and 10g for OTA Sample extraction: 30ml ACN/H_2O (60:40 v/v), belnded 10 min, 2ml extract diluted with 48ml PBS; Easi-extart AF IAC for AFs and Ochraprep IAC for OTA	HPLC – FLD with PCD	Mobile phase: ACN/H_2O/acetic acid (51:47:2, v/v/v) Flow-rate: 1ml/min Temperature column: 40°C Injection volume: 100µl λexcitation: 333nm λemission: 443nm	Spherisorb ODS2 150mmx4.6mm 5µm	-	0.2	-	Fernane *et al.*, 2010

Pistachios, walnuts, cashews, almonds, peanuts, seeds, etc.	AFB1	IAC	Sample quantity: 50g Sample extraction:100ml H_2O + 4g NaCl, 150ml MeOH, filtration,5ml filtrate+25ml PBS; IAC:10ml PBS, 30ml filtrate, wash 15ml H_2O, elution 0.5ml MeOH, 1ml H_2O; filtration if solution not clear.	HPLC – FLD with PCD	Mobile phase: H_2O/MeOH/ACN (42:29:17, v/v/v) Flow-rate: 1ml/min Temperature column: 40°C Injection volume: λexcitation: 362nm λemission: 456nm	Luna C18 25cm x 4.6mm, 5μm	-	0.2	0.6	Ulca, Evcimen e Senyuva, 2010
Almonds, walnuts, sunflower seeds, sesame seeds, peanuts, pistachios, hazelnuts and cashews	AFB1, AFB2, AFG1, AFG2, AFM1, AFM2	IAC	Sample quantity: 10g Sample extraction: 33%MeOH, filtration, 500μl filtrate + 500μl 33%MeOH	ELISA	Euroclon kit Absorbance at 450nm	-	-	-	-	Shadbad *et al.*, 2012
			Sample quantity: 10g Sample extraction: 1g NaCl+ 40ml MeOH/H_2O (80:20 v/v) + 20ml n-hexane, blended for 3 min, eliminate n-hexane phase, filtration; 7ml filtrate + 43ml PBS; IAC: 10ml PBS, 50ml filtrate, wash 20ml H_2O, dried with air, elution 2ml MeOH	HPLC-FLD with PCD	Mobile phase:ACN/MeOH/H_2O (17:29:54, v/v/v) Flow-rate: 1 ml/min Temperature column: Injection volume: 20ml λexcitation: 365 nm λemission: 435nm	Hichrom ODS 250mmx4.6mm 5mm	-	0.05 – 0.42	0.19 – 1.4	
Dried fruits (peanuts, almonds, walnuts, pistachios, hazelnuts) and seeds (sunflower,	AFBl, AFB2 AFG1, AFG2, OTA, FB1, FB2, T-2, HT-2, STE, CIT,	QuEChERS	Sample quantity: 2g Sample extration: 8ml H_2O + 10ml ACN: 5%FA; 4g $MgSO_4$ + 1g NaCl + 1g sodium citrate + 0.5g disodium hydrogen citrate	UHPLC – MS/MS	Mobile phase:(A) H_2O with 0.3% FA and 5mM ammonium formate, (B) MeOH with 0.3%FA and 5mM ammonium formate Gradient program: 0min:5%B; 1min:50% B; 2min:72%B;	Zorbax Eclipse Plus RRHD 50mm x 2.1mm 1.8 μm	-	0.17-9.68	0.57-32.6	Arroyo-Manzanares *et al.*, 2013

pumpkin, pine nuts)	DON, ZEN		sesquihydrate, centrifugation; DLLME for AFs: 2ml supernatant: evaporation and 1ml MeOH/H_2O (50:50), 4ml H_2O, 0.21g NaCl; injection 950µl ACN + 620µl chloroform		4min:80%B;and 6min:90%B, finally back to 5 B in 0.2min and maintained for 1.8min for column equilibration Flow-rate: 0.4 ml/min Temperature column: 35°C Injection volume: 5µl Ionization: ESI source in the positive mode Capillary voltage: 5 kV Collision gas pressure: 30psi Vaporizer temperature: 500°C Sheath gas pressure: 50psi Auxiliary gas flow:				
Walnuts, pistachios, hazelnuts, cashews, almonds	AFB1, AFB2, AFG1, AFG2	IAC	Sample quantity: 25g Sample extraction: 5g NaCl+ 125ml MeOH/H_2O (60:40 v/v), blended for 1min, filtration; 20ml filtrate + 20ml H_2O; IAC: 10 ml filtrate diluted, wash 10ml H_2O, elution 1ml MeOH	HPLC - FLD with PCD	Mobile phase: H_2O/ACN/MeOH (6:3:1, v/v/v) Flow-rate: 1ml/min Temperature column: Injection volume: 20µl λexcitation: 360nm λemission: 440nm	Spherisorb ODS C18 150mmx4.5mm 5µm	-	0.273-0.536	0.9-1.8	Tawila, El, Neamatallah e Serdar, 2013
Cereals and nuts (almond, peanut, pistachio)	AFBI, AFB2 AFG1, AFG2, OTA, OTB, T-2, HT-2, STE, CIT,	-	Sample quantity: 1g Sample extraction: 5ml ACN/H2O (85:15 v/v), shaking for 30min in higher speed with pulsation, centrifugation, 500µl extract + 20µl ISs + 480µl 20mM	UHPLC - MS	Mobile phase:(A) H2O with 0.3% FAand 5mM ammonium formate, (B) MeOH with 0.3%FA and 5mM ammonium formate Gradient program: 100%A at 0min, increase to 100%B at 8min, until 12min, then, return to	Hypersil GOLD aQ 100 x 2.1mm -	Isotope labeled ^{13}C	-	-	Liao *et al.*, 2015

	DON, ZEN, etc.		ammonium formate, vortex and filtration.		100%A in 8.5min, equilibration for 5.5min (t=18min) Flow-rate: 0.3ml/min Temperature column: 35°C Injection volume: 5µl Ionization: ESI source in the positive mode Capillary voltage: 4kV Collision gas pressure: Vaporizer temperature: 350°C Sheath gas pressure: Auxiliary gas flow: 15 l/min					
Pistachios, peanuts and walnuts (raw and roasted with salt)	AFB1, AFB2, AFG1, AFG2	-	Sample quantity: 10g Sample extraction: 50ml 33%MeOH, filtration, dilution 1:2 with 33%MeOH	ELISA	Clone total AF ELISA test kit, Absorbance at 450nm	-	-	-	-	Ostadrahimi *et al.*, 2014
Almond, hazelnuts, peanuts, pistachio, walnuts, brazil nuts, chestnuts and apricot	AFB1, AFB2, AFG1, AFG2	IAC	Sample quantity: 25g Sample extraction: 5g NaCl+ 125ml MeOH/H_2O (60:40 v/v), blended with hight speed 1min, sediment, filtration of supernatant; 20ml filtrate + 20ml PBS; IAC:20 ml diluted filtrate, wash MeOH/H_2O (25:75 v/v), elution 2ml MeOH+3ml H_2O	HPLC – FLD with PCD	Mobile phase:H_2O/MeOH/ACN (64:23:13, v/v/v) Isocratic program Flow-rate: 1ml/min Injection volume: 100µl λexcitation: 364nm λemission: 440nm	C18 150mmx4.6mm 5µm	-	-	0.4 - 1.3	Diella *et al.*, 2018

Peanuts, almonds and pistachios	AFBl, AFB2, AFG1, AFG2, OTA, FB1, FB2, T-2, HT-2, STE, CIT, DON, ZEN, etc.	QuEChERS	Sample quantity: 5g Sample extraction:10ml H2O, 10ml ACN:FA 0.1%; 4g MgSO4 +1g NaCl + 1g sodium citrate + 0.5g disodium hydrogen citrate sesquihydrate, centrifugation; d-SPE with EMR-lipid: activation with 5ml H_2O +5ml extrat, centrifugation, 5ml supernatant + 0.4g NaCl + 1.6g $MgSO_4$, centrifugation	HPLC-MS	Mobile phase: (A) H_2O with 0.1% FA, (B) ACN with 0.1% AF Gradient program:0–5 min 4% B, 5–20 min 100% B, 20–24 min 100% B, 24–28 min 2% B and this latest rate was maintained for 10 min (t=38min) Flow-rate: 200nl/min Ionization: ESI source in the positive mode Temperature column: 25°C Injection volume: 100nl Ionization: ESI source in the positive mode Capillary voltage: 2.2kV Collision gas pressure: Vaporizer temperature: 250°C Sheath gas pressure: Auxiliary gas flow:	Easy-Spray PepMap C18 nano 150mmx75μm 3μm	-	-	0.05-5	Alcántara-Durán *et al.*, 2019
Raw peanuts and roasted pistachios	AFB1; AFB2; AFG1; AFG2; OTA	QuEChERS	Sample quantity: 2.5g Sample extration: 10mL ACN + 10mL H2O with 0.2% FA, rotation for 30 min; 4g $MgSO_4$ +1g NaCl + 1g sodium citrate + 0.5g disodium hydrogen citrate sesquihydrate, centrifugation, follow by 2 extraction with 20ml hexane; d-SPE: supernatant +150mg C18 + 900mg $MgSO_4$, centrifugation,	LC – MS/MS	Mobile phase:(A) H_2O Gradient program: Flow-rate: 0.2ml/min Temperature column: 30°C Injection volume: 4μl Ionization: ESI source in the positive mode Capillary voltage: Collision gas pressure: 25psi Vaporizer temperature: 250°C	ODS C18 150mmx2.1mm 5μm	-	0.05-0.10	0.08-0.30	Alsharif, Choo e Tan, 2019

			wash 2x with 5mL ACN		Sheath gas pressure: Auxiliary gas flow: 14 L/min					
Almonds, hazelnuts, peanuts, pistachios, walnuts	AFB1, AFB2, AFG1, AFG2; ZEA	QuEChERS	Sample quantity: 2g Sample extraction: 10ml ACN/H_2O (80:20 v/v), rotation for 20 min, 4g Na2SO4 + 1g NaCl, centrifugation; d-SPE: 3ml supernant+100mg C18, centrifugation	UHPLC – MS/MS	Mobile phase: (A) H_2O with 0.1% FA, (B) ACN with 0.1% FA Gradient program: 25%A increased to 100% in 3.75min, reduction to 25%A in 6min (t=7.5min) Flow-rate: 0.2ml/min Ionization: ESI source in the negative mode Temperature column: 25°C Injection volume: 5µl Ionization: ESI source in the positive mode for AFs Capillary voltage: 3.5kV Collision gas pressure: 45psi Vaporizer temperature: 400°C Sheath gas pressure: Auxiliary gas flow: 11 L/min	C18, 100mmx2.1m, 1.8µm	-	-	0.5-1.0	Hidalgo-Ruiz *et al.*, 2019

Almonds, hazelnuts and pistachios	AFBI, AFB2 AFG1, AFG2, OTA, OTB, T-2, HT-2, STE, CIT, DON, ZEN, etc.	QuEChERS	Sample quantity: 1g Sample extraction: 5ml H_2O, 5mL ACN with 0,1%FA; 0.5g NaCl + 2 g $MgSO_4$, centrifugation; d-SPE: 1.5ml supernatant+ 50mg C18, centrifugation	UHPLC – MS	Mobile phase: (A) H_2O with 0.1% FA, (B)MeOH with 0.1% FA Gradient program:0%B for 1min, 95%B for 1.5min, 75%B for 2.5min, decrease to 60% in 1min, back to 0%B in 0,5min and held for 1.5min (t=8 min) Flow-rate:0,4 ml/min Ionization: ESI source in the posiive and negative mode Temperature column: 30°C Injection volume: 5 μl Ionization: ESI source in the positive and negative mode Capillary voltage: ± 4kV Collision gas pressure: Vaporizer temperature: 290°C Sheath gas pressure: 35 psi Auxiliary gas flow:	Luna Omega Polar C18, 50mmx2.1mm, 1.6μm	-	-	0.2–0.78	Narváez *et al.*, 2020

ACN - acetonitrile; **ADONs** - Sum of 3-acetyl and 15-acetyl-deoxynivalenol; **AFB1** - Aflatoxin B1; **AFB2** - Aflatoxin B2; **AFG1** - Aflatoxin G1; **AFG2** - Aflatoxin G2; **AFM1** - Aflatoxin M1; **AFM2** - Aflatoxin M2; **CIT** - Citrinin; **ELISA** - Enzyme-Linked Immunosorbent Assay; **ESI** - electrospray ionization; **FA** - formic acid; **FB1/FB2** - fumonisins; **HPLC-FLD** - High performance liquid chromatography with Fluorescence Detection; **IAC** - Immunoaffinity columns; **LC – MS** - Liquid Chromatography Mass Spectrometry; **OTA** - Ochratoxin A; **OTB** - Ochratoxin B; **MeOH** - methanol; **PCD** - post column derivatization; **PBS** - phosphate buffer saline; **QuEChERS** - Quick, Easy, Cheap, Effective, Rugged and Safe; **STE** - sterigmatocystin; **UHPLC-MS** - Ultra-High-Performance Liquid Chromatography tandem Mass Spectrometry; **SPME** - Solid-Phase Microextraction; **T-2/HT-2** - Trichothecenes; **ZEA** - Zearalenone.

3.1. Sample preparation

Mycotoxins are distributed heterogeneously and may only occur in a fraction of sample (Zheng, Richard e Binder, 2006). Thus, sampling and preparation of sample are crucial steps in the determination of these chemical contaminants, to ensure representativeness.

For determination of AFs in nuts "ready-to-eat", Codex Alimentarius recommend a sample of 10 kg of pistachio in-shell nuts or 5 kg shelled nuts, and the sample should be finely ground and mixed thoroughly using a process, to reduce particular size and disperse the contaminated particles evenly throughout the sample, ensuring homogenization, since distribution of aflatoxin and other mycotoxins is extremely non-homogeneous (FAO/WHO Codex Alimentarius Comission, 1995). During sample preparation, it is important to keep samples away from sunlight and also control temperature and humidity, in order to not favor mold growth and aflatoxin formation (FAO/WHO Codex Alimentarius Comission, 1995). Pre-treatment of sample is considered a fundamental and indispensable step in almost all analytical procedures, especially for analysis in complex food matrices (Yang *et al.*, 2020).

In Europe, sampling and analysis methods for the official control of the mycotoxins in food are established Regulation No. 401/2006. To analyze AFB1 and AFs in pistachios an overall sample of 30 kg is recommended, resulting from 10 to 100 elementary samples collected from different points of one lot, depending on the lot's weight. This sample is mixed and divided into two or three equal samples for laboratory with ≤10 kg before crushing. Then, each laboratory sample is separately finely ground and carefully mixed to ensure complete homogenization. In the case of lots in retail packaging, each package could be considered as one sample for analysis when it is less than 300 g.

The first step in sample preparation is extracting mycotoxins from the solid matrix to a liquid phase, separating them from other components. The extraction solvent is a mixture of an organic solvent with water, where the presence of water favors penetration of organic solvents into a matrix, and, in some cases, acids are used to break the bond of mycotoxins to other components, increasing the effectiveness of extraction (Yang *et al.*, 2020). The extraction solvent is chosen according to the characteristics of mycotoxins and matrices (FAO, 1990), and acetonitrile (ACN) is the organic solvent extraction more applied, follow by methanol (MeOH). Moreover, sodium chloride (NaCl) and n-hexane are usually added, in addition to solvent methanol:water (Shadbad *et al.*, 2012), due to higher fat content of pistachio.

The second step is clean-up to remove the interferers and impurities from the extract, such as lipids, proteins and other small molecules, to ensure sensitivity and selectivity. Solid-

liquid extraction techniques are often used, namely solid phase extraction (SPE), solid phase micro-extraction (SPME) and solid phase matrix dispersion (MSPD). However, in the pistachio nuts, researchers use immunoaffinity chromatographic columns (IACs) and the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method.

IAC is a very sensitive and selective technique because specific antibodies are used for mycotoxins. Affinity of antibody and reversibility of binding are very important because the aflatoxin-antibody complex has to be dissociated to release mycotoxins in the elution phase. The complex has to be stable enough for the washing steps (Katz e Siewierski, 1992). In a simplified way, the sample is applied into a column with anti-mycotoxins antibodies; then, the column washes, and the final step is the elution of mycotoxins. The eluate is evaporated until dryness to reduce volume and concentrate mycotoxin in the extract. Finally, the residue is redissolved into the mobile phase to follow chromatography analysis (Laranjeiro, Lino e Pena, 2015).

QuEChERS method, in a simplified way, is divided in two extraction stages. The first extraction step is based on the salting-out effect, with an organic phase in the presence of salts for extraction. Acetonitrile (ACN) is the most used extraction solvent, applicable to a wide range of organic compounds, without co-extraction of interferent molecules from the matrix (Pereira, Fernandes e Cunha, 2014) and easily parts from water in second phase (Perestrelo *et al.*, 2019). To increase efficiency, acidification with formic acid (FA) (Alcántara-Durán *et al.*, 2019; Arroyo-Manzanares *et al.*, 2013; Cunha, Sá e Fernandes, 2018), acetic acid (Bessaire *et al.*, 2019; Desmarchelier *et al.*, 2014) or citric acid (Wang, 2018) is frequently applied. In case of mycotoxins, combination of magnesium sulfate ($MgSO_4$) with sodium chloride (NaCl) in a 4:1 ratio is the most applied extraction salt. Magnesium sulfate allows the best salting-out of ACN and the best overall recoveries especially of polar analytes; however, $MgSO_4$ contributes to remaining parts of water in the acetonitrilic layer, so it helps to control the polarity of the extraction solvents and thus increases the selectivity of extraction (Rejczak e Tuzimski, 2015). In a second phase, the extract is cleaned with adsorbents to remove interferers. Generally, dispersive Solid Phase Extraction (d-SPE) is applied with primary secondary amine (PSA), octadecyl silica (C18) or graphitized carbon black (GCB) (Perestrelo *et al.*, 2019). More recently, new adsorbents have been available on the market, for example, Enhanced Matrix Removal-Lipid (EMR-Lipid) and Z-Sep. Alcántara-Dúran *et al.* (2019) compares two adsorbents: (1) EMR-lipid, remove lipids based on hydrophobic interactions and exclusion by size between long aliphatic chains of lipids and adsorbent (Perestrelo *et al.*, 2019); (2) PSA, which is useful for removing lipids, namely fatty acids, sugars, organic acids, and some pigments

and (3) C18, which is recommended for removal of high lipid content (Perestrelo *et al.*, 2019). The authors concluded that EMR-lipid presented the best results, with better percentage of recovery and lower matrix effect. Cunha *et al.* (2018) performed a clean-up with Z-sep$^+$ and C18. Z-sep$^+$ is composed of C18 and zirconia oxide bound to the same silica particle, removing fatty acids and pigments (Perestrelo *et al.*, 2019). Some authors select immunoaffinity chromatography for sample cleaning (Bessaire *et al.*, 2019; Desmarchelier *et al.*, 2014).

