TESE DE DOUTORAMENTO

DEVELOPMENT OF SCREENING AND CONFIRMATION METHODS FOR AFLATOXINS ASSESSMENT IN FISH FEED AND AQUACULTURE PRODUCTS

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ESCOLA DE DOUTORAMENTO INTERNACIONAL

PROGRAMA DE DOUTORAMENTO EN CIENCIA E TECNOLOXÍA QUÍMICA

SANTIAGO DE COMPOSTELA 2020

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DEVELOPMENT OF SCREENING AND CONFIRMATION METHODS FOR AFLATOXINS ASSESSMENT IN FISH FEED AND AQUACULTURE PRODUCTS

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"Nothing in life to be feared. It is to be understood"

Marie Curie

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ACKNOWLEDGEMENTS

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In the presence world of competition there is a race of existence in which those are having will to come forward succeed. Ph.D project is like a bridge between the theoretical knowledge and the practical issues in the society. With this willing, I joined this perfect project. Foremost, I would like to express my sincerely gratitude to my PhD advisors, **Prof. Antonio Moreda Piñeiro** and **Prof. Maria Raquel Domínguez González** for the continue support of my Ph.D study and research for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helps me in all the time of research and writing of this thesis. I could not have imagined have a wonderful advisors/mentors for my Ph.D study.

Besides my advisors, I would like to thank the rest of my **GETEE** research group members, **Prof. Pilar Bermejo-Barrera, Prof. Maria Carmen Barciela Alonso, Prof. Elena Pena Vazquez**, **Prof. Manuel Abole Somoza and Dr. Paloma Herbello Hermelo** for their encouragement, perceptive comments, and great supports to my studies. My sincere thanks also goes to AeMAT research group who are collaborative with GETEE group to success all of our research work.

I thank for my fellow lab-mates in GETEE group for their stimulate discussion, for sleepless night, we were working together for before deadline and all the fun we have had in the last three years. And also, I thank all the professors and lab-mates in LIDSA group for enlightening me, to success my Ph.D work.

Last, but not the least, I would like to thank my mother and father who alive in my heart until my death, to delivered me for the wonderful science world and gave me huge encouragement to face every challenge in spiritual throughout my whole life.

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ABBREVIATIONS

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μ-SPE	Micro solid phase extraction
AA	Air-assisted
ACN	Acetonitrile
AFs	Aflatoxins
AIBN	2-2 azobis (2-methyl-butyronitril)
AR%	Analytical recovery percentage
CE-LIF	Capillary electrophoresis with laser-induced fluorescence detection
CMC	Carboxymethyl cellulose
CNTs	Carbon nanotubes
CRM	Certified reference material
D-μ-SPE	Dispersive solid phase microextraction
DAD	Diode array detector
DESs	Deep eutectic solvents
DLLME	Dispersive liquid-liquid microextraction
DLPM	Dispersive liquid-phase microextraction
DMC	5,7 dimethoxycoumarine
DMSO	Dimethyl sulfoxide
D-SPE	Dispersive solid phase extraction
DVB	Divinylbenzene
EC	European Commission
EF	Extraction efficiency
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization
FD	Fluorescence detector
FDA	Food and drug administration
FT-IR	Fourier transform infrared spectrometry
GAP	Good agricultural practices
GC	Gas chromatography
GCC	Gulf corporation council
GI tract	Gastro-intestinal tract
GO-PVPP	Graphene oxide polyvinyl poly pyrroline
HACCP	Hazard analysis and critical control points
HF	Hollow fiber

HLLE	Homogenous liquid-liquid extraction
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
IARC	International Agency for Research on Cancer
IB	Inkjet based
Ils	Ionic liquids
ISVAEME	In-syringe vortex assisted emulsification micro extraction
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LDS	Low density solvent
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
MAA	Methacrylic acid
MALDI	Matrix-assisted laser desorption ionization MS coupled
TOF	Time of flight
MAS	Magnetic stirring assisted
MIPs	Molecularly imprinted polymers
MIT	Molecularly imprinted technology
MMIP	Magnetic molecularly imprinted polymer
MMIP-SB	Magnetic molecularly imprinted stir bars
MOFs	Metal-organic frameworks
MPLs	Maximum permitted levels
MRL	Maximum acceptable/residue level
MRM	Multi reaction monitoring mode
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
MWA	Microwave-assisted
NCs	Nanocrystals
NIPs	Non-imprinted polymers
OPLC	Overpressure layer chromatography
PDA	Polydopamine
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PIPES	Piperazine-NN-bis (2-ethane-sulfonic acid) di-sodium salt
PLE	Pressurized liquid extraction