QuEChERS has numerous advantages like reduction of the steps; simple and easy implementation; separation of a wide range of analytes and several samples in a short time; and use a smaller volume of samples and solvents, according to the principles of green chemistry (Cunha, Sá e Fernandes, 2018; Perestrelo *et al.*, 2019; Yang *et al.*, 2020). In addition, QuEChERS is also used in multiclass analysis with simultaneous analysis of multi-mycotoxin and multi-pesticides residues, for example, in cereals (Lacina *et al.*, 2012; Romero-González *et al.*, 2011; Zhang, Wu e Lu, 2013).

3.2. Detection and quantification

3.2.1. Chromatographic techniques

Chromatographic methods are based on the physical interaction between a mobile and stationary phase. Analytes are differently distributed between two phases, depending on their characteristics, resulting in different speed movement in the column, causing separation (Wacoo *et al.*, 2014). Thin-layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC) are used for analysis of mycotoxins. TLC is more use for specific identification of mycotoxins. GC was abandoned because it needs a derivatization step due to most of mycotoxins are being nonvolatile and polar substances.

In the case of confirmatory identity and quantitative determination of mycotoxins, namely in nuts, liquid chromatography (LC) is the most common technique, given its high precision, high sensitivity and low detection limit (Vargas Medina *et al.*, 2021). While reversed-phase elution and C18 columns are the mostly used, LC mycotoxins analysis is a flexible technique; it can use different elution modes, different column sizes, different particular sizes and different mobile phases compositions in order to improve mycotoxin separation. In recent years, different approaches have been applied to LC mycotoxin analysis, improving efficiency, and resolution, making it faster and cheaper. For example, the reduction of particle size or column diameter results in ultra-high liquid pressure chromatography (UHPLC) and capillary/nano-LC, respectively. Moreover, coupling two or more separation columns or using

enrichment/extraction first column to online sample preparation are new strategies (Vargas Medina *et al.*, 2021).

Previously, LC was combined with ultraviolet-visible detector (UV-Vis) and fluorescence detector (FLD) for AFs analysis due to their fluorescent properties (AFB1 and AFB2 exhibit fluorescence at 425 nm, AFG1 and AFG2 exhibit fluorescence at 450 nm); however, quenching occurred due to the mobile phase, hindering detection of AFs at lower concentrations, requiring derivatization (Zhang e Banerjee, 2020). For AF determination with FLD, derivatization step (pre- or post-column) is needed to promote sensitivity and resolution. Chemical derivatizations involve chemical reaction between AF and acid (trifluoroacetic acid) or halogen (bromine or iodine) molecules to improve fluorescence. Photochemical derivatization is based on derivatization of AF with UV radiation generated by a photochemical reactor, and there is no need to add any chemical reagents, which is more advantageous (Wacoo *et al.*, 2014; Yang *et al.*, 2020; Zhang e Banerjee, 2020). Photochemical derivatization is the most reported PCD for the determination of AFs in pistachios (Diella *et al.*, 2018; Fernane *et al.*, 2010; Shadbad *et al.*, 2012; Tawila, El, Neamatallah e Serdar, 2013), although some previously studies use bromination (Cheraghali *et al.*, 2007; Ulca, Evcimen e Senyuva, 2010). However, this derivatization step, especially with chemical derivatization, added complexity to analysis. In addition, other mycotoxins do not have these fluorescence proprieties, so this detection method is not suitable for multi-mycotoxins determination.

More recently, mass spectrometry (MS) was coupled as a detector, resulting in LC-MS based on a separation of analytes by LC and subsequent analysis of mass to charge (m/z) of ions in the gas phase, obtaining structural information that identifies molecules based on molecular weight (Wilson e Walker, 2010; Zhang e Banerjee, 2020). Nowadays, LC-MS is the most suitable technique recommended by the guidelines for identification, quantification and confirmation of multi-class mycotoxins, being highly sensitive and specific and one of the best options for this type of analytical determination in complex food matrices (Zhang e Banerjee, 2020). LC-tandem mass spectrometry (LC-MS/MS) is a powerful technique for mycotoxins because of its ability to detect multiple regulated, unregulated and emerging mycotoxins, with a need of precursors ions to correct identification and quantification (Vargas Medina *et al.*, 2021). LC-MS can be performed employing different MS analyzers to increase detection abilities, and provide different information and data treatment and emerging LC-High-Resolution Mass Spectrometry (HRMS). For example, there are classical, like triple quadrupole (QqQ) and time-of-flight (ToF), or hybrid modern detectors such as QqToF (double quadrupole-ToF) or Q-orbitrap (quadrupole-orbital ion trap) (Vargas Medina *et al.*, 2021).

While exhibiting high sensitivity, selectivity and mass accuracy, LC-HRMS is a very high-cost technique, and needs recurrent maintenance and to be regularly calibrated to maintain the high mass accuracy and resolution. In addition, its application depends on the training of users and data file storage because, when using HRMS in full scan mode for large numbers of samples, lots of information must be processed and stored (Zhang e Banerjee, 2020).

Recently, multi-mycotoxin methods have been developed to determinate a greater number of mycotoxins in a single chromatographic run. This progress is relevant since one food item may be contaminated by a fungus that produces different mycotoxins or can be contaminated by more than one species of fungus, resulting in co-occurrence (Malik, Blasco e Picó, 2010; Mbundi *et al.*, 2014). However, one of the challenges is the matrix effect; the signal is often suppressed due to co-elution with matrix components. Matrix-matched calibration, the addition of standard or use of internal standard are some of the solutions. Matrix-matched calibration uses calibration standards for fortifying "blank" samples (without mycotoxins of interest), with the addition of known mycotoxins concentration, and it is expected that the impact of matrix effect on the response of mycotoxins is similar in calibration and samples (Zhang e Banerjee, 2020). The internal standard (IS) allows greater flexibility in extraction techniques and conditions since it has previously been added to the sample. Moreover, IS allows correction of signal variations, measuring the relative response ratio between a mycotoxin and IS and associated recovery of method to final result (Zhang *et al.*, 2014). Some of the most commonly used IS in AFs determination are isotopes, such as ^{13}C-aflatoxin, and deuterated aflatoxin, since they will have characteristics similar to AF (Zhang e Banerjee, 2020). However, for correct analyses of multi-mycotoxins, a labelled compound for every single mycotoxin of interest should be use. Zearalanone (ZAN) is also an internal standard widely used (Berthiller *et al.*, 2005; Silva *et al.*, 2019; Spanjer, Rensen e Scholten, 2008), with chemical structure and chemical behavior during extraction and analysis similar to mycotoxins, but there is a risk of naturally contamination of sample.

In the scientific literature (**Table 2**), the widely used analytical column is C18 with 150 x 4.6mm, and particle size of 5 mm. Most recent studies with UHPLC used sub-2 mm diameter particles and permitted the reduction of LC column length to 100 x 2.1mm (Hidalgo-Ruiz *et al.*, 2019; Liao *et al.*, 2015) and 50 x 2.1mm (Arroyo-Manzanares *et al.*, 2013; Narváez *et al.*, 2020). Towards the mobile phase, the most used solvents are water, acetonitrile and methanol, with the addition of formic acid, acetic acid or ammonium formate, in different proportions and mixtures. Regarding LOD, the methods just for AFs present lower LODs, as Nonaka *et al.* (2009) with the lower LOD of 0.02 μg/kg, also Shadbad *et al.* (2012) and Alsharif

et al. (2019) with 0.05 μm/kg. Concerning multi-mycotoxins methods, the lowest LOD is 0.17 μg/kg from Arroyo–Manzanares *et al.* (2013), and lowest LOQ is 0.05 μg/kg from Alcántara-Durán *et al.* (2019).

Chromatographic techniques have been commonly used in the determination of aflatoxins and other mycotoxins, with good results, in particular excellent sensitivity and the ability to detect multiple analytes in low levels in complex matrices, but require expensive equipment and trained personnel and high maintenance costs, and may not be a technique accessible to all countries and/or laboratories (Chu, 1984; Wacoo *et al.*, 2014). There is a need to develop faster, cheaper and simpler methods (Zheng, Richard e Binder, 2006), to improve and facilitate the control of mycotoxins in order to ensure food safety.

3.2.2. Immunoassays

Immunochemical methods are emerging as new methods for the determination of mycotoxins, based on the specific and high affinity reaction between the antigen (the target (bio)analyte) and antibody (Chu, 1984). Enzyme linked immuno-sorbent assay (ELISA) is one of the immunoassays with antibodies fixed on a solid base, able to distinguish the three-dimensional structure mycotoxins, causing the specific bond (Zheng, Richard e Binder, 2006). This technique requires antibodies produced by immunizing animals with mycotoxins, including rabbits and goats. However, mycotoxins with low molecular weight do not produce immune response by themselves. Therefore, mycotoxins are conjugated with a carrier protein or polypeptide before immunization in order to stimulate immunological response and production of antibodies (Chu, 1984). Conjugation depends on the chemical structure and the functional groups of mycotoxins. AFs do not have a reactive group, so a carboxylic group is primarily introduced (Chu, 1984) and later conjugation with bovine serum albumin-BSA (Turner *et al.*, 2015). Cross-reactivity of antibodies, that is, the ability of antibodies to react with other antibodies, influences the accuracy of the assay (Sinha, 1999). For example, Leszczyńska *et al.* (2018) demonstrated that all antibodies used to determine total aflatoxins tested positive for cross-reactivity (AFB1 100%, AFB2 200%, AFG1 15%, AFG2 16%, AFM1 63%). Most of monoclonal antibodies produced against AFs are highly specific to AFB1 and have a partial cross-reaction with AFG1 (Mehan, V.K., and Gowda, 1997). Other compounds with similar chemical groups can also interact with antibodies, due to low molecular weight, resulting in underestimations or overestimates (Zheng, Richard e Binder, 2006).

ELISA has two main steps: (1) reaction between antibody and antigen and (2) enzymatic reaction between enzyme and substrate. The assay occurs in a well of a test plate, which

contains antibodies selective to antigen of interest immobilized in a solid phase. Then, another antibody, conjugated with an enzyme, binds to immobilized antigens. The enzyme substrate is added, and a reaction occurs that involves color change measured and compared with calibration curves, allowing quantification of antigens (Wilson e Walker, 2010).

There are variations of this assay, depending on the characteristics of the antigen and matrix. Competitive ELISA assay is based on competition for antibody binding sites(Sinha, 1999). There are two versions of competitive ELISA: direct and indirect (**Figure 4**).

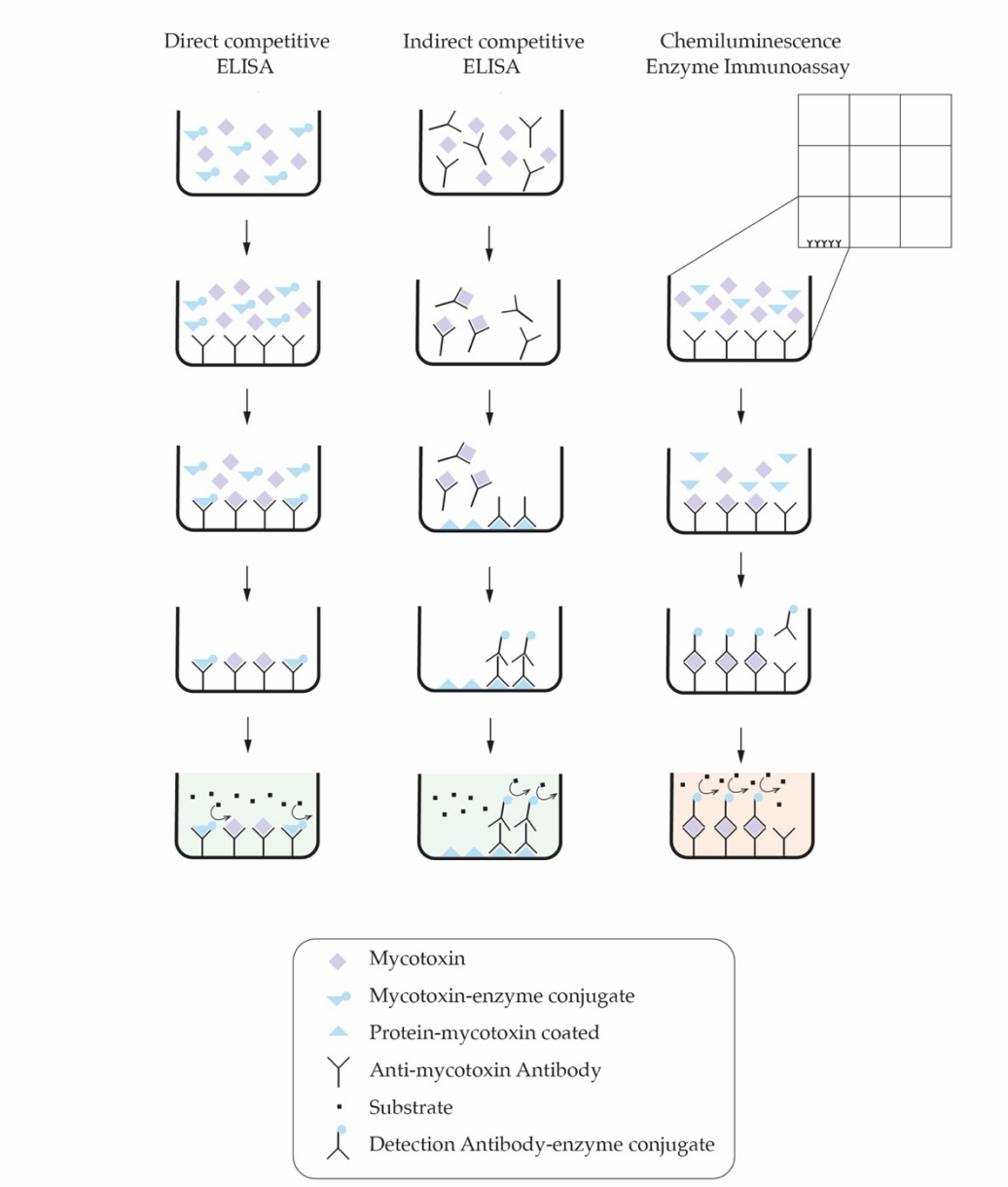


Figure 4. Schematic illustration of Immunoassays: (a) Direct competitive ELISA; (b) Indirect Competitive ELISA and (c) Chemiluminescence Enzyme Immunoassay.

Direct ELISA uses a mycotoxin-enzyme conjugate that competes for the available spaces on the coating antibody layer, while indirect ELISA involves a protein-mycotoxin conjugate immobilized on the microplate that competes with mycotoxin present in the sample (Sinha, 1999; Wilson e Walker, 2010). The most commonly used enzyme is horseradish peroxidase (HRP) and alkaline phosphatase (ALP) (Wacoo *et al.*, 2014; Zheng, Richard e Binder, 2006). In direct competitive ELISA, the sample solution or mycotoxin standards are mixed with a mycotoxin coupled enzyme and are added to wells coated with antibody. Thus, there is competition of mycotoxins with mycotoxin conjugated by binding to the antibody. This is followed by a washing step to remove any unbound enzyme conjugate. After that, an enzymatic substrate is added; enzyme converts substrate into a color product. The reaction is interrupted by adding a stop solution and color intensity is measured spectrophotometric with an absorbance filter of 450 nm (Ono *et al.*, 2017; Wilson e Walker, 2010; Zheng, Richard e Binder, 2006). In indirect competitive ELISA, antibody is added with sample solution containing mycotoxins. Next, the solution is added to wells coated with protein-mycotoxin conjugate, and the remaining free antibodies bind to mycotoxins in wells. After washing, a second antibody labelled with an enzyme detected the first antibody (Ono *et al.*, 2017; Wilson e Walker, 2010; Zheng, Richard e Binder, 2006). Then, the enzymatic substrate is added, and the enzyme converts the substrate into a color product. In these assays, color intensity is inversely proportional to the concentration of mycotoxins in the sample (Zheng, Richard e Binder, 2006); that is, the higher concentration of mycotoxin, the lower signal generated, since there is less mycotoxin conjugated with the enzyme or less second antibody labelled with an enzyme.

While direct ELISA uses a single conjugate, requires one less incubation step and, consequently, one less washing step (Mehan, V.K., and Gowda, 1997), indirect ELISA is more sensitive and flexible since more than one second antibody can be bound per primary antibody (Wilson e Walker, 2010). On the market, ELISA kits based on the direct competitive assay for the test of aflatoxins in different food matrices are already available, including in nuts. In pistachio nuts, ELISA is used for rapid methods for mycotoxins detection. Lee *et al.* (2004) developed rapid direct competitive ELISA for monitoring aflatoxin AFB1 at 10 μg/kg in pistachio and other nuts and cereals. Bensassi *et al.* (2010) studied the contamination of pistachio nuts in two years of storage, screening levels of AFB1 by ELISA combined with an immunoaffinity step. Some biosensors based on indirect competitive immunoassay for detection of AFB1 have been developed for different matrices, like cereals (Kong *et al.*, 2017; Sapsford *et al.*, 2006), and peanuts (Azri *et al.*, 2018; Sapsford *et al.*, 2006).

Several studies have compared the determination of mycotoxins by ELISA and HPLC method, since HPLC is considered a reference method and widely used (Wacoo *et al.*, 2014). For example, Azer & Cooper (1991) analyzed 178 food samples for total aflatoxins, including nut and nut products, obtaining a determination coefficient of 0.999, i.e., there is a high degree of agreement between the two methods. It should also be noted that the ELISA method demonstrated a high degree of precision, useful for rapid testing, in a concentration range of 15 to 50 μg/kg. Moreover, Shadbad *et al.* (2012) and Ostadrahimi *et al.* (2014) used the ELISA method to determinate AFs in pistachio and other nuts, and the results were favorably confirmed by HPLC. Contrary to HPLC, ELISA is not useful in multi-mycotoxin determination because it requires different assays with different antibodies specific to each mycotoxin (Bensassi *et al.*, 2010; Lee *et al.*, 2004) or group of mycotoxins (Xu *et al.*, 2016), becoming more expensive and more time consuming.

3.2.3. Biosensors

The chromatographic methods are expensive and require trained personnel and procedures are, in general, complex and slow for multiclass residues. For these reasons, a new technology is necessary to detect simultaneously detect different compounds including mycotoxins.

In general (bio)sensors provide fast, reliable screening, with good sensitivity and selectivity, and low detection limits and are relatively economic, especially if applied to a large number of routine analysis. The detection of mycotoxins by biosensors mostly relies in two types of detection methods: optical and electrochemical (Yang *et al.*, 2020). The current trend are the optical biosensors based on chemiluminescent methods, which can be divided into CLIA (Chemiluminescent immunoassay) and CLEIA (chemiluminescent enzyme immunoassay) (**Figure 4**).