PP	Polypropylene
PVDF-HFs	Polyvinylidene fluoride hollow fibers
QDs	Quantum dots
QuEChERS	Quick, easy, cheap, effective, rugged and safe
RSD	Relative standard deviation
RTP	Room temperature phosphorescence
SBSE	Stir bar sorption extraction
SDME	Single-drop microextraction
SEM	Scanning electron microscope
SFE	Supercritical fluid extraction
SLM	Supporting liquid membrane
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
Ss	Switchable solvents
SUPRA	Supramolecular solvents
TEM	Transmission electron microscopy
TIC	Total ion chromatogram
TLC	Thin-layer chromatography
UA	Ultrasound-assisted
USFDA	United States Food and Drug Administration
UV	Ultraviolet detection
VA	Vortex assisted
XRD	X-ray diffraction spectrometry

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ABSTRACT

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Abstract

Aflatoxins (AFs) are a class of mycotoxins produced as secondary metabolites by fungi belonging to several *Aspergillus* species, and AFs named as AFB1, AFB2, AFG1, and AFG2 have been identified as the most dangerous and the most frequently found in food and feed. Among them, AFB1 is metabolized into a variety of hydroxylated derivatives, including AFM1, which are less toxic than the parent compounds. Human and animal exposure to AFs is a big health issue since these substances have proved to be carcinogenic, mutagenic and hepatotoxic, and to promote immunosuppression, and impairment of fertility in living beings.

Besides facing the complexity of many food/feed and environmental matrices, current analytical methods must be sensitive enough to detect and determine contaminants at very low levels. Sample pre-treatment techniques can be therefore useful approaches for a further interference-free and sensitive determination by modern analytical methods. The sample pre-treatment should be selective, fast, and repeatable procedures that avoid analytes losses and guarantee analyte integrity. This Thesis deals with the optimization of several sample pre-treatment techniques for AFs extraction from fish feed, fish muscle and liver, as well as the development of fast screening methods for AFs determination, and the assessment of AFs *in vitro* bioavailability in fish. Therefore, an overall strategy for studying the risks of AFs from cultured fish consumption (AFs contents in fish feed used in aquaculture facilities and in cultured fish species produced in Galicia, and AFs bioavailability from raw and cocked fish) has been developed.

The first chapter of the Thesis has been devoted to the synthesis and application of Mn-doped ZnS quantum dots (QDs) coated with molecularly imprinted polymers (MIPs) as a phosphorescent nanosensor for a sensitive and selective screening of AFs in fish feed. The synthesized nanocomposites exhibit an intense room-temperature phosphorescence (RTP), and AFs quench the RTP when interacting with the recognition cavities of the MIP layer attached to the QDs. The developed method showed a limit of detection (LOD) and a limit

of quantification (LOQ) lower than the AFs levels set in the EU regulation and allows a fast and low-cost determination of AFs.

The second and third chapters have been focused on the synthesis of MIPs for the selective micro-solid phase extraction (μ-SPE) of AFs from extracts from fish feed and fish flesh/liver. Before MIPs-based extraction/pre-concentration, an optimized ultrasound assisted extraction (UAE) procedure was applied for target (AFs) isolation. The first μ-SPE method (Chapter 2) consisted of MIPs enclosure inside a polypropylene membrane (porous membrane-protected molecularly imprinted polymer micro-solid-phase extraction, MIMSPE), whereas the second proposal (Chapter 3) was based on a vortex assisted molecularly imprinted polymer dispersive micro-solid phase extraction (D-μ-MISPE). Both μ-SPE showed high pre-concentration factors, good analytical recoveries and precision, as well as high selectivity for AFs and re-usability of the absorbent/absorbent device. The sample pre-treatments were applied to fish feed and cultured fish (flesh and liver) samples.