CLIA detection is the result of a very selective (bio)chemical reaction between the antigen (the target (bio)analyte) and an antibody specific to detection of the target (bio)analyte. The reaction mechanism is based on oxidation and reduction reactions that yield changes in chemiluminescence, depending on the amount of target analyte that can be monitored by optical detection methods. The most commonly utilized chemiluminescent (CL) compound in aqueous solution is luminol or isoluminol. In the presence of a catalyst (enzyme, metal-containing molecule or metal), luminol interacts with hydrogen peroxide in alkaline solution to produce 3-aminophthalate in an excited electronic state, which returns to the ground state with the emission of light. The signal is then detected by an optical detection system. To increase the lifetime and the amplitude of the signal, a substance known as an "enhancer" (for

example, 4-iodophenol) is added to the reaction medium. At the end of an immunoenzymatic experiment, this luminous reaction can be used to detect antigen–antibody binding (Rahman *et al.*, 2019).

CLEIA (combines chemiluminescence (CL) and enzyme immunoassay) detection techniques are currently the most sensitive in immunoassay research. CLEIA is becoming increasingly popular for the detection of trace compounds due to its great qualities of high specificity, lower limit of detection, good linearity range and environmental friendliness (Yu *et al.*, 2016). The main two label enzymes used in CLEIA are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). Due to the low cost and the ease of access, horseradish peroxidase is considered the most used. While the luminescence efficiency of the horseradish peroxidase system can be increased by using a suitable enhancer (Yu *et al.*, 2016), it is quite poor when compared to the ALP system (Yu *et al.*, 2016). In any case, CL substrates, such as the luminol/peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase, can efficiently detect enzyme labels.

One of the advantages of the CLEIAS is the possibility of application of advanced nanotechnology. For example, Freitas, Barros, Brites, Barbosa, and Silva (2019) used the Evidence Investigator Biochip Array Technology (BAT) (Randox, Crumlin, UK) in a semi-quantitative methodology in the analysis of mycotoxins in maize. In this case, Biochips were used, composed of 9 mm square-shaped solid substrate with a panel of discrete test regions (DTR) where each DTR consists of different antibodies or other reactive species specific (multiplexing) to each assay. The advantage of being able to detect and semi-quantify, in a single analysis, multiple analytes, makes CLEIA a powerful screening tool in several matrices.

While (bio)sensors are a trend and numerous have been developed during the last years, there is a lack for application of this methodology to determinate mycotoxins, especially in pistachio. Kumaniaris *et al.* (2020) developed an electrochemical immunosensor for the determination of AFB1 in pistachio based on the immobilization of the AFB1 antibodies on the surface of gold screen printed electrodes. This method presented good sensitivity (LOD=1 ng/mL) showing potential as a screening method, but also as a quantitative method since it successfully determines AFB1 concentrations in the range of 4.56–50.86 ng/mL in unknown pistachio samples.

Spectroscopy techniques have been applied for rapid and real-time analysis for mycotoxins, with little or no sample preparation, without destroying the sample(Orina, Manley e Williams, 2017). Paghaleh *et al.* (2015) developed a method based on the laser induced fluorescence spectroscopy, using a UV laser (λ = 308 nm) for in line measurement of the concentration of

AFs in pistachio nuts, without sample preparation, and results are in agreement with the HPLC method. Wu and Xu (2019) developed a multiplexing fiber optic laser induced fluorescence spectroscopy for detection of AFB1 in pistachios, using five wavelengths between 440 and 564 nm because physical and chemical characteristics of pistachios at different positions of contaminated products are unequal or nonuniform. Results show an accuracy of 97% and low levels of AFB1 (50 ppb). Valasi *et al.* (2021) used diffuse reflectance infrared Fourier transform spectroscopy with chemometrics for screening AFs in pistachios using four spectral regions to classify AF-contaminated from non-contaminated pistachios and results show that this methodology correctly separated 80% of test samples.

4. Occurrence of mycotoxins in pistachios

In pistachio nuts, AFs are the most frequently found mycotoxins (**Table 3**). The occurrence of AF contamination is sporadic and very dependent on environmental conditions (CAST, 2003). In nuts, FAO indicates that *Aspergillus flavus* and *A. parasiticus* do not grow or produce aflatoxins at temperatures below 10°C, relative humidity below 70% and water activities (aw) lower than 0.7 (JECFA, 2005). According to Baazeem *et al.* (2021), *A. flavus* grows in pistachio when incubated at between 25 to 35°C and with aw ranging from 0.95 to 0.98, in vitro and in situ studies, but AFB1 was optimum produced at 30°C and aw >0.98. These mycotoxins are predominant in Africa, Asia and North and South America, where environmental conditions are more favorable. However, due to globalization and climate change, AFs can be found all over the world (Miraglia *et al.*, 2009). Besides nuts, AFs occur in various other foods, namely cereals (corn, rice, wheat), spices (pepper, turmeric, ginger), oilseeds (peanuts, soybeans, sunflower), legumes, among others (Bhat, Rai e Karim, 2010).

AFs were first identified in England, in the 1960s, where an outbreak arose, known as "Turkey X disease", which caused the death of more than 100,000 turkeys due to consumption of peanut flour contaminated by fungi, namely species such as *Aspergillus flavus* and aflatoxins (Aiko e Mehta, 2015). The first outbreak of aflatoxicosis in humans occurred in 1974 in India, caused 106 deaths due to consumption of contaminated maize from environmental causes that occurred before harvest (Benkerroum, 2020). In Kenya, in 2004, one of the largest and most severe outbreaks of aflatoxicosis occurred in humans, which caused the death of 125 people due to liver failures due to consumption of contaminated maize, with more than 300 cases of abdominal pain, pulmonary edema and liver necrosis (Probst, Njapau e Cotty, 2007).This outbreak was due to incorrect storage of maize in a humid and hot environment, providing

for the growth of fungi, combined with a poor diet amoung the low socio-economic population and also a lack of medical resources (Tan, 2020).

The vast majority of studies summarized in **Table 3** present high values of positive samples; however, sampling is reduced and may not be representative of the global market. Cheraghali *et al.´s* (2007) study comprises a greater number of samples, collected between March 2002 and February 2003 in Iran; 37% of samples were contaminated with AFB1 and 11.8% were above maximum levels in country (5 μg/kg), higher than that legislated in Portugal and Europe. About 28% of the samples were contaminated with all AFs. AFB1 is the most frequently found and most concentrated. In some samples, the maximum levels were exceeded, constituting a risk to the health of the population, particularly in the study by Diella *et al.* (2018) which presented the highest levels of AFB1 and sum of AFs, El Tawila *et al.* (2013), Alsharif *et al.* (2019) and Cheraghali *et al.* (2007). El Tawila *et al.* (2013) showed that AFB1 content in pistachios nuts has the highest amplitude, ranging from 1.9 to 411 μg/kg, and in the study by Diella *et al.* (2018) values of AFs are between 8.8 and 387.3 μg/Kg.

In Europe, the occurrence data on food as submitted to EFSA, resulting from samples collected between 2003 and 2018 to reflect the current contamination levels in European countries, show that the food category "Legumes, nuts and oilseeds" is one of the greatest contributors to dietary exposure to AFs and AFB1, and the highest AF mean concentration are in pistachio, peanuts and other seeds (Schrenk *et al.*, 2020). Previously, pistachio also had the highest level of AFs compared with other tree nuts (EFSA, 2007). In Iran, the main producing country, the mean concentrations of AFs in pistachio was 54 μg/kg and considering the maximum level of 4 μg/kg and 20 μg/kg, 40 and 60% of pistachio samples were rejected, respectively (JECFA, 2007). JECFA conclude that pistachios were the main contributor to dietary AF exposure from tree nuts, ranging from 0.2 to 0.8 ng/kg bw per day (JECFA, 2007).

Table 3. Occurrence of mycotoxins in pistachios worldwide.

Reference	Country	Number samples	Myctoxin	N° positive samples	Positive samples (%)	Average concentration (μg/Kg)	Min-max (μg/Kg)
Cheraghali *et al.*, 2007	Iran	10 068	AFB1	3 699	37	5.9	-
			AFs	2 852	28	7.3	-
Fernane *et al.*, 2010	Algeria	31	AFs	2	6	-	0.4 - 0.7
			OTA	1	3	170	-
Coronel *et al.*, 2012	Spain	70	OTA	2	3	0.228	0.134 - 0.321
Shadbad *et al.*, 2012	Iran	32	AFB1	17	53	-	9.5 - 43.8
			AFB2			-	0.9 - 9.4
			AFG1			-	nd - 19.7
			AFG2			-	nd - 7.1
Set e Erkmen, 2010	Spain	70	AFs	14	20	8.9	n.d. – 108
Tawila, El, Neamatallah e Serdar, 2013	Saudi Arabia	53	AFS	18	34	16.6	-
		9	AFB1	9		-	1.9 - 411
			AFB2			-	nd - 10.7
			AFG1			-	nd - 4.6
			AFG2			-	nd - 0.8
Varga *et al.*, 2013	Austria	8	AFs	0	0	-	-
			H-T2	0	-	-	-
			OTA	1	13	<LOQ	-
			T2	0	-	-	-
			ZEA	0	-	-	-
Liao *et al.*, 2015	USA	10	AFB1	2	20	-	0.5-1.2
			AFB2	1	10	0.,9	-
			AFG1	1	10	0.5	-
			AFG2	0	-	0.0	-
			DON	0	-	-	-
			FB1	0	-	-	-
			FB2	0	-	-	-
			OTA	3	30	1.4	1.0-6.6
			T2	0	-	-	-
			ZEA	0	-	-	-
Diella *et al.*, 2018	Italy	8	AFB1	4	50	31.9 (median)	8.2 - 354.5
			AFs		50	33.9 (median)	8.8 - 387.3
Alsharif, Choo e Tan, 2019	Malaysia	10	AFB1	4	40	7.10	5.30 - 10.15
			AFB2	3	30	2.18	1.46 - 3.47
			AFG1	4	40	2.45	1.90 - 3.31
			AFG2	2	20	0.86	0.81 - 0.90
			OTA	0	-	-	-
Kulahi e Kabak, 2020	Turkey	50	OTA	2	4	0.527	0.198 - 0.850

AFs - Aflatoxins (AFB1, AFB2, AFG1 and AFG2); AFB1 - Aflatoxin B1; AFB2 - Aflatoxin B2; AFG1 - Aflatoxin G1; AFG2 - Aflatoxin G2; FB1 and FB2 - Fumonisins; OTA - Ochratoxin A; DON - Desoxynivalenol; T-2/HT-2 - Trichothecenes; ZEA - Zearalenone; LOQ - limit of quantification; n.d. - not detected.

Few studies have evaluated AFs in nuts and derivatives with different types of processing. Ostadrahimi *et al.* (2014) determined AFs in raw pistachios and roasted with salt, demonstrating that samples toasted with salt contained higher AFs than unprocessed samples, in order of 22.02 μg/kg. This fact may be due to prolonged storage time with conditions suitable for fungal growth in addition to thermoresistance and stability of AFs at processing temperatures. AF occurrence were sometimes different from study to study depending on the characteristics of the samples analyzed. Some studies have not detected AFs in the food matrices (Nonaka *et al.*, 2009), but other studies reported high levels of contamination. This is justified by the different origins of products (not mentioned in many cases), different storage conditions or type of processing.

Several studies evaluated the OTA levels in pistachio nuts (Alsharif, Choo e Tan, 2019; Coronel *et al.*, 2012; Fernane *et al.*, 2010; Kulahi e Kabak, 2020; Liao *et al.*, 2015; Varga *et al.*, 2013) and the results show low percentage of positive samples for OTA contamination. In the study by Liao *et al.* (2015), three of the pistachio samples were contaminated with OTA, between 1.0 and 6.6 μg/kg. Moreover, Varga *et al.* (2013) and Zinedine *et al.* (2007) noticed the presence of OTA, but at levels lower than LOQ.

Fernane *et al.* (2010) conclude that pistachio can be highly contaminated with aflatoxin- or ochratoxin-producing isolates but the presence of mycotoxins is not high. In fact, out of 31 samples, only two samples were contaminated with AFs and only one sample had OTA.

None of the studies in this review mentioned the presence of other mycotoxins, such as DON, FB1, FB2, ZEA, T2 or HT2, in pistachios. In summary, the available data is still scarce in pistachio nuts and evaluation of the effect of processing is lacking.

Regarding emerging mycotoxins, few studies have evaluated the presence of these mycotoxins in pistachios. Liao *et al.* (2015) detect 1.9 μg/kg of BEA in one sample of roasted pistachios, out of a total of ten samples. Tolosa *et al.* (2013) surveyed the occurrence of ENNs and BEA in nuts and dried fruits in Spain, studying three samples of pistachio. Results show that no presence of BEA and ENNs in pistachio fruit is detected, but, in pistachio shell, ENA, ENA1 and ENB are found at concentrations of 0.326 μg/kg, 0.015 μg/kg and 0.209 μg/kg, respectively, explained by protective effect of the shell. FUS is produced by *Fusarium proliferatum*, *F. subglutinans*, and *F. verticillioides* and occurs in grains and grain-based foodstuff (Gruber-Dorninger *et al.*, 2017). STC, an *Aspergillus* mycotoxin, mainly occurs in grain, green coffee beans, spices, nuts and cheese, but information is still limited. Concerning *Alternaria* mycotoxins, TeA is the most frequently found in nuts like almonds, hazelnuts, peanuts, and

pistachio (Mujahid *et al.*, 2020) and are probably associated with negative effects on protein biosynthesis (Gruber-Dorninger *et al.*, 2017).

5. Biomonitoring

Biomonitoring is an important method for assessing the real exposure to aflatoxins by humans, determining concentrations of mycotoxins, their metabolites or reaction products in biological fluids (Martins *et al.*, 2019). It involves the collection of biological samples from individuals such as blood, urine, saliva, breast milk, as well as hair and nails. To do this, it is necessary to have knowledge of toxicokinetics, especially biotransformation, to identify possible biomarkers of exposure. Biomonitoring is currently an area under development; determination of AFs in food does not constitute a true assessment of exposure since individuals are exposed to multiple food sources with aflatoxins, in addition to other routes of exposure, such as inalatory and dermal (Al-Jaal *et al.*, 2019).

In case of AFs, biomonitoring can be performed by analyzing presence of AFB1 metabolites in blood, milk and urine. In addition, excreted DNA and protein adducts in blood can also be monitored (Bennett e Klich, 2003). AF metabolite evaluation in biological fluids is usually performed through liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Based on studies in humans and animals, adduct AFB1-N7-guanine in urine represents the most reliable biomarker for exposure to aflatoxin, but reflects only recent exposure. In addition, AFB1-albumin adduct is also considered a biomarker for prolonged blood exposure to AFB1 due to the half-life time of albumin of 20 days. AFM1 can be found in human breast milk, which can be considered as a biomarker of maternal and infant exposure to AFB1. It is also excreted in urine and can be considered a biomarker, however, only for recent exposure to aflatoxins (Al-Jaal *et al.*, 2019; Bennett e Klich, 2003).

In Portugal, Martins *et al.* (2019) evaluate the exposure of the population to mycotoxins, between 2015 and 2016, through analysis of 37 biomarkers in 24 h urine samples and first morning urine, to estimate probable daily intake and perform risk characteristics. From this study, it was concluded that the Portuguese population is more exposed to six mycotoxins: deoxynivalenol, zearalenone, ochratoxin A, alternariol, fumonisin B1 and citrinin. These levels are above safety limits, representing a public health problem. In this study, exposure to aflatoxins was not evaluated.

6. Prevention and control

Due to the risks of aflatoxins to human health and economic losses, strategies have been developed to reduce *Aspergillus* and AFs contamination. Prevention of fungal contamination is the most effective and preferable measure.

Reducing levels of AFs in pre-harvest begins with plant selection, planting and harvesting dates, plant density and crop rotation, as well as soil treatments, irrigation and pest management (FAO/WHO, 2018). AF contamination can be prevented using seeds genetically modified to be resistant to *Aspergillus* infection and/or environmental stress (Rushing e Selim, 2019). However, plant breeding could not be effective because resistance is conferred by multiple genes and environmental pressure is an uncontrollable factor (JECFA, 2017).

Another biological pre-harvest strategy to reduce AFs is using non-toxigenic/atoxigenic *A. flavus* isolates to competitively exclude aflatoxin-producing strains during crop colonization or physically displacement. To ensure efficacy, atoxigenic fungi must be (1) selected from local environments, (2) highly competitive and (3) predominant relative to the toxigenic strain in agricultural environments. This strategy shows a reduction in AF contamination between 70 and 90% in cotton, maize and peanuts, and it has also been implemented in pistachios with reductions ranging from 20 to 45% (Doster, Cotty e Michailides, 2014; JECFA, 2017; Yin *et al.*, 2008). Several of the atoxigenic *A. flavus* strains have been developed into biopesticides for the management of AF contamination and they are already used in the USA in pistachios, such as AF36 coated into a carried sterile grain (Moral *et al.*, 2020). However, there are uncertainties regarding their use, mainly (1) the impact of the addition of biocontrol strains in *Aspergillus* population, like a decrease of *A. flavus* followed by an increase in *A. niger* (Michailides *et al.*, 2018) and (2) the possibility of atoxigenic strains reverting back to toxin producers (FAO/WHO, 2018). Some *Aspergillus* strains could be also effective in post-harvest AF mitigation (Yin *et al.*, 2008).

While the pre-harvest contamination with AFs in more common in nuts than post-harvest contamination, in post-harvest, control of moistures, temperature, mechanical damage, insect damage and aeration can prevent mycotoxin contamination (JECFA, 2017).

Another prevention strategy is predictive modelling, using large volumes of data and various correlate environmental factors with the potential for *A. flavus* growth and consequently aflatoxin production in entire food chain (FAO/WHO, 2018). In pistachio storage, Marín *et al.* (2012) applied models to predict the growth of *A. flavus* and AF production as functions of moisture and temperature and results show that the model correctly predicts

the presence of *A. flavus* in 90% of cases and AFs in 89% of cases. Aldars-García *et al.* (2015) also attempted to model growth and AFB1 production by *A. flavus* in storage and transport in order to support decisions on ventilation timing and refrigeration adequation, respectively; the model correctly predicted the presence of AFB1 in 70 to 81% of cases. While post-harvest modelling is more developed, preventing contamination in pre-harvest is also a good perspective. Kaminiaris *et al.* (2020) developed mechanistic model considering a tree's phenology and meteorological data which correctly predicted 75% of AFB1 contamination in pre-harvest; the authors suggested that this model could indicate the appropriate time for harvest, supporting agricultural systems, and also the pistachios with the highest risk of contamination due to the prediction of field conditions. Predictive modelling has also been applied to OTA in pistachio by Marín *et al.* (2008) who built a probability model function of moisture and temperature, correctly predicting 90% of the cases.

Recently, metabolomics was applied to future prevention of mycotoxins. This new science analyze metabolomes, all the low molecular weight metabolites in biological sample, as a result not only of cell's genome but also of the environment interaction. Metabolomics is useful for understanding the chemical interactions between plant, toxigenic fungus and microbiota, and the influence of biotic and abiotic stress in biosynthesis of mycotoxins and modified forms of mycotoxins as result of biological or chemical modifications, such as food processing. The knowledge of determinants and factors that govern fungus infection and mycotoxin production allows the development of new efficient strategies to mitigate the occurrence of mycotoxin in food (Richard-Forget, Atanasova e Chéreau, 2021).

7. Decontamination

In order to reduce or eliminate AF contamination and to ensure food safety, decontamination methods can be physical, chemical or biological. The effectiveness depends on several factors, such as the chemical stability of mycotoxins, the nature of the process, the type and the interaction with the food matrix and the interaction with multiple mycotoxins (Park, 2002). It should always be taken into account that these methods: (1) inactivate, destroy or remove the toxin; (2) not be able to produce or leave toxic residues; (3) necessarily maintain the nutritional value of the food; (4) not change the acceptability or technological properties of the food and, (5), if possible, destroy fungal spores, preventing the proliferation and production of new mycotoxins (Park, 2002).

Table 4 presents the outcomes from studies on aflatoxin B1 decontamination by physical, chemical, and biological methods.

Table 4. Summary of studies using decontamination methods to degrade AFB1 in pistachio nuts.

Method	Treatment	Assay conditions	Reduction AFB1	Reference
Physical	Heat/Roasting	150°C for 30min	63%	Yazdanpanah *et al.*, 2005
	Gamma radiation	10 kgy	68%	Ghanem, Orfi e Shamma, 2008
Chemical	Ozonation	0.9 mg/L for 420min	23%	Akbas e Ozdemir, 2006
	Seed extract *Trachyspermum ammi*	37°C for 24h	91%	Velazhahan *et al.*, 2010
	Leaf extract *Adhatoda vasica*	37°C for 24h	96%	Vijayanandraj *et al.*, 2014
	Leaf extract *Corymbia citriodora*	30°C for 72h	95%	Iram *et al.*, 2015
	Leaf extract *Ocimum basilicum*	30°C for 72h	90%	Iram *et al.*, 2016
Biological	Kefir-grains	30°C for 6h	97%	Ansari *et al.*, 2015
	Bacillus subtilis UTBSP1	35°C for 5 days	95%	Farzaneh *et al.*, 2012
	Saccharomyces cerevisiae	-	40-70%	Rahaie *et al.*, 2010
Others	Heat + Acidification	15ml lemon juice 6g citric acid 120°C for 1h	49%	Rastegar *et al.*, 2017

7.1. Physical decontamination

Physical processes of decontamination include separation of the density-contaminated fraction and the reduction/inactivation of AFs by cooking, boiling, toasting, microwave heating, or irradiating contaminated food. However, AFs are highly heat stable and are not easily destroyed, so it is necessary to heat at high temperatures to effectively decrease the levels of aflatoxins (Rushing e Selim, 2019), depending on time, temperature and moisture content (Aiko e Mehta, 2015). Some studies indicate that roasting aflatoxin-contaminated pistachios at 150°C for 30 min reduced AFB1 levels by 63%; when the same process was performed, for 120 min, more than 95% of the AFB1 was degraded, but changes were caused in the appearance and taste of pistachios (Sataque Ono *et al.*, 2011; Yazdanpanah *et al.*, 2005). Ostadrahimi *et al.* (2014) determined AFs in raw and roasted with salt pistachios, demonstrating that the samples roasted with salt contained higher content of AFs (mean: 22.02 □g/kg) than the unprocessed samples (mean: 0.48 μg/kg). This fact, according to the authors of the study, may be due to prolonged storage time with conditions suitable for fungal growth. According to Yazdanpanah *et al.* (2005), the effect of toasting on the reduction of AFs in pistachios was evaluated to define the optimum conditions. It was found that the treatment of

samples at 150°C for 30 min significantly reduced the levels of AFs, without alteration of organoleptic characteristics. Heat treatment is widely applied in the food industry, for biscuits, pasta, cereals, snacks, etc. (Ismail *et al.*, 2018).

Recently, non-thermal processes like Cold Plasma treatment, electron beam irradiation and pulsed electric field have been applied to reduce mycotoxin contamination with good results in different foodstuffs(Aron Maftei *et al.*, 2014; Bosch *et al.*, 2017; Janić Hajnal *et al.*, 2019; Mousavi Khaneghah *et al.*, 2020). These techniques are processed at near room temperature, and so do not significantly affect the nutritional status or the organoleptic properties, constituting alternatives to the conventional decontamination techniques for pistachio (Adebo *et al.*, 2021; Sipos *et al.*, 2021; Udomkun *et al.*, 2017). Cold plasma treatment (CP) is an interesting tool to reduce mycotoxins due to both fungi reduction and mycotoxin degradation in all food chains (Wu, Cheng e Sun, 2021; Yousefi *et al.*, 2021). CP was already applied by Tasouji, (2018) in pistachio nuts to reduce *Aspergillus flavus* and results showed a reduction of 67% with 10 min of irradiation time, without alteration of the texture. The CP technique was also performed to reduce AFB1 in hazelnuts and reduced 70–73% of spiked AFB1 (Sen, Onal-Ulusoy e Mutlu, 2019). This decontamination process is possible for industrial implementation because it is eco-friendly, energy efficient, low cost and fast. Gamma (γ) and ultraviolet (UV) radiation are also applied for destroying AFS because they are photosensitive (Aiko e Mehta, 2015). Ghanem *et al.* (2008) studied the effect of gamma radiation on the inactivation of AFB1 and concluded that at a dose of 10 kGy there was a reduction of 68.8% and 84.6% in shelled and in-shell pistachios, respectively, and degradation was positively correlated with the increase of dose. There is a significant difference between shelled and in-shell pistachios; the authors explain this due to the fact that in in-shell pistachios the fungal growth was limited to the surface of the peel and limited *Aspergillus* entrance into the kernel itself. These techniques are applicable to different food matrices. However, due to the associated risks to human health, more studies are needed (Rushing e Selim, 2019).

Currently, mechanical separation based on size and density by gravity systems removes small and shriveled pistachios. Additionally, manual sorting of stained shells, discolored shells and defective pistachios is also applied in industry. Both methods are applied to reduce AF contamination in pistachios (Adibian, 2016; Georgiadou, Dimou e Yanniotis, 2012; Hadavi, Feizi e Gheibi, 2017; Ismail *et al.*, 2018). Several studies indicate a positive correlation between physical properties (size, color, shape, density and fungal damages) and AF contamination. Doster and Michailides (1999) reported that pistachio nuts with oily shells, crinkled shells or shell discoloration had more kernel decay and NOW infestation, and consequently more AF

contamination. Shakerardekani, Karim and Mirdamadiha (2012) concluded that pistachios with yellowish-brown and dark-greyish stains have the highest levels of AFs, and, after removing those stained nuts, there is a contamination reduction of between 95 and 99%, depending on pistachio cultivar. Manual sorting is a more time-consuming and tedious task, so sorting using new technologies has been studied. McClure and Farsaie (1980) reported the elimination of pistachios contaminated with AFs by fluorescence sorting. Özlüoymak and Güzel (2020) develop an image processing technique to measure and analyze color by irradiating pistachios at a wavelength of 365 nm and the contaminated pistachios exhibited bright-greenish yellow fluorescence. This method can be applied at a new real-time determination and separation system.

Furthermore, some pistachios could look healthy on the outside but have necrotic spots resulting from stigmatomycosis disease, which has a positive correlation with higher aflatoxin contamination. In addition, S.Yanniotis *et al.* (2011) developed a method based on X-ray imaging for the detection of necrotic spots in pistachios and rejecting these nuts results in a reduction of AFs of 60%. This methodology could be applied in an automatic separation machine at industrial levels to reduce AF contamination.

7.2. Chemical decontamination

Chemical decontamination methods use chemical compounds that degrade the structure of AFs. These methods may result in toxic degradation products that may harm the consumer's health, and/or unacceptable changes in the quality of the final product, both nutritional and sensory (CAST, 2003). Within the chemical methods, three are highlighted: acidification, ammonization and ozonation.

Acidification is a way to prevent fungal growth or inactivate AFs. Lactic acid, citric acid, tartaric acid or hydrochloric acid are used more frequently and the use of salicylic, benzoic, boric, oxalic or propionic acids has been shown to be effective in reducing the content of AFs. In the case of AFB1, the use of acids results in the conversion to AFB2, AFB2a, AFD1 and less toxic forms (Aiko e Mehta, 2015; Ismail *et al.*, 2018; Kumar, 2018; Nazhand *et al.*, 2020). This is a simple method, without the need for equipment or specialized people, and only require the contact of food matrix with acid for a certain period of time (Rushing e Selim, 2019), and is low-cost technique.

Ammonization is the most efficient technique, with a reduction of about 99%, but more common in animal feed decontamination. Ammonium is used in gaseous or hydroxide form,

degrading AFB1 in AFD1 in alkaline, and consequently reducing mutagenicity. This technique requires more complex infrastructure (Rushing e Selim, 2019).

Ozonation is one of the most promising methods, using gaseous ozone, a potent oxidant, for short periods of time. It is very effective in different types of food matrices and is accepted to be used at the industrial level (Ismail *et al.*, 2018; Rushing e Selim, 2019). Akbas e Ozdemir (2006) study the efficiency of ozone in degradation of AFs in pistachios, and the results indicated that ozonation at 0.9 mg/L for 420 min reduce AFB1 and AFs in 23% and 24%, respectively, which indicated that AFB1 is more sensitive to this method than the other aflatoxins (AFB2, AFG1 and AFG2). In addition, no significant changes occur in color, fatty acid composition or organoleptic properties of pistachio.

Another method for decontamination is the association of two or more types of process. Rastegar *et al.* (2017) use physical and chemical methods through roasting 50 g pistachio nuts at 120°C for 1 h with 15 mL lemon juice and/or 6 g of acid citric to remove AFB1. The level of AFB1 was reduced by 49% without a noticeable change in desired appearance of pistachios. The reduction was higher (93%) using 30 mL lemon juice but desired physical properties were altered.

It is also worth mentioning the use of aqueous extracts of plants, since they are rich in bioactive compounds such as tannins, terpenoids, alkaloids and flavonoids, with antifungal properties (Ismail *et al.*, 2018; Rushing e Selim, 2019). Several studies have indicated the high efficiency in the degradation of AFs with the use of plant extracts. The authors indicate that detoxification is related to the modification of the lactone ring structure of the AFs (Iram *et al.*, 2016; Velazhahan *et al.*, 2010). The extracts will have high molecular weight compounds, be soluble in water and be thermolabile (Velazhahan *et al.*, 2010; Vijayanandraj *et al.*, 2014). In the case of *A. vasica* extract, alkaloides appear as a principle of detoxification of aflatoxins (Vijayanandraj *et al.*, 2014). All studies reveal a reduction in the contents of other aflatoxins, especially AFB2 (Iram *et al.*, 2016; Velazhahan *et al.*, 2010; Vijayanandraj *et al.*, 2014). While this technique is more time consuming, it is simple since the sample is incubated with plant extract in specific time and temperature conditions, it has high efficacy and, because it is considered "natural", it is more acceptable to the consumer. These extracts have compounds that are biodegradable, environmentally friendly, safe and low cost, constituting an alternative to other synthetic chemical compounds (Ismail *et al.*, 2018). However, the standardization of the extract activity must be assured, since the composition of the extract is influenced by edapho-climatic conditions, and different cultivars. A drawback is the influence in the

organoleptics characteristics of the foodstuffs that can be overcome by micro or nanoencapsulation (Christman *et al.*, 2018; Laokuldilok *et al.*, 2016; Vijayanandraj *et al.*, 2014).

7.3. Biological decontamination

Biological methods use bacteria, yeasts or enzymes to degrade or inactivate AFs, or, in some cases, for adsorption of mycotoxins. Lactic acid bacteria, such as *Lactobacillus*, and *Saccharomyces cerevisiae*, are among the most studied for this process, especially in fermented products and beverages. The food is inoculated with the microorganism, so this method is more complex and time consuming, because it requires the growth of the microorganism. The enzyme peroxidase decomposes hydroperoxides and free radicals are generated that react with aflatoxins (Kumar, 2018). For example, kefir-grains are a symbiotic association of microorganisms and Ansari *et al.'s* (2015) study indicated 96.8% reduction of AFG1 in pistachio with kefir-grains pre-treated in 70°C and incubated during 6h at 30°C. *Bacillus subtilis* UTBSP1 have ability to reduce AFB1 by 95% as shown in Farzaneh *et al.* 's (2012) study, resulting from incubation at 35-40°C for five days, and its degradation activity was likely due to the extracellular enzymes. Yeasts are also studied for the decontamination, for example, *Saccharomyces cerevisiae* has the ability to surface binding aflatoxin in 40% and 70%, depending on the initial AF concentrations (10 ppb and 20 ppb, respectively) and the study also showed that acid and heat treatments increase this ability to 60-73% and 55-75%, respectively. This treatment had no effect on qualitative characteristics of pistachio nuts, such as color and texture (Rahaie *et al.*, 2010).

PART II

EXPERIMENTAL PART

Notes:
Part II was published as a research article: MATEUS, A.R.S.; BARROS, S.; PENA, A.; SILVA, A.S. **Development and Validation of QuEChERS Followed by UHPLC-ToF-MS Method for Determination of Multi-Mycotoxins in Pistachio Nuts**. Molecules. 26:19 (**2021**), 5754. https://doi.org/10.3390/molecules26195754
Also as a poster communication: MATEUS, Ana Rita Soares.; BARROS, Silvia; PENA, Angelina; SILVA, Ana Sanches - **Mycotoxins in pistachio (Pistacia vera L.): occurrence and trends in analytical methodologies**. In: International Conference on Food Contaminants (ICFC 2021), 4, Lisbon. 26 and 27 of September 2021.
And as an oral communication: MATEUS, Ana Rita Soares.; BARROS, Silvia; PENA, Angelina; SILVA, Ana Sanches - Development and validation of QuEChERS followed by UHPLC-ToF-MS method for determination of multi-mycotoxins in pistachio nuts. International Scientific Conference Food & Climate Changes, Croatia, October 2021 (accepted).

1. Materials and methods

1.1. Chemicals and reagents

Methanol, acetonitrile (ACN), both HPLC gradient grade, and formic acid were purchased from Merck (Darmstadt, Germany). Water was purified by Milli-Q plus system from Millipore (Molsheim, France) with resistivity of 18.2 MΩ x cm.

Mycotoxins standards and internal standard (zearalenone, ZAN) were purchased from Sigma–Aldrich (Madrid, Spain) and were dissolved in acetonitrile (AFB2, AFG1, ZEA, T2 and ZAN), methanol (AFB1, AFG2 and OTA) or acetonitrile:water (50:50, v/v) (FB1 and FB2). Stock solutions were prepared with a concentration of 1 mg/mL, except T2, which presented a concentration of 25 mg/mL. These stock solutions were subsequently used to prepare working solution for calibration.

Calibration work solution were prepared in acetonitrile with concentration of 10 ng/mL of AFB1; 20 ng/mL of AFB2, AFG1 and AFG2; 15 ng/mL OTA and 1 μg/mL of FB1, FB2, T2, HT2 and ZEA. All standard solutions were stored in amber vials in the dark at – 20°C, for at least 2 years (Silva *et al.*, 2019), and before use, they were kept at room temperature for 15 min.

For QuEChERS, trisodium citrate dihydrate and anhydrous magnesium sulfate were purchased from PanReac (Barcelona, Spain). Sodium chloride was purchased from Fluka (Seelze, Germany). Sodium citrate dibasic sesquihydrate were purchased from Sigma-Aldrich (Madrid, Spain). For clean-up procedures, EMR-Lipid d-SPE tubes were purchased from Agilent Technologies (Santa Clara, CA, USA) and Z-Sep from Supelco - Merck (Darmstadt, Germany). For clean-up testes, primary secondary amine-bonded silica (PSA) and C18 were acquired from Agilent Technologies (Santa Clara, CA, USA). Sep-Pak columns of C18 (1 g and 500 mg) were purchased from Waters (Woods Hole, MA, USA).

1.2. Samples and sampling procedure

Sixteen samples of pistachio nuts (raw or roasted, salted or natural, conventional and biological products, packaged and bulked) were randomly purchased in different supermarkets in Portugal between February and April of 2021 for determination of mycotoxins. The characteristics of the different samples are summarized in Table 5. Samples are origin from Iran, United States of America (USA), and Spain. In-shell pistachios were pealed. Pistachio kernels and pistachio shells samples (500-1000 g) were ground (Retsch rotor mill SK 300 with a sieve of trapezoid holes of 1.00 mm), mixed thoroughly to assure complete homogenization and preserved at -20°C until analysis (Figure 5).

Table 5. Pistachio samples and their characteristics

Sample	Pistachio	Roasted	Salted	Agriculture	Packaged	Expiration date	Origin country
A	In-shell	Yes	Yes	Conventional	Yes	07/2021	USA
B	In-shell	Yes	Yes	Conventional	No	*	Nd
C	In-shell	Yes	Yes	Conventional	Yes	11/2021	Nd
D	In-shell	Yes	Yes	Conventional	Yes	*	Nd
E	In-shell	Yes	Yes	Conventional	No	*	Iran
F	In-shell	Yes	Yes	Biological	Yes	07/2021	Spain
G	In-shell	Yes	Yes	Biological	Yes	*	Spain, Iran or USA
H	In-shell	Yes	Yes	Conventional	Yes	10/2021	nd
I	In-shell	Yes	No	Conventional	Yes	05/2021	USA
J	In-shell	Yes	No	Conventional	Yes	08/2021	USA
K	Kernel	Yes	Yes	Conventional	Yes	07/2021	USA
L	Kernel	Yes	No	Conventional	Yes	07/2021	USA
M	Kernel	Yes	No	Conventional	Yes	02/2022	Spain
N	Kernel	No	No	Conventional	Yes	02/2022	USA or Spain
O	Kernel	No	No	Conventional	Yes	11/2021	Iran
P	In-shell	Yes	Yes	Conventional	No	*	nd

* Expiration date was not mention.

Figure 5. Sample preparation: ground and homogenization of pistachio kernel and pistachio shell.