Moreover, the developed UAE was combined with a vortex assisted dispersive liquid-liquid micro-extraction (DLLME) procedure (Chapter 4), and results from applying this solvent-based micro-extraction technique were compared with those achieved by sorbent-based micro-extraction techniques (MIMSPE and D-μ-MISPE). The developed DLLME method was successfully applied to pre-concentrate AFs from fish extracts. In addition, DLLME was found to be highly robust for AFs pre-concentration from dialysates after performing an *in vitro* bioavailability approach when assessing human bioavailability of AFs from fish.

In a final chapter (Chapter 5), an *in vitro* bioavailability study based on a dialysability approach has been applied for AFs bioavailability from cultured fish flesh, and chicken and rabbit liver. In addition to AFs bioavailability in raw food, the influence of the culinary process (frying and steaming) on AFs bioavailability was also considered, and moderate AFs dialysability ratios were found for raw and cocked fish. Findings have also showed that AFB2 is partially transformed into other compounds during the in vitro dialysability process, and attempts based on high resolution mass spectrometry

were carried out for elucidating some transformation products from AFB2 in the dialyzable (dialysate) and non-dialyzable (residue) fractions.

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I. INTRODUCTION

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1. AFLATOXINS: EXTRACTION, DETECTION AND BIOAVAILABILITY

1.1 AFLATOXINS

Moulds from *Aspergillus* genus are the most important causes of food and feed spoilage and they can produce mycotoxins as toxic secondary metabolites under adequate environmental condition. Aflatoxins (AFs) are a group of mycotoxins that are categorized by the International Agency for Research on Cancer (IARC) as class 1A human carcinogens [1]. AFs were firstly found in 1960, and it was known as "turkey X disease". The disease was caused by the widespread death of more than 10,000 turkeys on a poultry farm in the south and east of England. As later reported, the disease was shown to be instigated by AFB1 contaminated feed imported from Brazil [2].

The AFs are classified chemically as furonocoumarin derivatives (Figure 1), and the biosynthesis occurs through the polyketide pathway [3]. Primarily, AFs are produced by two species of *Aspergillus* fungi named as *A. flavus* and *A. parasiticus* which are especially found in areas with hot and humid climates. *A. flavus* is an universal fungi, favouring the leaves and flowers like aerial parts of plants and produces only type B aflatoxins (AFB1 and AFB2). *A. parasiticus* is more adapted to the soil environment and has more limited distribution, and produces both B and G AFs (AFB1, AFB2, AFG1 and AFG2) [4]. Other *Aspergillus* species such as *A. ochraceoroseus*, *A. nomius*, *A. bombycis*, *A. pseudotamarii*, *A. tamarii*, and *A. oryzae* are known to produce AFs but the toxicological level is significantly low [5-7]. Aflatoxins M1 and M2 are the hydrolated metabolites from B1 and B2, and may be found in milk and milk products obtained from livestock after consumption of contaminated feed [8]. This mycotoxin is found in food and feed as a result of fungal contamination in both pre and postharvest. The degree of contamination depends on various factors such as humidity (water activity), temperature, nutrition sources and pH, sun light radiation (light and dark conditions), the substrate (age of the culture), storage conditions, etc. [9]. Aflatoxins have been found in a variety of

agriculture commodities, but most prominent contamination has been encountered in maize, corn, barley, nuts and oilseeds. These plant ingredients are commonly used to prepare the feeds for animals, and some of them are directly consumed by humans. Therefore, AFs represent a health risk to animals and humans. The current study is mainly focused on developing screening and confirmation methods for AFs analysis in fish feed and fishery products.

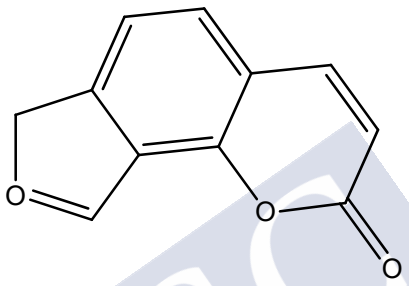

Figure 1: Furanocoumarin structure

1.2 TOXICOLOGICAL EFFECT OF THE AFLATOXINS

The acute effect of the AFs was well studied since they were isolated from peanut feeds during the investigation of the epizootic of "Turkey X disease" in England. Four major types of AFs (B1, B2, G1 and G2) and their metabolites (M1 and M2) have been recognized as hepatotoxins and hepatocarcinogenic. In addition, Q1 is the major metabolite from B1, and it was found in *in vitro* liver preparations of other higher vertebrates [10]. Currently, more than 14 AFs are known, but B1 is the most toxic and the most highly monitored and regulated AFs in foodstuff and feeds [11].