1.3. Extraction procedure

Mycotoxin extraction was performed according to a QuEChERS procedure: about 5 g of pistachio (5.0 ± 0.1 g) was weighted in 50 mL polypropylene tubes. First, 250 μL at 10 μg/mL of zearalanone (ZAN) was added. Afterward, samples are hydrated with 10 mL of ultrapure water with 0.1% of formic acid and 10 mL of acetonitrile is added. Then, the sample and the extractant was mixed for 1 min in vortex. Next, mixture of extraction salts for liquid–liquid partitioning step (4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate and 0.5 g of disodium hydrogen citrate sesquihydrate) were added and mixed for 1 min in vortex, following by centrifugation at 12,669 x g for 5 min at 5 °C. Finally, organic phase was used to carry out the d-SPE procedure, testing different sorbents:

Experiment 1: EMR sorbent in 15 mL falcon tube was first activated with 5 mL of ultrapure H_2O and vortexed for 30 s. After, 5 mL of organic extract were added, vortexed for 1 min and then centrifuged at 12,669 x g for 5 min at 5°C. Then, supernatant was decanted for 15 mL falcon tube with 1.6 g of anhydrous magnesium sulfate and 0.4 g of sodium chloride to obtain a phase separation between H_2O and ACN, followed by vortex for 1 min and centrifugation at 12,669 x g for 5 min at 5°C. Afterwards, 4 mL of the extract was transferred to a 15 mL falcon tube and evaporated to dryness under a gentle stream of nitrogen at 40°C.

Experiment 2: 5 mL of organic phase were transferred into a 15 mL falcon tube with 100 mg of Z-Sep. The mixture was shaken for 1 min in vortex and then was centrifuged at 12,669 x g for 5 min at 5°C. To compare with EMR-Lipid procedure, supernatant was decanted for 15 mL falcon tube with 1.6 g of anhydrous magnesium sulfate and 0.4 g of sodium chloride, followed by vortex for 1 min and centrifugation at 12,669 x g for 5 min at 5°C. After, 2 mL of the extract was transferred to a 15 mL Falcon tube and evaporated to dryness under a gentle stream of nitrogen at 40°C.

Finally, residues from EMR-lipid and Z-Sep d-SPE procedure were redissolved with 500 μL of acetonitrile 40% (v/v), vortexed for 30 s follow by 15 min in an ultrasonics bath and filtered through a PVDF mini-uniprep™ for injection into the UHPLC-ToF- MS system.

1.3.1. Clean-up experiments

Different clean-up sorbents were evaluated, namely the C18, PSA, Z-Sep and $MgSO_4$ in different proportions and mixtures, and EMR-Lipid, using a 5 mL of pistachio extract from QuEChERS spiked with 1 mL of calibration work solution (**Figure 6**).

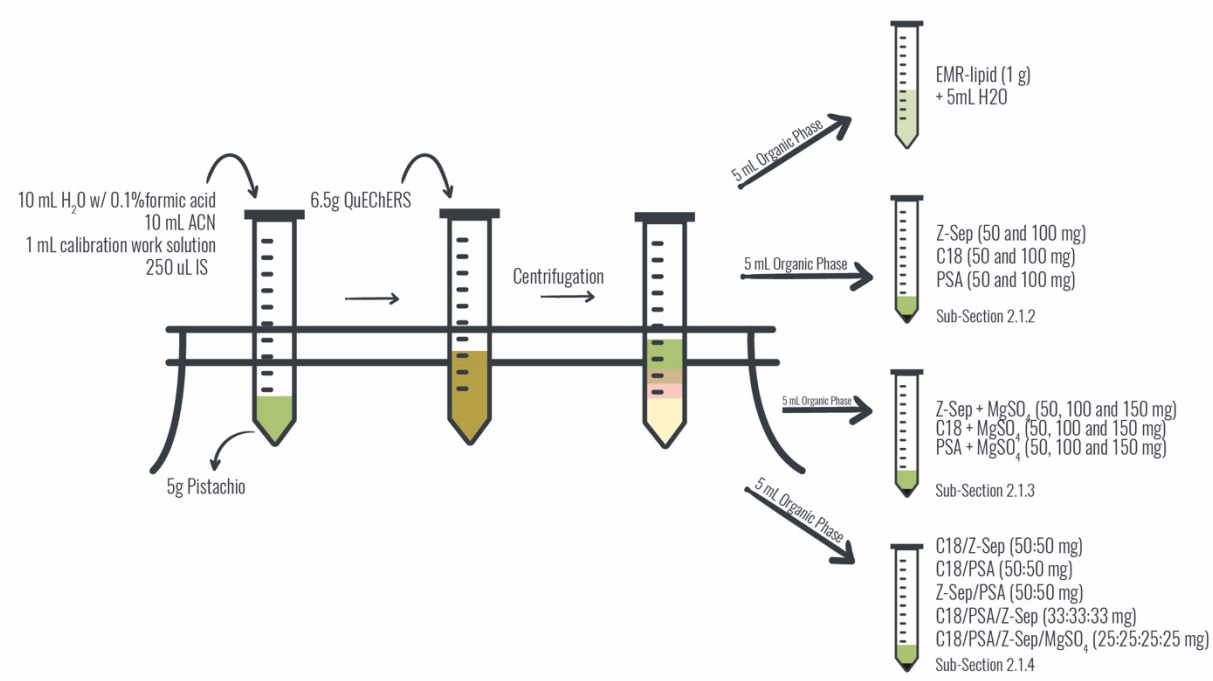


Figure 6. Diagram of different dispersive solid-phase extraction (d-SPE) clean-up procedure experiments.

1.3.2. Spiking experiments

To determine the recovery of the target analytes, spiking experiments were performed. The matrix-matched calibration was prepared by spiking blank sample of pistachio (5 g) with 7 different levels, using 0.0625 mL to 2 mL of calibration of the work solution (sub-Section 1.1) to obtain a concentration range between 0.125 to 4.0 μg/mL of AFB1; 0.250 to 8.0 μg/mL of AFB2, AFG1 and AFG2; 0.19 to 6.0 μg/mL of OTA; 12.5 to 400.0 μg/mL of FB1, FB2, ZEA, T2 and HT2. Subsequently, extraction was performed as described in sub-Section 1.3. This concentration levels include the maximum levels imposed for mycotoxins in EC Regulation No. 1881/2006 for nuts (European Commission, 2006). Even though there is no legislation for *Fusarium* mycotoxins (FBs, ZEA, T2 and HT2) in nuts, there is an EC Recommendation of 27 march of 2013 for the presence of T-2 and HT-2 toxins in cereals and cereal products (European Commission, 2013) and EC Regulation No. 1881/2006 establishes maximum levels for ZEA and FBs for cereals for direct human consumption (European Commission, 2006). For validation purposes, the concentration range considered was 12.5 to 400 μg/kg to include all the levels found EC Regulations and Recommendations for cereals, because we considered cereals as a possible reference to pistachio nuts due to the fact that both matrices are solid

with some similarity in water composition (raw pistachios: <5% water (USDA Database pistachio, 2019) and cereals: mean of 12% (PortFIR, 2019, 2019, 2019, 2019; USDA Database pistachio, 2020)), although lipid content is higher for pistachio.

Before method development, 3 different pistachio samples were analysed to ensure that any mycotoxin would not be present, using modify method based on Sanches Silva *et al.* (2019) method with two-step extraction with acetonitrile 80% (v/v). Thus, the selected blank samples were analysed by this new method, and none of the studied mycotoxins were detected.

1.3.3. Matrix effect

To evaluate the influence of co-extracted compounds on analytical signals, the matrix effect (ME) was determined by the signal suppression-enhancement (SSE). ME was the mean of ME at three concentration levels comparing peak areas from calibration standard solution and peak areas from matrix-matched calibration curve with fortified pistachio samples according with equation (1):

$$ME = \frac{Peak\ area_{\ matrix-matched\ calibration\ curve}}{Peak\ area_{\ calibration\ standard\ solution}} \times 100, \qquad (1)$$

Signal enhancement was considered when SSE >100%, inexistence of the matrix effect when SSE= 100% and signal suppression when SSE <100%. According to several authors (Alcántara-Durán *et al.*, 2019; Ferrer Amate *et al.*, 2010), matrix effect could be classified as negligible ([0%]–[±10%]), soft ([±10%]–[±20%]), medium ([±20%]–[±50%]) and strong ([±50%]).

1.4. UHPLC–ToF-MS parameters

Detection and quantification were performed with a Nexera X2 Shimadzu UHPLC coupled with a 5600+ ToF-MS detector (SCIEX, Foster City, CA, USA) equipped with a Turbo Ion Spray electrospray ionization source working in positive mode (ESI+).

In terms of chromatographic conditions, a column Zorbax Eclipse Plus C18 (2.1 mm x 50 mm, 1.8 μm) was used and kept at 30°C, the autosampler was maintained at 10°C to refrigerate the samples and a volume of 20 μL of sample extract was injected in the column. The mobile phase consisted of 0.1% formic acid [A] and acetonitrile [B] with a flow rate of 0.5 mL/min and with the following gradient program: 0–12 min from 90% to 30% [A]; 12–13 min from 30% to 10% [A] and kept until 14 min; back to 90% [A] from 14 to 15 min until the end of the run. The total run time was 17 min.

In terms of mass spectrometry, the acquisition was performed in full-scan from 100 to 750 Da using the Analyst® TF software (SCIEX, Foster City, CA, USA) and with the following settings: ion source voltage of 5500 V; source temperature 575°C; curtain gas (CUR) 30 psi; Gas 1 and Gas 2 of 55 psi; declustering potential (DP) of 100 V. Every 7 injections the ToF-MS detector was calibrated in the mass range of the method, to guarantee the accurate mass resolution.

1.5. Identification of mycotoxins

The identification and data processing of mycotoxins were made through the PeakView™ and MultiQuant™ software (SCIEX, Foster City, CA, USA).

The isotope match is presented automatically by the PeakView™ software, and regarding identification criteria of mycotoxins, three parameters and their corresponding equations (equation (2)-(4)) were used: (1) maximum relative retention time deviation (ΔRRT) of 2.5% (equation (2)); (2) difference in the isotope pattern with a tolerance of 10% (equation (3)); and (3) exact mass deviation (Δm) with a tolerance of 5 ppm (equation (4)).

$$\mathrm{RRT} = \frac{\mathrm{RT_{analite}}}{\mathrm{RT_{internal\ standard}}}, \qquad (2)$$

where $RT_{analite}$ is the retention time of analite, and the $RT_{internal\ standard}$ is the retention time of internal standard (zearalanone).

$$\Delta\mathrm{RRT} = \left(\frac{\mathrm{RRT_{spiked\ samples}} - \mathrm{RTT_{standard}}}{\mathrm{RRT_{standard}}}\right) \times 100, \qquad (3)$$

$$\Delta\mathrm{m\ (ppm)} = \left(\frac{\mathrm{Exact\ mass} - \mathrm{Detected\ mass}}{\mathrm{Exact\ mass}}\right) \times 10^6. \qquad (4)$$

1.6. Validation of UHPLC-ToF-MS method

The method was validated by the determination of concentration range, linearity, limit of detection (LOD), limit of quantification (LOQ) and accuracy by determining precision (repeatability and precision inter-day) and trueness by recovery assays at different levels. According to Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of result, when

certified reference materials are not available, trueness of measurements can be assessed through recovery of additions of known amounts of the analytes to a blank matrix (European Commission, 2002).

LOD and LOQ were determined as the concentration that originates a signal-to-noise ratio (S/N) ≥3 and ≥10, respectively. For the determination of repeatability (RSD_r) and precision inter-day (RSD_R), blank samples of pistachio were spiked at different levels (n=6) take in account the ML of each mycotoxin. In the case of RSD_R extraction was carried out in three different days by two different operators.

2. Results and discussion

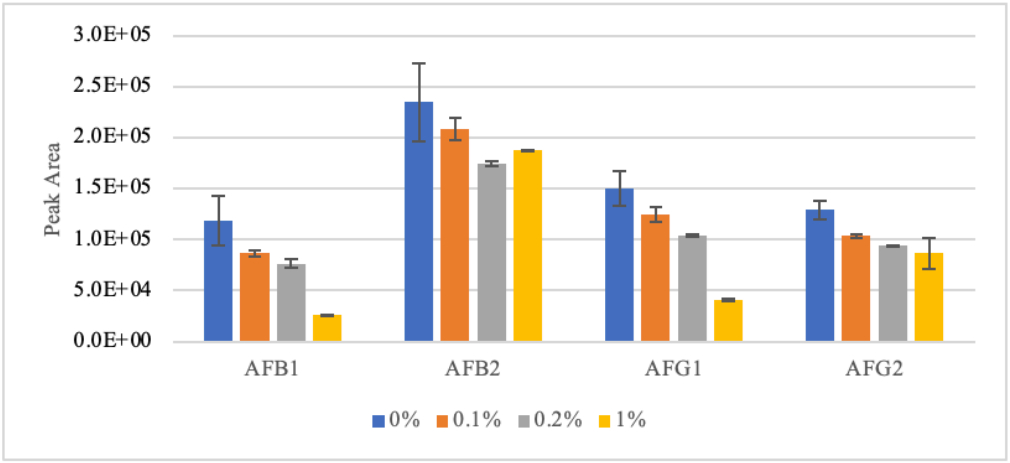
2.1. Extraction and clean-up optimization

2.1.1. Effect of water acidification

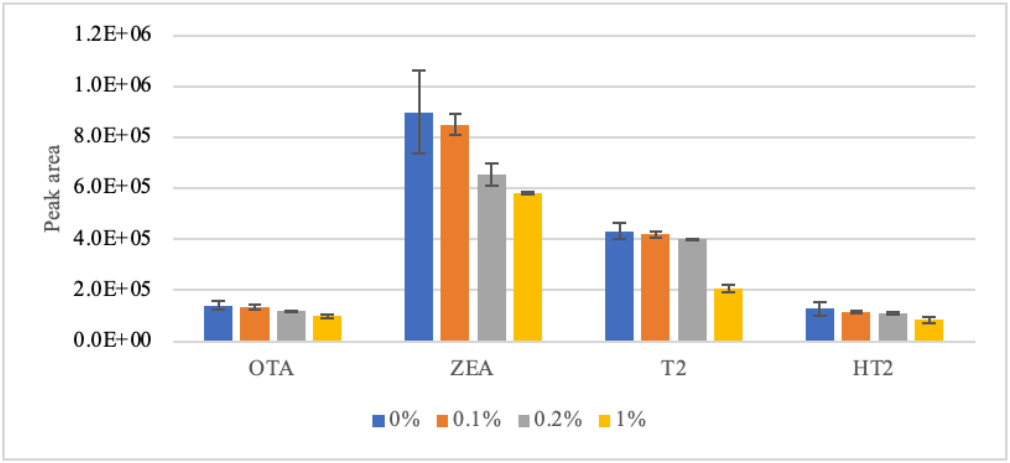
QuEChERS method was used for the extraction of mycotoxins from pistachio nuts. The procedure involved the extraction of 5 g pistachio with 10 mL acetonitrile after shaking the sample with 10 mL of water acidified with 0.1% of formic acid (FA). In fact, acetonitrile/water extraction (in different percentages) is one of the most common mixtures used for mycotoxin analysis in nuts because solubility of lipids in acetonitrile is limited, thus lipid co-extraction with this solvent is relatively low. In addition, ACN is compatibility with the chromatographic applications (Rejczak e Tuzimski, 2015). Different amounts of formic acid (0%, 0.1%, 0.2% and 1% v/v) in water were tested to assure the best results. For these tests, blank samples of pistachio were spiked with 1ml of calibration work solution, resulting on 2 μg/kg of aflatoxin B1 (AFB1), 4 μg/kg of aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), 3 μg/kg of ochratoxin A (OTA) and 200 μg/kg of fumonisin B1 (FB1), fumonisin B2 (FB2), zearalenone (ZEA), toxin T2 (T2) and toxin HT-2 (HT2).

Results show that for AFs, the major peak areas are achieved using pure water (**Figure 7**). With addition of FA, peak areas reduce, and the lower areas are obtained using 1% FA. The same conclusion was observed for OTA, ZEA, T2 and HT2. Contrary, major peak areas for fumonisins (FB1 and FB2) are achieved with 0.2% of FA, however, using 1% of FA reduced peak area, suggested that fumonisins needs only slight acidification (**Figure 7**).

In conclusion, for optimal results in multi-mycotoxins analysis, addition of 0.1% of FA in water was used in this study, performing the best results, because fumonisins need acidification (Spanjer, Rensen e Scholten, 2008) but other mycotoxins have similar peak areas with pure water or 0.1% formic acid. In fact, 0.1% of FA to extract mycotoxins from pistachio samples has previously been reported in the literature (Alcántara-Durán *et al.*, 2019; Alsharif, Choo e Tan, 2019; Narváez *et al.*, 2020).



(a)



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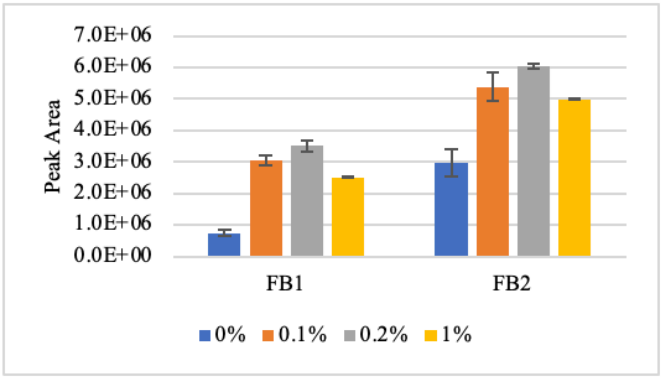


(c)

Figure 7. Effect of different levels of acidification of water with formic acid on the extraction of **(a)** AFs (AFB1 - Aflatoxin B1; AFB2 - Aflatoxin B2; AFG1 - Aflatoxin G1; AFG2 - Aflatoxin G2) and **(b)** Ochratoxin A (OTA), Zearalenone (ZEA), toxin T2 (T2) and toxin HT-2 (HT2) and **(c)** FBs (Fumonisins, FB1, and FB2) on average peak areas (n=2).

2.1.2. Influence of C18, PSA and Z-Sep sorbents

In this study, different clean-up sorbents were evaluated, namely the C18, PSA, Z-Sep and $MgSO_4$ in different proportions and mixtures, and EMR-Lipid, using a 5 mL of pistachio extract from QuEChERS spiked with 1ml of calibration work solution (**Figure 6**). Conclusions about clean-up efficiency were based on the peak area of each mycotoxin.

Results for the single use of sorbents show that, in generally, using 100 mg PSA or Z-Sep result in greater peak areas then 50 mg (**Figure 8**). Exception is the use of 100 mg C18 that increased peak areas of AFB1, AFB2 and AFG1 when compared with 50 mg, but decrease peak areas for AFG2, FBs, OTA, ZEA, T2 and HT2. This increasing analytical performance using increase amounts of C18 and PSA sorbents was also reported by Zhao *et al.* (2016), and the best results are achieved using 200 mg of C18 for 16 mycotoxins in vegetable oils.