Health effects caused by AFs are mainly divided into acute and chronic toxicity, and carcinogenicity is most studied chronic effect [12]. Moreover, a number of other chronic health effects such as reproductive toxicity [13, 14], impaired growth of children [15], compromised immunity [16], neurotoxicity [17] and intestinal malfunctions [18, 19] are nowadays well-known.

The acute toxicity of AFs has been studied many times and the epidemiologic, experiment, and clinical studies proved that an exposure to above 6000 mg of AFs by oral ingestion can cause a

severe intoxication. Acute toxicity may cause direct liver damage, and the subsequent illness or death [20-22]. Lipid infiltration from the liver cells causes liver cirrhosis, necrosis and hepatocyte death. The symptoms of aflatoxicosis at the whole organism level regards mainly the reduction of critical blood proteins, vomiting, edema, icterus and abdominal pain [23]. Despite most of animals are not immune to the acute effects of AFs, some few animal, such as rats, have been shown to have a great tolerance to some extent. But, susceptible species such as rabbits and ducks have a very low lethal dose (0.3 mg kg^{-1}); whereas, chicken has a median lethal dose (18 mg kg^{-1}) [24]. Adult humans usually have a high tolerance for AFs, however, children are more sensitive to acute AFs intoxications, and acute poisonings (even children death) has been reported [25].

1.3 AFLATOXIN CONTAMINATION IN FOOD AND FEED

The occurrence and production of the AFs are dependent on geographical and climatic conditions, and also on the previous presence of the fungus in the same harvesting area, which implies an increase of fungus (and mycotoxins) in successive harvests. AFs were firstly identified in animal feed in 1960 [2]. *Aspergillus* contamination and AFs production can happen in crops themselves or supported by insect actions [26]. It occurs during transportation and storage, and it is promoted by several factors such as insect activity, poor timing of harvest, stress and damage conditions of the crops, heavy rain at the harvesting time, and inadequate drying of crops before storage [27].

These toxins have great thermal stability and persist in some cooked foods. However, some food processing methods such as boiling, roasting, baking and steaming destroy AFs to some extent. Moreover, AFs remain in the food after the fungi have disappeared and freezing has a little effect on their presence in food.

AFs contamination represents a major issue not only in food, but also in feeds. There are two types of feeds: animal origin feeds and plant origin feeds, the later are the most common and it is based on maize, barley, wheat, cottonseed, corn and oat. A good level of hygiene of the animal food products, the moisture content and temperature of the feed are needed for avoiding fungi growth and the

subsequent AFs production. Factors promoting AFs formation in the feed depends on the type of feed and the quality of feed storage [28, 29]. Contamination can occur during processing of products, pre and post-harvest stages of the ingredients (improper drying of grains after harvest), poor storage, insect attack, and non-use of mould inhibitors [30]. Natural contamination in feed has been studied extensively for cattle, swine, fish and poultry [31]. In general, studies on the presence of AFs in several animal feeds were reported in past decades. Kichou and Walser (1993) analysed 315 samples of poultry feeds made of corn, wheat, soybean meal, sunflower meal, cottonseed meal and sorghum as feed ingredients. They reported AFs contamination levels within the 20-200 μg kg^{-1} range, and four samples reached AFB1 levels from 2000 to 5625 μg kg^{-1}. They observed later that the highly contaminated feed samples were linked to clinical aflatoxicosis in chicken [32]. Several studies have also shown the presence of AFM1 in the milk of sheep, cows, goats, camels and buffaloes [33-35]. The relationship between AFB1 and AFM1 in excreted in milk was found to be varied with the animal breed, milk production, and frequency of the daily milking.

Moreover, several studies were carried out for investigating the presence of AFs in fish feed [36, 37]. Foodstuffs such as fish meal, soybean, peanut, rice bran, corn, sunflower meal, cottonseed cake and other cereals have been used for fish feed preparation [38]. Feed contaminated with AFs trends to cause growth obstructions, increase the mortality of the cultured fish, decreased the productivity and quality of the fishery products [39]. In addition, feed contamination indirectly causes to risk for human health through the transformation of the AFs residues from fishery products [40].

Due to the potential health hazardous of AFs, efforts have been done to completely eliminate the toxins or to reduce its content in food and feed. There are many techniques including extraction with solvents, such as hexane, water, acetone, ethanol, and methanol [41] for removing AFs in oilseed before used to feed preparation. On other occasions, AFs reduction in food and feeds was achieved by adsorption in bentonite clays [37] and hydrated sodium calcium aluminosilicate [42]; by gamma rays [43], and by ultraviolet [44] and

solar irradiation [45]; and by thermal treatments [46]. Moreover, the specific lactic acid bacteria strains (physically bound in liquid media) has been found to efficiently remove AFs from foodstuff [47, 48]. All these detoxification technologies are not applicable for most food and feed, but the bentonite clay and the lactic bacteria methods are cost-effective and capable to AFs detoxification without adverse side effects, and they could offer significant benefits in human and animal health [49, 50]. The application of good agricultural practices (GAP) and the establishment of hazard analysis and critical control points (HACCP) system are also essential for the control of AFs in feeds. In addition, the disinfection of the feed millers and the use fungal growth inhibitors to feed are other useful methods for AFs control. However, the restriction of fungicide added in organic farming may affect the quality of the feed ingredients used [28].

1.4 LEGISLATION FOR AFLATOXINS

AFs are subject to regulations at the national, regional, and international level. The main objectives of the regulations are to protect public health and guarantee the safety of food and feedstuff envisioned for human and animal consumption. An effective legal framework should include 5 major components. There are:

1) Regulatory limits
2) Monitoring to ensure compliance with limits
3) Guidance to industry
4) Cooperation between agencies on food safety
5) Enforcement action

In 1969, the United Nation Food and Drug Administration (USFDA) issued the action levels for AFs. This is the firstly issued document in the world. In 1998, the European Union (EU) introduced the most extensive and detailed regulation regarding AFs in various foodstuffs and feedstuff [51]. Based on the Food and Agriculture Organization (FAO) assessment regarding the AFs, lots of countries have prepared regulations for mycotoxins [52, 53].

1.4.1 European Union Regulation

Foodstuff containing contaminants shall not be placed on the market since their consumption can affect public health. Moreover, contaminant levels shall be kept as low as reasonably achievable levels through all stages such as production, processing, treatments and storage. Due to the fact that AFs occur naturally in food, it is not possible to impose a total ban on them. Therefore, restriction action was necessary to establish their maximum concentration but to minimize the health risk. The council regulation (EEC) No. 315/93 [54] was published based on the legal framework on the contaminants in foods. Later, the commission regulation EC No. 466/2001 was published regarding the maximum levels of certain contaminants in foodstuffs including AFs. In order to kept the contaminants levels as low level by following good practises at all stages of food processing, the subsequent amendments of EC No.1882/2003 and EC No. 569/2009 where necessary to confirm the maximum tolerance for specific contaminants established by EC [55]. Due to the hazardous effect of the AFs, it is necessary to take the restriction action on their maximum concentration levels and minimizing any risk to human health. Replacing the former EC No. 466/2001 regulation [56], the commission regulation EC No. 1881/2006 [51] setting maximum levels for AFs in food and feed, and it is amended by the EC 165/2010 [57] which justifies the potential increase of health risk by possible increase of the existing maximum levels for AFs in pistachios, hazelnuts and almonds, and their derived products. According to the EC No 1881/2006 and EC No. 165/2010 regulations, maximum levels for certain AFs and other contaminants in foodstuff and feedstuff have been highlighted. The regulation includes also general rules regarding prohibitions on use, mixing and detoxifications, reference regulations for sampling and analysis (official control of the maximum level), and rules for monitoring and reporting.

In addition, the foodstuff listed in the annex of the regulation cannot be placed on the market where they contain a contaminants (AFs) at a level exceeding the maximum level set out [51]. Maximum levels for AFs set by the EU should be subjected to a range of foods and feed ingredients. As an example, the maximum level of AFB1 in

tree nut, oilseeds, and cereals (rice, corn and other cereals) should be 2 μg kg^{-1} (the maximum level of total AFs should be 2-8 μg kg^{-1}). Similar as food, feedstuff for animals are subject to tolerance level for AFs, and the maximum level of AFB1 in the feedstuff should be 20 μg kg^{-1}. EU legislation on AFs in food and feed is regularly amended and updated with new scientific evidences, and it is essential in order to safeguards public health, to place in the market safe and healthful products, and safeguard the important role of trade.