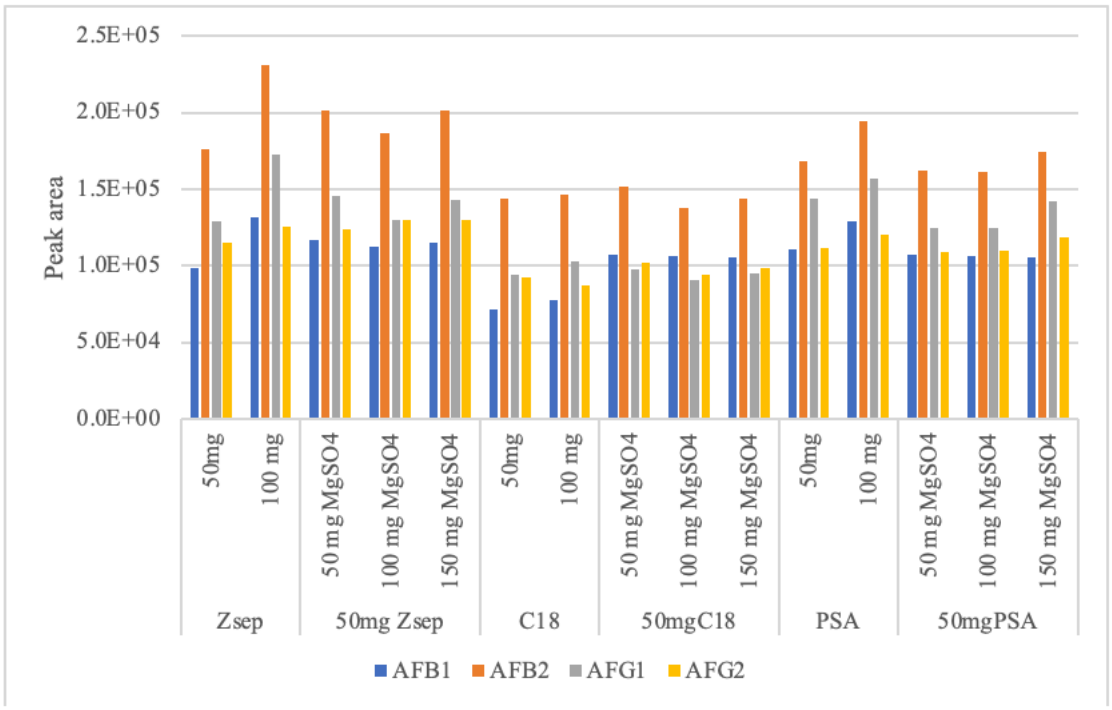
Using PSA and C18 caused a significant loss in the analytical signal of OTA, especially using 100 mg of PSA where OTA is not detected. In other way, using 100 mg PSA originated greater signal for ZEA, T2 and HT2 among traditional sorbents.

2.1.3. Influence of magnesium sulfate addition to C18, PSA and Z-Sep sorbents

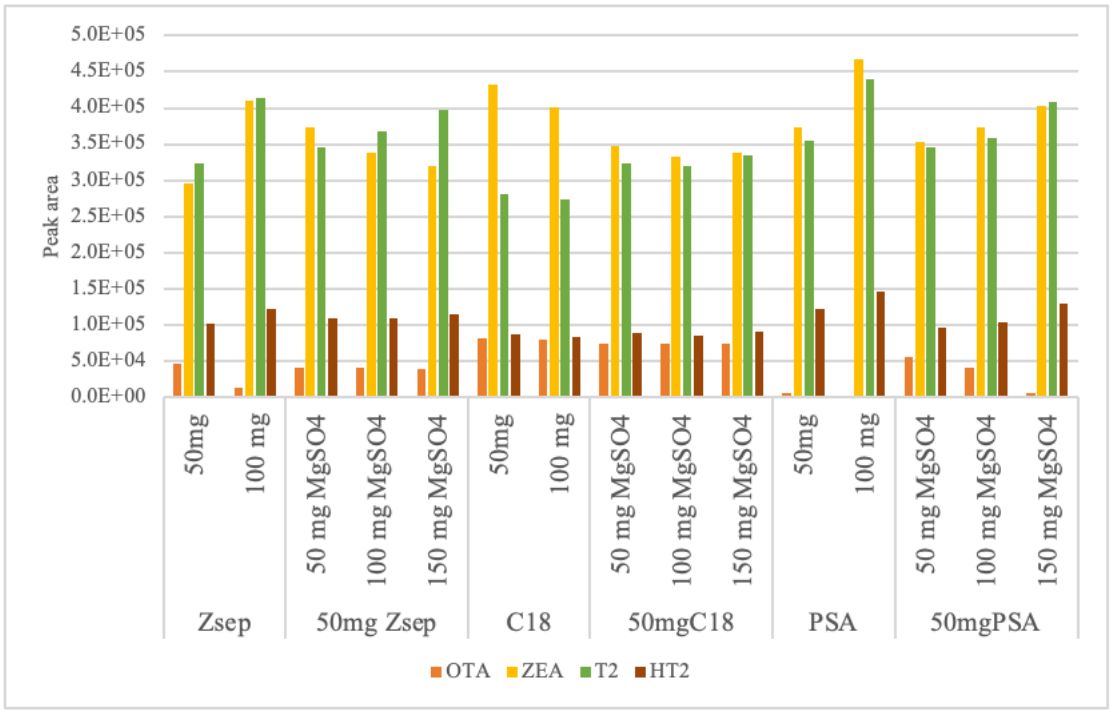
Magnesium sulfate ($MgSO_4$) has been used in clean-up to remove H_2O (Rejczak and Tuzimski, 2015). Then, 50, 100 and 150 mg of $MgSO_4$ was mixed with 50 mg sorbents. For AFs, addition of $MgSO_4$ to C18 results in slight increase of peak area. Regarding PSA, only addition of 150 mg $MgSO_4$ give better analytical signals for AFB2, AFG2, OTA, ZEA, HT2 and T2. Addition of $MgSO_4$ to Z-Sep give better analytical signals for all mycotoxins comparing to 50 mg Z-Sep, except for OTA. Still, using 100 mg of sorbents is always a better option, despite that for AFB2 the addition of 100 or 150 mg $MgSO_4$ and, for T2 addition of any quantity of $MgSO_4$ give better analytical signs even better than 100 mg of Z-Sep or 100 mg of C18, respectively. This small increase in peak areas could be less noteworthy because to compare all sorbents, procedure with EMR-Lipid was considering the standard and this method have an additional "polish step" with $MgSO_4$ and NaCl (4:1 w/w) for water removal.

2.1.4. Influence of combination of different sorbents

Yet, combination of different sorbents in the same proportion was tested (**Figure 9).** For AFs and T2, mixture of C18, PSA, Z-Sep and $MgSO_4$ (25:25:25:25 w/w) presented the major peak areas. The combination of C18: Z-Sep presents the best analytical signal for OTA, ZEA and HT2.

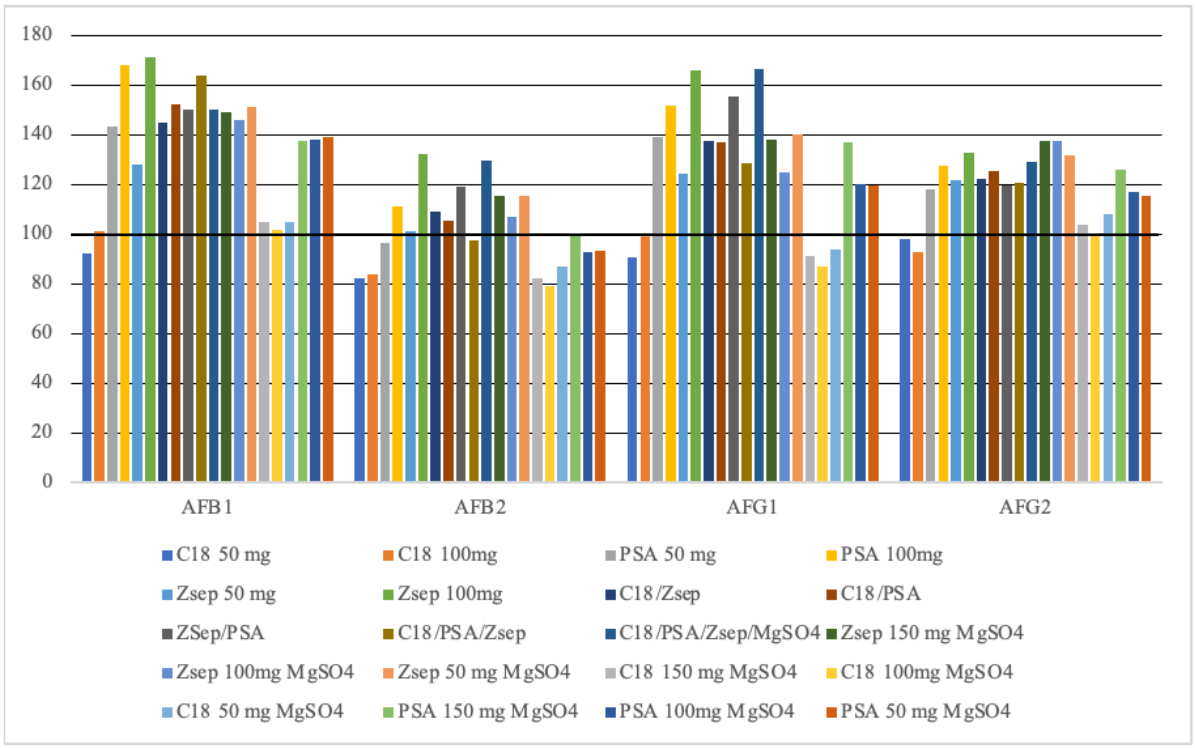


(a)

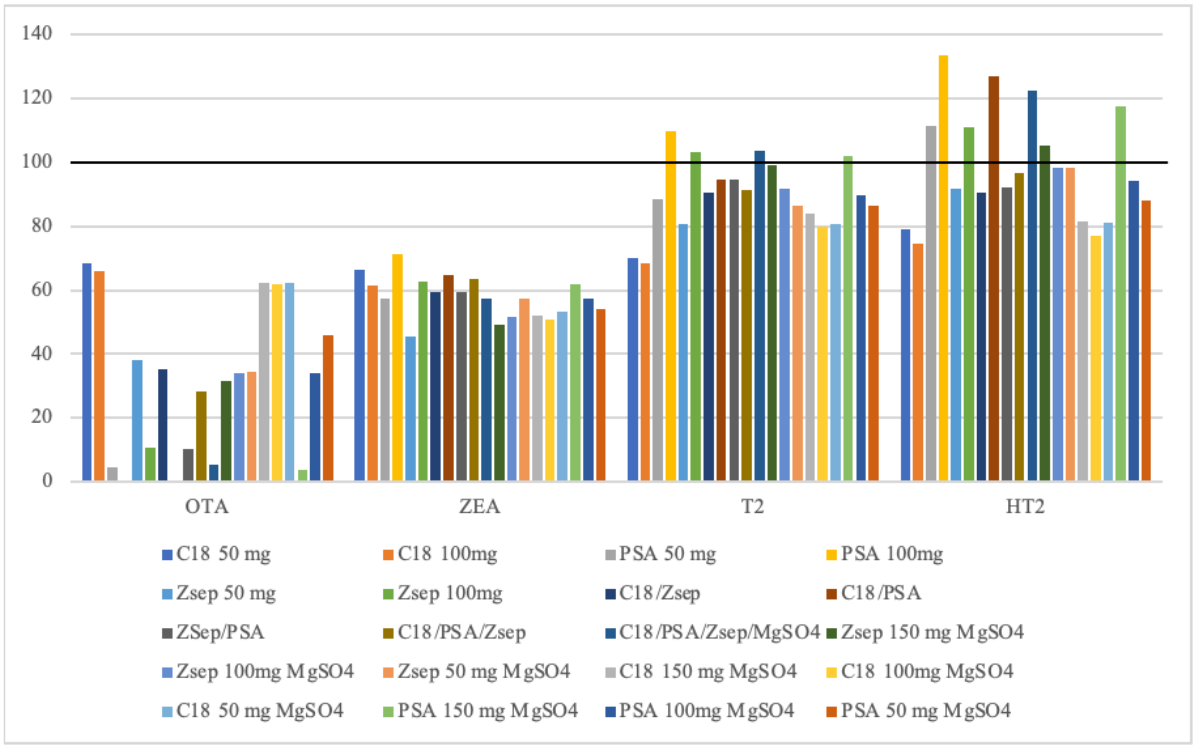


(b)

Figure 8. Average peak areas (n=2) using silica gel with zirconium oxide (Z-Sep), octadecyl modified silica (C18), primary secondary amine (PSA), and magnesium sulfate ($MgSO_4$) sorbents in d-SPE clean-up of **(a)** AFs (AFB1 - Aflatoxin B1; AFB2 - Aflatoxin B2; AFG1 - Aflatoxin G1; AFG2 - Aflatoxin G2) and **(b)** Ochratoxin A (OTA), Zearalenone (ZEA), toxin T2 (T2) and toxin HT-2 (HT2)



(a)



(b)

Figure 9. Effect of different sorbents in dispersive solid-phase extraction (d-SPE) clean-up, considering peak areas from enhanced matrix removal-lipid (EMR-Lipid) for comparison (100%) of **(a)** AFs (AFB1 - Aflatoxin B1; AFB2 - Aflatoxin B2; AFG1 - Aflatoxin G1; A AFG2 - Aflatoxin G2) and **(b)** Ochratoxin A (OTA), Zearalenone (ZEA), toxin T2 (T2) and toxin HT-2 (HT2).

2.1.5. Solid phase extraction (SPE) with C18 cartridges

Solid phase extraction (SPE) on two different C18 cartridges (500 mg and 1 g of C18) was also tested for clean-up (**Figure 10**). Results show 1 g C18 cartridges increase peak areas from all mycotoxins when compared with 500 mg of C18, however, analytical signal was always lower than EMR-Lipid clean-up (**Figure 11**). Also, OTA and FBs are present in second elution using 1 g C18, and ZEA and T2 are present in second elution of both columns. It is important to mention that SPE method needs vacuum, use more solvents to condition of column and elution of analytes, and is difficult to apply on large number of samples, so, d-SPE have more advantages because is faster and cheaper (Rejczak e Tuzimski, 2015).

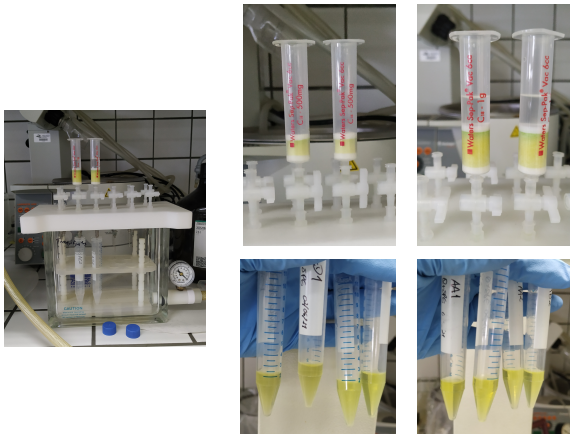


Figure 10. Solid phase extraction (SPE) with 500 mg and 1 g of octadecyl modified silica (C18) cartridges.

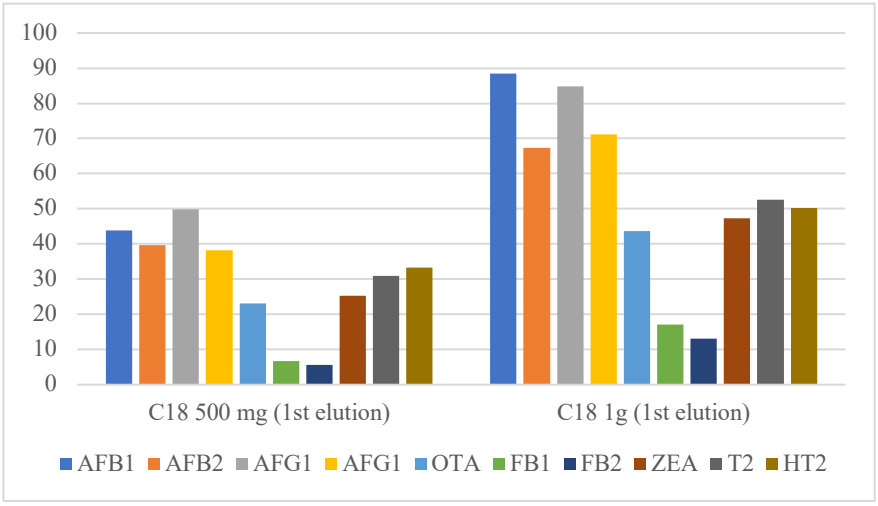


Figure 11. Effect of Solid phase extraction (SPE) clean-up with octadecyl modified silica (C18) cartridges (500 mg and 1 g), considering peak areas from enhanced matrix removal-lipid (EMR-Lipid) for comparison (100%) of AFs (AFB1 - Aflatoxin B1; AFB2 - Aflatoxin B2; AFG1 - Aflatoxin G1; A AFG2 - Aflatoxin G2), Ochratoxin A (OTA), Fumonisins (FB1 and FB2), Zearalenone (ZEA), Toxin T2 (T2) and toxin HT-2 (HT2).

2.1.6. Conclusions regarding clean-up optimization

Among all combinations assayed and considering EMR-lipid peak area as 100% to compare with other sorbents, the results given in **Figure 9** showed that better analytical signals were achieved when the 100 mg Z-Sep was used as sorbent for d-SPE. In fact, multi mycotoxins methods are a challenge because mycotoxins have different chemical proprieties, resulting in differences in peak areas and consequent concentration with different sorbents. For mycotoxins, excepting AFs, EMR-Lipid provides the highest analytical signals. Given these results, 1 g of EMR-lipid and 100 mg of Z-Sep were selected to perform clean-up step in mycotoxins analysis.

Concerning fumonisins, using PSA or Z-Sep there is no signal for FB1. Although EMR-Lipid gives better analytical signal for both fumonisins, 50 mg of C18 also give good analytical signal. The use of 100 mg of C18 or the addition of $MgSO_4$ decrease peak areas (Figure 12). In the study carried out by Jo *et al.* (2021) in feedstuffs, fumonisins B1 and B2 were also not detected by PSA, while C18 provides analytical signal for all 13 mycotoxins tested.

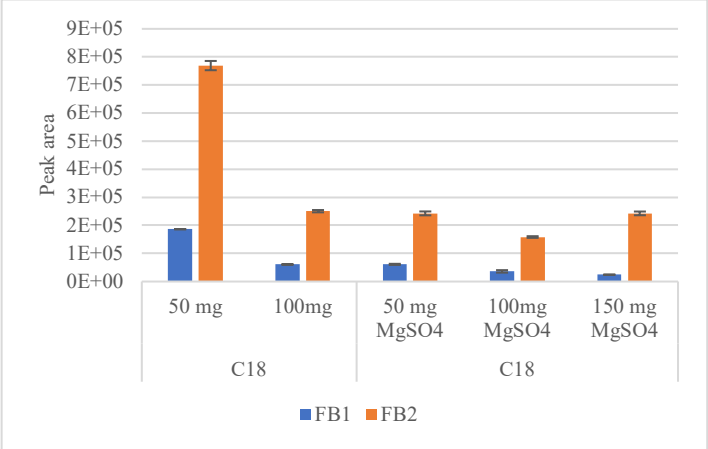


Figure 12. Average peak areas (n=2) using octadecyl modified silica (C18) and magnesium sulfate ($MgSO_4$) in dispersive solid-phase extraction (d-SPE) for Fumonisins (FB1 and FB2).

In some studies, for determination of mycotoxins in pistachio nuts, d-SPE clean-up step is not applied (Spanjer, Rensen e Scholten, 2008; Varga *et al.,* 2013) because, according to authors, clean-up step reduces the number of mycotoxins analyzed. However, there is a decrease in sensitivity, with higher LOQs. Extract clean-up is important to reduce co-extracts which can negatively affects LC-MS/MS equipment and could rapid degradation of the analytical performance of column. D-SPE with EMR-lipid or Z-Sep provides chromatogram with lower background levels as show in **Figure 13**.

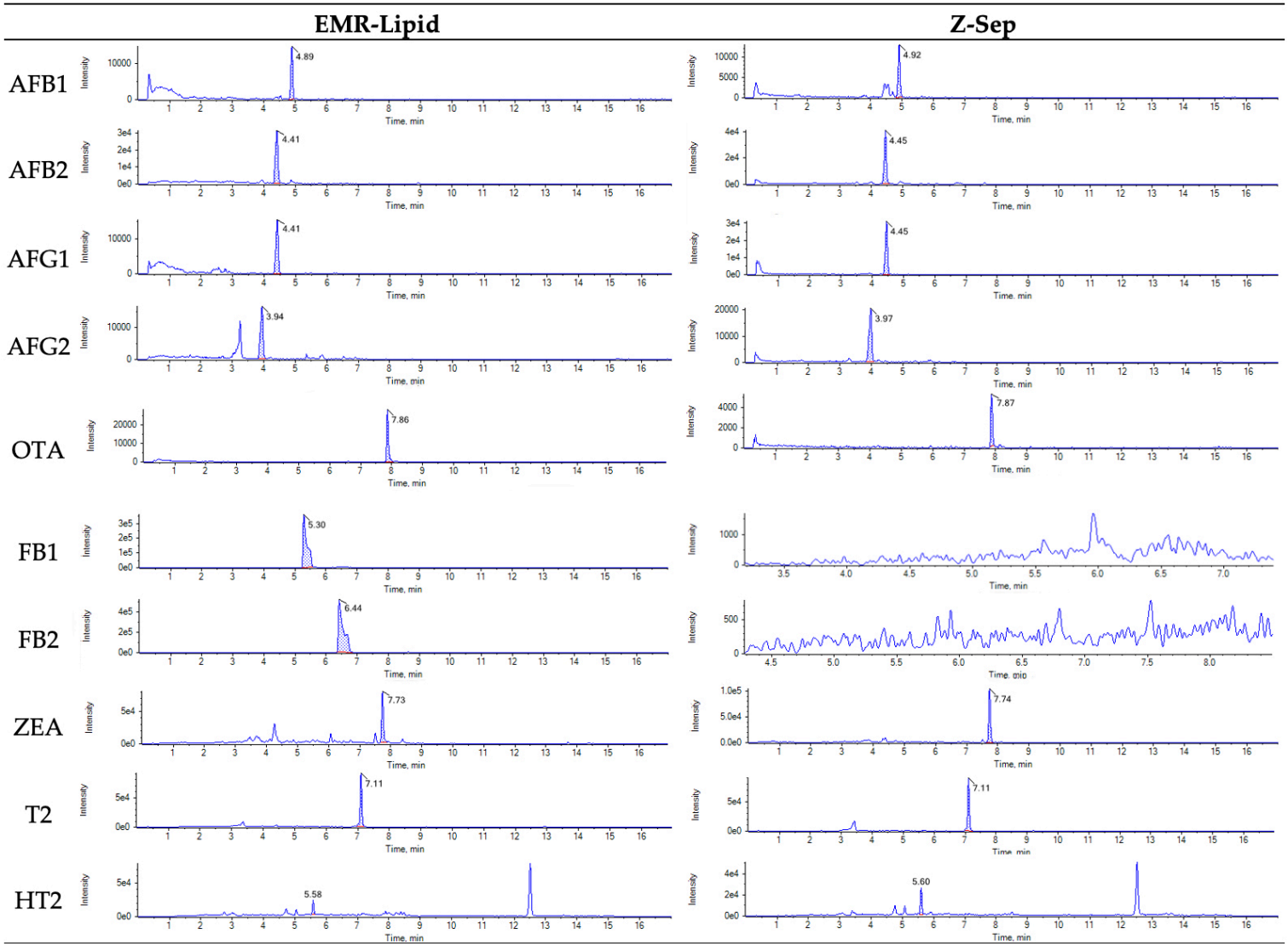


Figure 13. Chromatograms of blank pistachio sample spiked with 2 μg/kg of Aflatoxin B1 (AFB1), 4 μg/kg of Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), 3 μg/kg of Ochratoxin A (OTA), 200 μg/kg of Fumonisin B1 (FB1), Fumonisin B2 (FB2), Zearalenone (ZEA), toxin T2 (T2) and toxin HT-2 (HT2), with enhanced matrix removal-lipid (EMR-lipid) and zirconium oxide (Z-Sep) sorbents in dispersive solid-phase extraction (d-SPE) clean-up.

2.2. Validation of the analytical method

Linearity was evaluated by matrix matched calibration curves in different ranges for different mycotoxins (**Table 6**). Determination coefficients (r^2) of calibration curves were always higher than 0.99, indicating suitability to quantify mycotoxins in the selected calibration range for both methods. However, determination coefficient was higher for AFB1, AFG1, AFG2, ZEA, T2 and HT2 when Z-Sep is used as sorbent in clean-up step, especially for AFB1 (r^2=0.9993) and ZEA (r^2=0.9994).

Table 6. Linearity and sensitivity of UHPLC-ToF-MS method for the simultaneous determination of mycotoxins in pistachio.

Mycotoxin	LOD (μg/kg)		LOQ (μg/kg)		Linear range (μg/kg)		Calibration curve parameters					
							r^2		Slope		Interception	
	EMR	Z-Sep	EMR	Z-Sep	EMR	Z-Sep	EMR	Z-Sep	EMR	Z-Sep	EMR	Z-Sep
AFB1	0.125	0.125	0.5	0.125	0.5-4.0	0.125-2.0	0.9901	0.9993	32491.9	60259.9	4765.9	571.7
AFB2	0.25	0.25	0.5	0.25	0.50-8.0	0.25-4.0	0.9989	0.9973	42436.2	49476.5	-2191.6	1238.5
AFG1	0.50	0.25	1.0	0.25	1.0-8.0	0.25-4.0	0.9929	0.9974	17067.9	38603.0	11613.0	-967.9
AFG2	0.50	0.25	1.0	0.25	1.0-4.0	0.5-8.0	0.9931	0.9976	22299.4	30307.8	6944.3	2158.1
OTA	0.19	0.75	0.38	1.50	0.38-3.0	1.5-6.0	0.9934	0.9914	32398.2	6792.0	1900.9	-2529.1
ZEA	12.5	12.5	25	12.5	25-200	12.5-400	0.9958	0.9994	1504.5	1910.7	-3896.5	-1000.2
T2	12.5	12.5	25	25	25-200	25-400	0.9938	0.9979	1644.7	1570.9	12400.9	3811.1
HT2	25	25	25	25	25-400	25-400	0.9976	0.9979	287.6	403.1	7879.4	7252.4
FB1	12.5	*	25	*	25-200	*	0.9961	*	16960.4	*	-33602.9	*
FB2	12.5	*	12.5	*	12.5-200	*	0.9983	*	28671.6	*	-1339.8	*

AFB1 - Aflatoxin B1; AFB2 - Aflatoxin B2; AFG1 - Aflatoxin G1; AFG2 - Aflatoxin G2; FB1/FB2 - Fumonisins B1 and B2; OTA - Ochratoxin A; T2/HT-2 -Trichothecenes; ZEA - Zearalenone
*FB1 and FB2 are not detected when using Z-Sep as sorbent.

The sensitivity of the method was expressed as LOD and LOQ and results are compiled in **Table 6**. LOD and LOQs are much lower than the requirement imposed by EU regulations for the ML of aflatoxins (AFs and AFB1) in pistachio and sensitive enough to detected other mycotoxins not regulated for nuts. Z-Sep clean-up method provide more sensitivity, LODs and LOQs are lowest, especially for AFs. For ZEA, T2 and HT2, LODs and LOQs are the same for both methods.

LOQs are lower than those reported by Narváez *et al.* (2020) for AFB1, AFB2, AFG2 (0.39 μg/kg) and for AFG1, T2 and HT2 (0.78 μg/kg) using C18 for clean-up followed by UHLPC-Q-Orbitrap MS and lower than those reported by Cunha *et al.* (2018) for AFs (1.25 μg/kg) and for OTA (5 μg/kg) using C18 and Z-Sep$^+$ for clean-up followed by HPLC-Quattro Micro triple quadrupole-MS. Our results only indicate higher LOQ for T2 and HT2 than Cunha *et al.* (2018) (1.25 μg/kg). The same EMR-Lipid method with nano flow HPLC-MS allows lowest LOD, for example, 0.05 μg/kg for AFG1, AFG2 and ZEA; 0.5 μg/kg for AFB1, AFB2, FB1 and OTA and 5 μg/kg for FB2, T2 and HT2 (Alcántara-Durán *et al.*, 2019). However, regarding OTA, EMR-Lipid method is more sensitivity with LOD of 0.19 μg/Kg and LOQ of 0.38 μg/kg.

Table 7. Results of the validation for different mycotoxins, including recovery (Rec), relative standard deviation repeatability (RSD_r) and relative standard deviation of precision inter-day (RSD_R) at different spiking levels with enhanced matrix removal-lipid (EMR-Lipid) and zirconium oxide (Z-Sep) sorbents in dispersive solid-phase extraction (d-SPE) clean-up.

			EMR-Lipid				Z-Sep			
Mycotoxin	Ion	Retention time (min)	Spiked level (μg/kg)	Rec (%) (n=6)	RSD_r (%)	RSD_R (%)	Spiked level (μg/kg)	Rec (%) (n=6)	RSD_r (%)	RSD_R (%)
AFB1	313.07066 $[M+H]^+$	5.00	0.50	119.1	10.68		0.125	93.3	3.59	
			1.0	89.1	8.27	3.70	0.250	98.1	6.99	3.77
			1.5	101.9	10.73		0.50	98.9	4.97	
			2.0	101.9	10.35		1.0	100.7	5.29	2.56
			4.0	100.2	11.90	8.80	1.5	97.3	5.06	
							2.0	101.4	3.59	
AFB2	315.08631 $[M+H]^+$	4.52	0.50	111.4	4.85	3.54	0.25	102.8	8.69	
			1.0	102.6	6.16		0.50	98.0	6.09	5.67
			2.0	100.5	6.90	3.56	1.0	95.3	6.65	
			3.0	100.4	2.97		2.0	99.9	5.56	4.12
			4.0	97.9	4.83		3.0	100.3	6.68	
			8.0	97.5	2.43	2.79	4.0	96.5	5.64	
AFG1	329.06558 $[M+H]^+$	4.53	1.0	77.3	25.25		0.25	105.7	5.71	
			2.0	76.9	24.74	6.76	0.50	112.0	6.83	8.17
			3.0	97.9	7.70		1.0	92.1	9.62	
			4.0	104.6	6.68		2.0	101.8	6.72	5.62
			8.0	101.7	12.98	3.25	3.0	99.3	5.04	
							4.0	97.6	6.01	
AFG2	331.08123 $[M+H]^+$	4.04	1.0	86.0	20.66		0.50	119.7	5.37	9.69
			2.0	95.4	11.78	26.75	1.0	100.4	4.82	
			3.0	100.1	12.04		2.0	103.6	5.83	2.96
			4.0	104.3	7.02	9.37	3.0	96.3	9.92	
							4.0	98.0	4.10	
							8.0	99.4	9.44	2.21
OTA	404.08954 $[M+H]^+$	7.97	0.38	95.0	10.30		1.50	105.9	9.26	
			0.75	97.5	11.95	9.85	2.25	88.7	6.97	7.85
			1.50	100.4	14.74		3.0	109.4	7.31	
			2.25	108.5	4.81	9.01	6.0	82.6	3.99	6.35
			3.0	102.3	10.04					
ZEA	319.154 $[M+H]^+$	7.83	25	93.9	1.61	10.05	12.5	106.0	2.56	
			50.0	96.3	10.21		25	102.4	7.18	4.47
			100	112.9	16.38	7.41	50.0	97.1	4.77	
			150	92.1	14.25		100	94.7	8.54	2.49
			200	99.6	5.43		150	97.1	4.13	
							200	98.8	5.48	
							400	98.8	2.74	3.97
T2	489.2095 $[M+Na]^+$	7.21	25	80.8	2.40	9.42	25	100.2	5.89	7.82
			50.0	99.1	1.30		50.0	97.3	3.75	
			100	100.6	3.41	6.33	100	105.6	3.38	7.92
			150	106.9	6.98		150	98.3	1.85	
			200	96.7	3.33		200	98.86	5.29	
							400	97.9	7.24	1.77
HT2	425.217 $[M+H]^+$	5.69	50.0	118.9	15.23	10.81	25	75.2	3.89	8.89
			100	90.7	9.12		50.0	91.2	7.42	
			150	108.3	11.35	7.42	100	105.0	6.88	6.82
			200	109.8	10.79		150	108.2	1.21	
			400	102.2	2.69		200	103.8	2.12	
							400	99.8	3.70	7.75
FB1	722.396 $[M+H]^+$	5.32	25	104.0	4.01	2.58				
			50.0	94.4	5.36					
			100	99.3	2.18	4.56	na	na	na	na
			150	101.9	5.66					
			200	99.2	4.65					
FB2	706.401 $[M+H]^+$	6.48	12.5	101.3	4.67					
			25	100.1	3.17	3.66				
			50.0	91.1	8.28					
			100	103.5	1.02	3.43	na	na	na	na
			150	100.0	3.56					
			200	99.4	2.98					

na-not applicable.

Table 7 shows the results of recovery, repeatability, and precision inter-day for the different mycotoxins in a blank pistachio sample spiked at 7 different concentration levels. The results regarding the validation of method (**Table 7**), show that for some mycotoxins there is not linear range when using all the 7 spiking levels, because LODs are higher due to signal-to-noise ratio, or at higher concentration levels there is a loss of linearity. Concerning recovery, Z-Sep provides good recoveries for all mycotoxins within the appropriated range established by the Commission Regulation EC No. 401/2006, ranging between 78 to 119%. These recoveries are comparable to other studies, for example, the recoveries reported by Cunha *et al.* (2018) using C18 and Z-Sep+ in clean-up step (57-102%) and by Alcantara *et al.* (2019) also using EMR-Lipid (70-120%). For EMR-Lipid method, good recoveries are also achieved ranging 79 to 120%.

Repeatability of the method was evaluated by the Relative Standard Deviation (RSD_r) for all mycotoxins, using the same sample, same operator in a short time and the values are acceptable considering criteria established by Commission Regulation EC No. 401/2006 (European Commission, 2006), ranging between 1.30 to 25.25% and 1.21 to 9.92% for EMR-Lipid and Z-Sep method, respectively, considering the eight validated mycotoxins for both methods, excluding FBs. The highest RSD_r is for AFG1 at spiked level of 1.0 μg/kg (25.25%) but this value is in accordance with criteria established by Commission Regulation EC No. 401/2006. Regarding each mycotoxin, method using Z-Sep has best repeatability for AFB1, AFG1, AFG2, OTA, ZEA and HT2 and similar repeatability to EMR-lipid for T2. However, for AFB2 the best repeatability was achieved with EMR-Lipid.

Precision inter-day of the method was evaluated by the Relative Standard Deviation (RSD_R) at 3 different days of analysis, 2 or 3 different concentration levels with different operators and the values are acceptable, ranging between 2.8 to 26.8% and 1.8 to 9.7%, for EMR-Lipid and Z-Sep method, respectively. For precision inter-day, clean-up using Z-Sep presented the best results for all mycotoxins, except for AFB2.

Matrix effect (ME) is caused by the alteration of ionization efficiency of target analytes in the presence of co-eluting compounds, affecting negatively analytical performance (Zhou, Yang e Wang, 2017). Z-Sep cause a signal enhancement for all mycotoxins, excepting OTA. ME was negligible for AFB1(SSE =107.0%), AFB2 (SSE = 107.1%), AFG1 (SSE = 103.3%) and HT2 (SSE = 108.1%) in Z-Sep clean-up, soft to AFG2 (SSE = 112.5%) and ZEA (SSE = 116.1%), and medium to T2 (SSE = 128.7%). For seven mycotoxins, Z-Sep gives the lowest matrix effect varying between negligible to medium (103 to 129%). This signal enhancement in AFs was found by Hidalgo-Ruiz *et al.* (2019) in pistachio using C18 (ME = 42-67%).