1.4.2 United State Food & Drug Administrative Regulation

Economic losses in the livestock and poultry sectors in the United States (US) were associated with the presence of mycotoxins (especially AFs), which led to lower animal production, animal illness and deaths. Moreover, this is a big issue for humans since foodstuff derived from contaminated livestock and poultry. US government was strict regarding the feed control for large scale animal production units [58]. The case of AFs appears in the food drug and cosmetic act (FD&C act) under the section of 402 (a) (1) known as "adulterated" whereas naturally occurring food and feed contaminants. The Food and Drug Administration (FDA) is responsible for the safety of all foods and they evaluate whether adulteration of food or feed (domestic or imported) has taken place and developed the regulations and guidance [59].

To reduce the naturally occurred contaminants in foodstuff and feedstuff, FDA issues policy guidance consists of

1) Advisory form – Guidance to the industry concerning levels for the contaminants present in food and feed
2) Action level – Precise level of contamination at which the agency is prepared to take the regulatory action
3) Regulatory level –Valid regulations under the public notice

For AFs, FDA has established action levels in human food, animal feeds and animal feed ingredients and some of them are shown in Table 1.

Table 1: Action levels for AFs in some human foods, animal feeds and feed ingredients

Products	Intended used	AFs level (ppb)
Milk	Direct consumption for human	0.5
Food, peanut and peanut products, Brazil nuts, pistachio nuts	Direct consumption for human	20
Corn and peanut products	Finishing for beef cattle	300
Cottonseed meal	Beef cattle, poultry, swine	300
Corn and peanut and other animal feed and feed ingredients	Immature animals	20
Peanut products, cottonseed meal, and other animal feeds and feed ingredients	Dairy animals, animal not listed above, or when the intended use is unknown	20

FDA also ensures that imported products meet US standards by regulating importers' responsibility to verify that their foreign suppliers have adequate preventive controls in place and checking the third-party quality certification (assurance the food quality) which complies with US food standards.

1.4.3 Regulations for AFs in other countries

Except for the EU and the US some other counties also followed the regulations for AFs in foodstuff and feedstuff [60]. Other countries such as Turkey, Bosnia, Herzegovina, and Switzerland seem to be inspired by the EU regulation and tend to have comprehensive legislation controlling the level of aflatoxins. Maximum limits for AFB1 and total AFs in several foodstuff have been published by India, Canada, Australia, New Zealand, Gulf Corporation Council (GCC) including Saudi Arabia, United Arab Emirates, Kuwait, Bahrain, Oman, Yemen, and Qatar, and Nigeria. Total aflatoxin limits in some countries are listed in Table 2 [61].

Table 2: Total aflatoxin limits in some different foodstuff

Country	Foodstuff	Total AFs (μg kg^{-1})
Australia/New Zealand	Peanuts/ Tree nuts	15
South Africa	Peanuts	15
Canada	Nut and nut products	15
Nigeria/GCC	Almonds, hazelnuts pistachios, shelled Brazil nut (direct consumption)	10
	Peanuts, almonds, shelled brazil nut, etc. (further processing)	15
India	Wheat, maize, sorghum, rice, groundnut kernels, and other cereals and foods	30
Vietnam	Peanut and other oilseeds as raw materials for foods	15
China/Egypt	Peanuts (ready to eat)	10

It is evident that the established maximum tolerance levels vary to a great extent among countries. This indicates the great difference in the risk assessment of each country and region. Most of developing countries which are located in tropical areas encounter greater contamination of AFs and other mycotoxins in food and feed, but most of these countries export their products to developed countries. In that context, developing countries need to balance their policy taking into account trade interests, food security, and food safety issued. However, countries may establish different limits upon scientifically based evidence and maintaining their own regulations for foreign and local markets.

1.5 SAMPLE EXTRACTION METHODS FOR AFs

Sample pre-treatment is the main time factor in analysis, and it will affect the final choice of the detection procedure. The extraction method and the extraction solvent for AFs isolation are dependent on the sample matrix [62]. The purity of the analyte affected directly the sensitivity of the determinations and therefore, clean-up protocols are

important to ensure that the trace amount of analyte is not masked by interfering compounds from the sample matrix, chemicals and/or solvents used [63]. Several methods have been proposed for AFs extraction and some of those are briefly described in the next sections.

1.5.1 Liquid-based extraction methods

Liquid-Liquid Extraction (LLE) involves two different phases such as an aqueous phase and an immiscible organic phase. AFs (target compound) extraction into an organic solvent must guarantee that the matrix components remains in aqueous phase [64]. Solvents such as hexane and cyclohexane are used to remove non-polar contaminants such as lipids and cholesterol from biological samples [65]. However, this procedure is time-consuming and is matrix dependent. Some miniaturized LLE methods such as homogeneous LLE [66], Liquid-Phase Microextraction (LPME) techniques such as Hollow Fiber Liquid-Phase Microextraction (HF-LPME) [67], and Dispersive Liquid-Phase Microextraction (DLPME) [68,69] have been introduced. These techniques are promising tools in Analytical Chemistry and are appealing procedures for sample clean-up because of the low detection limits offered (high pre-concentration factors), and they are environmentally friendly approaches.

1.5.2 Solid based extraction methods

Solid-Phase Extraction (SPE) techniques are by far the most popular technique for AFs extraction [70]. The principle of this technique is the analyte interaction with a solid sorbent, which is eluted with an appropriate organic solvent after a washing step. Sorbents such as C-18, silica gel, and phenyl or amino-propyl bonded phases are commonly used for SPE (column and cartridges operation mode) [71]. The main drawbacks of SPE are low selectivity and the high usage of the solvents. Solid-based microextraction techniques based on the use of a solid sorbent became widely used procedures and many new approaches has been introduced in Analytical Chemistry. Solid-Phase Microextraction (SPME) was used to extract AFs from different sample matrices [72,73]. Novel nanocomposite based Hollow Fiber

SPME (HF-SPME) was also applied for extracting AFs in cereals. Advantages of HF-SPME compared with traditional extraction techniques are mainly the simplicity, the reduction in extraction time, and cost, and that can be applied for analyzing the AFs at very low level [74].

Stir Bar Sorption Extraction (SBSE) is another solid-based microextraction technique which has been also used for AFs extraction, and which is inexpensive, simple and efficient [75]. Micro Solid Phase Extraction (μ-SPE) is a novel technology that has been used for AFs extraction in combination with Molecularly Imprinted Polymers (MIPs) for increasing the selectivity and ability to treat complex matrices [76].

1.5.3 Combined extraction techniques

There are several problems that must be faced when assessing trace amounts of toxic and essential compounds. As an example, co-extraction of other components of the sample can affect the quality of measurements since the high background signals and interferences and the low concentration of the target analytes, mainly in solid materials. To overcome these problems, several attempts have been focused on combining two sample pre-treatments, the first process for targets extraction, and the second one for targets pre-concentration [77].

On other occasions, two sample pre-concentration methods can be combined and regarding AFs, there are several developments of extraction techniques such as dispersive micro SPE (D-μ-SPE), and dispersive SPE (D-SPE, QuEChERS) combined with DLLME [78]. In addition, most of pre-concentration methods, such as DLLME and D-μ-SPE, can be assisted (speed up) with vortex stirring, ultrasound, and air, and several examples for AFs can be found in the literature [79].

1.6 ANALYTICAL METHOD FOR AFS DETERMINATIONS

For the purpose of the determination of AFs, different screening and confirmatory analytical methods have been developed in the last decades. Most of these analytical methods have to be performed after using the appropriate pre-treatment method. As screening methods, the Enzyme-Linked Immunosorbent Assay (ELISA) is a semi-

quantitative, fast, repeatable, and sensitive procedure for AFs determination [80, 81]. Other screening methods recently proposed have been based on molecularly imprinted polymer Mn-doped ZnS quantum dots (MIP-QDs) composite as a phosphorescence probe for the determination of total AFs [82]. The results indicate that a phosphorescence nanosensor is a versatile tool which has excellent sensitivity and selectivity.