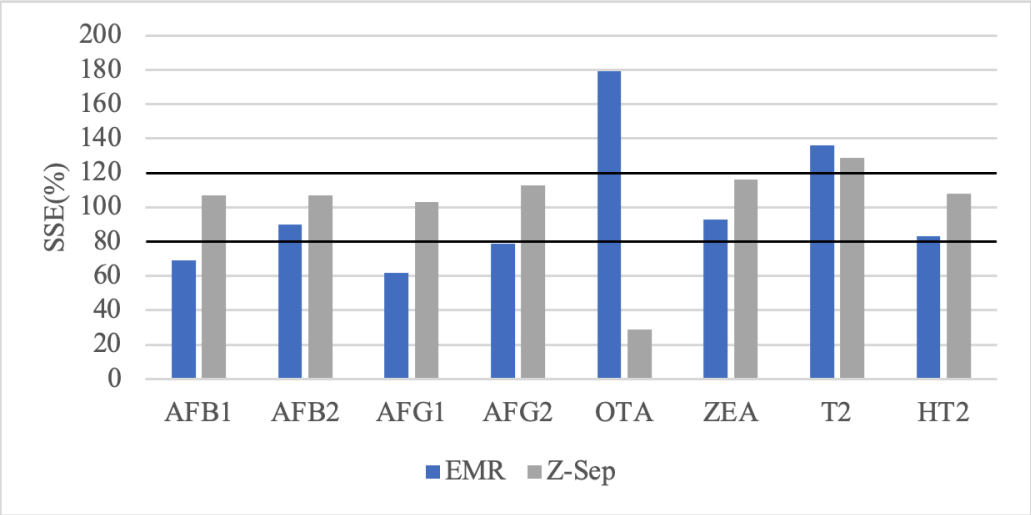
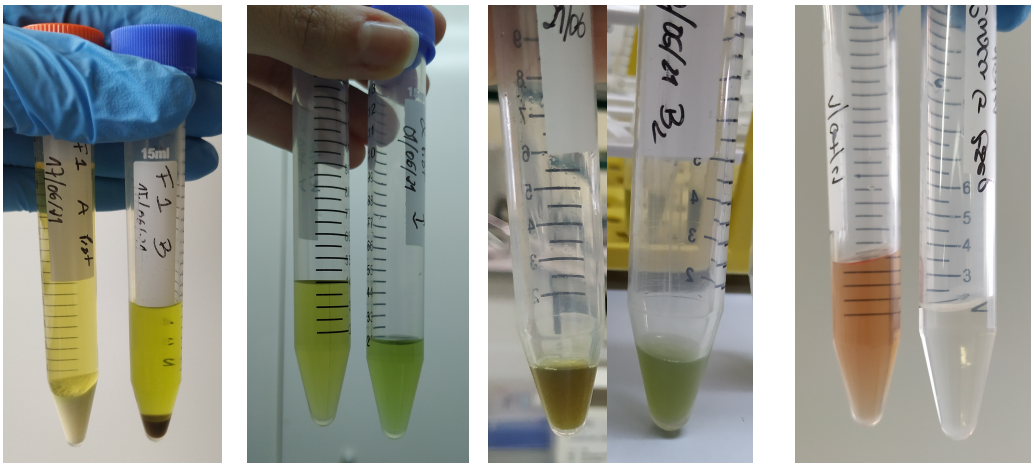


Figure 14. Matrix effect with enhanced matrix removal-lipid (EMR-lipid) and zirconium oxide (Z-Sep) as dispersive solid-phase extraction (d-SPE) sorbents for ten mycotoxins.

However, EMR-Lipid provided signal suppression for AFB1 (SSE = 69.3%), AFB2 (SSE = 90.1%), AFG1 (SSE = 62.4%), AFG2 (SSE = 79.7%), ZEA (SSE = 93.1%) and HT2 (SSE = 83.5%), only for HT2 are a medium signal enhancement (SSE =136.3%). However, in Alcántara-Durán *et al.* (2019) study, EMR-Lipid sorbent displays negligible matrix effect in all mycotoxins in pistachio samples (between 0 to 6%). It was noticed that, after the d-SPE clean-up step, Z-Sep sorbent gave a greener extract comparing to EMR-Lipid sorbent with yellow tone, so using Z-Sep as sorbent indicate a higher amount of pigment remained in the extract (**Figure 15**).



(a) (b) (c) (d)

Figure 15. Difference of colors among (a) extracts after the addition of sorbent and centrifugation at blank pistachio sample spiked with 1 mL of work calibration solution; (b) pistachio sample extracts before the evaporation step; (c) pistachio sample extracts redissolved with 500 μL of acetonitrile 40% (v/v) and (d) one samples of pistachio shell before the evaporation step with enhanced matrix removal-lipid (EMR-lipid) (Falcon tube at left) and zirconium oxide (Z-Sep) (Falcon tube at right) sorbents in dispersive solid-phase extraction (d-SPE) clean-up.

In case of OTA, it was a found a strong matrix effect, higher than 50%, using both sorbents, although Z-Sep provides a signal suppression (SSE = 28.8%) and EMR-Lipid a signal enhancement (SSE = 179.3%). The same strong matrix effect for OTA was reported by Cunha, Sá e Fernandes (2018) using 50 mg C18 and 50 mg Z-Sep+ as sorbents in nuts samples, ranging between 174.9 to 231.0%. Similar results are obtained by Arroyo-Manzanares *et al.* (2013) who found ME of -65.6% applying dispersive liquid–liquid microextraction (DLLME) in edible nuts; SSE of 194.1% in maize by Silva *et al.* (2019) and SSE = 180% in vegetable oils using C18 by Zhao *et al.* (2016).

Comparing methods for fumonisins (FB1 and FB2) is not possible because there is only analytical signal for clean-up with EMR-Lipid. The determination coefficient is good ($r^2 > 0.99$) between 25.0 to 200.0 mg/kg and 12.5 to 200.0 μg/kg, for FB1 and FB2, respectively. This method has good recovery (94.4 to 104% for FB1 and 91.1 to 103.5% for FB2), with good values for repeatability (2.2 to 5.7% for FB1 and 1.0 to 8.3% for FB2) and precision inter-day (between 2.6 and 4.56%) (**Table 2**). EMR-Lipid sorbent causes a strong signal enhancement, this fact as already reported by in maize samples with SSE = 123.6% (Tebele *et al.*, 2020) and SSE = 125.4% (Silva *et al.*, 2019).

Numerous multi-mycotoxin methods for pistachio, nuts and other foodstuffs based on QuEChERS methodology have been published (Abreu *et al.*, 2020; Alcántara-Durán *et al.*, 2019; Alsharif, Choo e Tan, 2019; Arroyo-Manzanares *et al.*, 2013; Hidalgo-Ruiz *et al.*, 2019; Jettanajit

e Nhujak, 2016; Jo *et al.*, 2021; Narváez *et al.*, 2020; Pantano *et al.*, 2021). The main difference among those methods is the clean-up step using Immunoaffinity Chromatographic Columns (IACs) or d-SPE with different mixtures of sorbents. IACs is very sensitive and selective technique due to specific of antibodies to mycotoxins, but (1) uses more solvents in washing and elution steps; (2) there is a possibility of cross antibody reaction; and (3) it depends on the availability of columns in the market concerning mycotoxins and matrices. In d-SPE, other authors include a freezing step that increases the time of analysis (Abreu *et al.*, 2020; Pantano *et al.*, 2021) or uses more than one sorbent which has a higher cost (Jettanajit e Nhujak, 2016; Jo *et al.*, 2021; Pantano *et al.*, 2021). Recently, some methods are based on "diluted and shoot" approach, a "no clean-up" technique that could affect the performance of the chromatographic equipment (Abreu *et al.*, 2020). So, QuEChERS with d-SPE using Z-Sep as sorbent, is a simple, rapid and easy technique for application to large number of samples in a short time, with less use of reagents, solvents and materials, allowing effective extraction of mycotoxins and removing lipids and other compounds present in pistachios that can interfere with the HPLC system.

Also, this validated UHPLC-ToF-MS method provides high sensitivity and specificity for identification, quantification and confirmation of multi-class mycotoxins, where identification of molecules is based on molecular weight. This MS detector has advantages when compared, for example, with previously used fluorescence detection (FLD) (Cheraghali *et al.*, 2007; Diella *et al.*, 2018; Fernane *et al.*, 2010; Shadbad *et al.*, 2012; tawila, El, Neamatallah e Serdar, 2013; Ulca, Evcimen e Senyuva, 2010) which is only applicable for AFs due to their fluorescent properties (AFB1 and AFB2 exhibit fluorescence at 425 nm, AFG1 and AFG2 exhibit fluorescence at 450 nm) (Kumar *et al.*, 2017) and required a derivatization step to increase resolution and sensitivity (Wacoo *et al.*, 2014; Zhang e Banerjee, 2020).

It is also important to refer that QuEChERS protocol involving EMR-Lipid sorbent was more time-consuming due to the two extra steps to active sorbent with water to achieve better efficiency and then step with $MgSO_4$ and NaCl to obtain a phase separation between H_2O and ACN (Hernández-Mesa e García-Campaña, 2020). In this study, to better compare between EMR-Lipid and Z-Sep sorbents, this second extra step was also applied, but in literature using Z-Sep there is no need (Hernández-Mesa e García-Campaña, 2020).

So, it could be concluded that Z-Sep sorbent is the most efficient way to remove matrix interferents, easier and faster, providing best analytical performance for multi-mycotoxins method ranging AFs, ZEA, T2 and HT2. However, for OTA, EMR-Lipid is the best option for clean-up due to the lowest LOD and LOQ and since mycotoxins are present at low

concentration in pistachio, Z-Sep provides better precision (repeatability and precision inter-day). For fumonisins, Z-Sep is not a good sorbent. In this case, EMR-Lipid provide good analytical performance.

It should be highlighted that although no legal limits have been defined for mycotoxins other than AFs in pistachio, climate changes have an impact on abiotic factors as temperature, water activity (a_w), relative humidity, and CO_2, known as critical factors to fungal growth and mycotoxins' production in field and/or during storage (Baazeem *et al.*, 2021; Marroquín-Cardona *et al.*, 2014; Medina, Rodríguez e Magan, 2015; Peter Mshelia *et al.*, 2020). Due to this fact, new multi-mycotoxin methods for determination of mycotoxins in pistachio nuts should be validated, in order to detect simultaneously more mycotoxins to ensure food safety.

2.3. Occurrence of mycotoxins in pistachio

In order to show the applicability of the method, sixteen samples of pistachio were analyzed using Z-Sep as sorbent for d-SPE clean-up and also using EMR-Lipid to determine fumonisins (**Figure 16, Figure 17**). Each sample was extracted in duplicate.

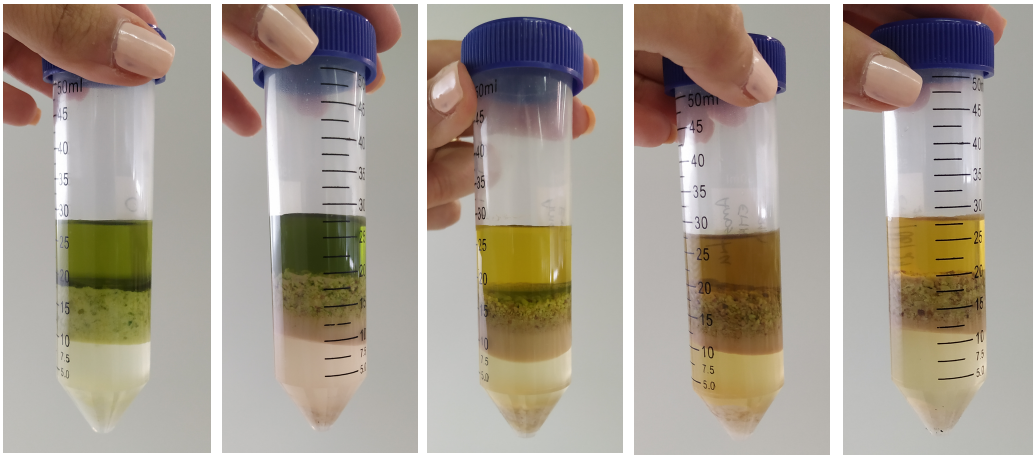


Figure 16. Example of 5 pistachio kernels samples after QuEChERS procedure.

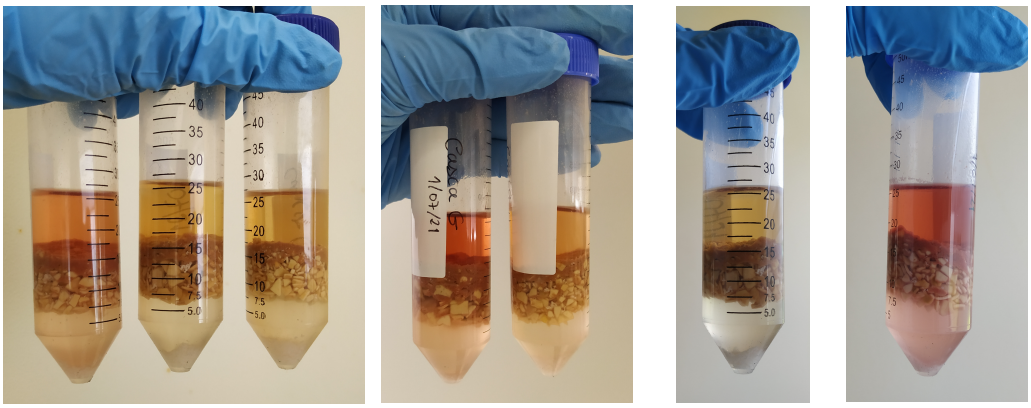


Figure 17. Example of 7 pistachio shells samples after QuEChERS procedure.

One sample, corresponding to a raw pistachio from Iran, was detected with 0.20 µg/kg of AFB1 (Figure 18). It should be noted that this concentration is following the current ML established by the EU for aflatoxins in nuts. Alcántara-Durán *et al.* (2019) also detected one sample with AFB1, but above LOQ and Liao *et al.* (2015) report two samples with 0.5 µg/kg and 1.2 µg/kg of AFB1.

AFB2 was detect in another sample at 0.73 µg/kg, lower than ML established for the sum of AFB1, AFB2, AFG1 and AFG2 (4 µg/kg) (Figure 18). Similar results are obtained by Liao *et al.* (2015) which detected in one out of ten pistachio samples at 0.9 µg/kg of AFB2. In this study, pistachio shells were analyzed and in two samples AFB2 was quantified (0.53 and 0.56 µg/kg).

HT2 was found in three sample at 50.63 µg/kg, 67.37 µg/kg and 71.56 µg/kg (Figure 18). These pistachios samples are in bulk sale, indicating that temperature and/or relative humidity conditions are not optimum to storage. Fumonisin B1 was detected in one pistachio kernel and shell from USA, but at a concentration lower than the LOQ (25 µg/kg) (Figure 18). However, no limits are established for fumonisins or HT2 in nuts.

Various studies reported occurrence of mycotoxins in pistachio nuts. Aflatoxins, especially AFB1, and OTA are the most frequently detected, and none of the studies from the last two decades show contamination with FBs, ZEA, T2 or HT2.

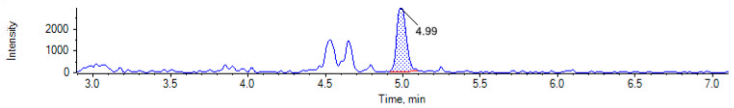
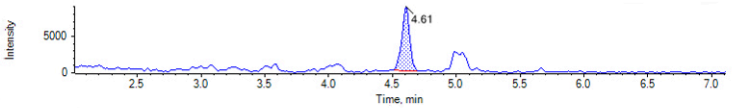
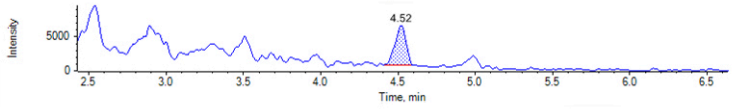
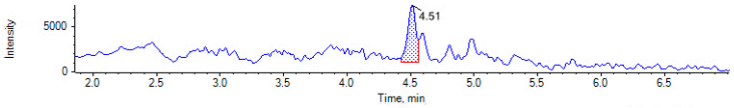
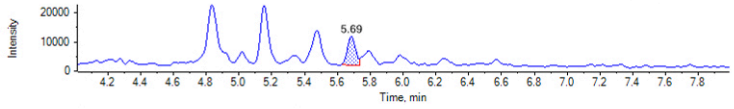
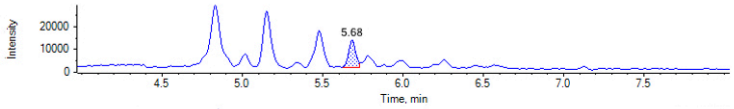
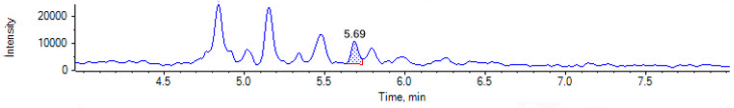
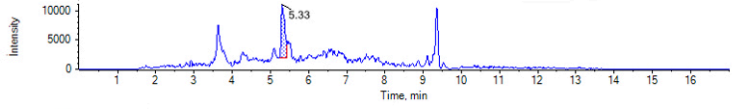
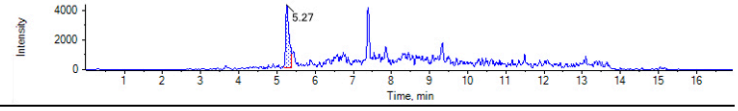
Pistacho sample	Mycotoxin	Chromatograms
O (kernell)	AFB1	
K (kernell)	AFB2	
C (shell)	AFB2	
E (shell)	AFB2	
B (kernell)	HT2	
E (kernell)	HT2	
P (kernell)	HT2	
I (kernell)	FB1	
I (shell)	FB1	

Figure 18. Chromatograms of pistachio samples with Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), toxin HT-2 (HT2) and Fumonisin B1 (FB1).

CONCLUSIONS

Pistachio (*Pistacia vera* L.) is considered a "healthy food", due to its nutritional level and its health benefits, but is also an important source of exposure to mycotoxins, especially aflatoxins, as a result of fungal contamination mostly in the field due to the early split of shell at the end of maturation. Research to monitor the levels of mycotoxins' contamination in pistachio is of utmost importance to ensure consumers health.

An analytical method based on QuEChERS followed by Ultra-High-Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry was validated for the simultaneous detection of eight mycotoxins in pistachios. For matrices with high lipid content, like pistachio nuts, it becomes evident that the clean-up step is fundamental for reducing interferences in the analysis and allowing a smaller number of maintenances in analytical equipment. The optimization of the extraction procedure included the evaluation of different sorbents, and lastly EMR-Lipid and Z-Sep are compared.

It was concluded that the use of 100 mg of Z-Sep provided best analytical performance, with good recovery (79 to 120%), repeatability (RSD_r <10%) and precision inter-day (RSD_R <10%) in agreement with criteria established by Commission Regulation EC No. 401/2006 for mycotoxins analysis. The LODs for AFs ranged from 0.125 to 0.25 μg/Kg, which are lower than the maximum levels in nuts regulated by the EU. Although for OTA, LOD and LOQ are lower using EMR-lipid, precision (RSD_r and RSD_R) is better using Z-Sep. Z-Sep procedure is easier and faster, comparing to EMR-Lipid sorbent which had to be active with water before clean-up. Method with EMR-Lipid sorbent also gives good performance for determination of mycotoxins, including fumonisins, according to criteria in Commission Regulation EC No. 401/2006. But, considering AFs as the mycotoxins of greatest interest in pistachios, contrary to FBs, Z-Sep sorbent provides more advantages. Furthermore, 6 of 16 real samples of pistachios were found to be contaminated with one mycotoxin (AFB1, HT2 or FB1) but at low concentrations. The concentration of AFB1 was lower than the permitted according to EU legislation. Also, AFB2 and FB1 were detected in pistachio shells.

In the near future, the results of this dissertation should be complemented and compared with pistachio samples from different countries, including a large sampling plan with other countries with a Mediterranean diet. Additionally, the developed method could be validated in other matrices as peanuts, dried figs and raisins, which have a higher consumption and play an important role on mycotoxins human exposure. Furthermore, the development of new methodologies for decontamination of pistachio nuts is needed, for example, a packaging with antifungal proprieties, to decrease the exposure of consumers to mycotoxins.

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