Methods for single AFs quantification required sophisticated separation techniques such as High-Performance Liquid Chromatography (HPLC) [83], Gas Chromatography (GC) [84]. HPLC and GC are usually hyphenated with Mass Spectrometry (MS), and HPLC-MS, HPLC-MS/MS and GC-MS are the separation/detection techniques of choice [76,85]. HPLC with Fluorescence Detector (FD) has been also reported as a highly efficient, sensitive and resolution method for AFs determination. However, HPLC-MS and HPLC-MS/MS offer the lowest limits of detection for AFs.

1.7 BIOAVAILABILITY OF AFs

Bioavailability is an important assessment that is used to understand the absorption of trace elements or any other compounds in the foodstuff that are theoretically released in the gastrointestinal tract and become available for intestinal absorption [86]. Bioavailability is a combination of several steps, such as digestion, absorption, transport, utilization, and elimination [87]. There are two types of bioavailability approaches such as *in-vitro* and *in-vivo* methods. An *in-vivo* method is performed by using isotopes in humans (especially trace elements), and it gives the best estimation of bioavailability. Bio-accessibility is the simplest *in-vitro* method that indicates the maximum fraction of a substance in food that can theoretically be released from the foodstuff in the Gastro-Intestinal tract (GI tract) (bio-accessible fraction) and becomes then available for intestinal absorption (enter into the bloodstream) [88].

Bioavailability and bio-accessibility of the AFs have been evaluated by several authors in several foodstuffs [89-91]. It is very

important to know how much of compound absorption to the intestine and enter the bloodstream.

In addition, several strategies, based on bioavailability approaches, studies have been discussed to reduce the absorption of AFs by livestock and poultry since there is no commercially available treatments for effectively destroy AFs and fungi toxins in feed [89]. Galvano *et al*. have proposed the prevention of mycotoxicosis in livestock through non-nutritional adsorbents in the diet which are bind with mycotoxins in the gastrointestinal tract. The suggested technology has capable of reducing mycotoxin bioavailability [92]. Moreover, a wide range of adsorbents such as activated carbon, hydrated calcium, zeolites, bentonite, and aluminosilicates has been tested to check the potential in feed to overcome mycotoxicosis. Those adsorbent materials in the livestock industry have led to the introduction of a wide range of new products, but Kolossova *et al*. have reported that there is not an approved status in the EU market regarding the use of mycotoxin binders in animal feed [93].

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CRITICAL REVIEW OF MICRO EXTRACTION TECHNIQUE FOR AFLATOXIN DETERMINATION IN FOOD AND FEED

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Critical review of microextraction technique for aflatoxin determination in food and feed

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ABSTRACT

Aflatoxins (AFs) are the secondary metabolite compounds that grow on the foodstuff and animal feeds which affect to causing the disease and death in humans and animals. In this review, the authors summarized the innovative sample pre-treatment method for extract and clean-up the AFs in food and feed samples. These efforts resulted in the innovation of sorbent based and liquid-based, and combine microextraction techniques, which are considered the field of green chemistry. Both sorbent based and liquid-based microextraction techniques are used for AFs extraction and liquid-based microextraction (LPME) technology is more popular due to the effective extraction of target compounds form different sample matrix. Solid phase microextraction (SPME), hollow fiber SPME and stir bar sorption extraction (SBSE) are the main techniques used for AFs extraction. There are three types of LPME techniques named on single-drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME), and hollow fiber liquid-phase microextraction (HF-LPME). Among these LPME techniques, DLLME is a mostly used AFs extraction technique and it has a range of applications for different sample matrix. Compare to the other techniques, DLLME is characterized by the most simplicity of the operation, low cost, low time consuming, high enrichment factor,

good recovery, which makes is as to be one of the most widely used LPME technique for AFs analysis.

Keywords: Aflatoxins, Green chemistry, Microextraction, Dispersive, Liquid-liquid microextraction, Solid-phase microextraction

2.1 INTRODUCTION

Food and feed contaminated with mycotoxins has a negative impact on the economies of the agricultural sector in developing countries where harvest and post-harvest technologies of the moulds (fungi) growth prevention are not adequately implemented [1]. Grains including maize, oat, barley, wheat, and groundnut are the main source of contaminants especially mycotoxins which represent a significant part of human and animal diets and play a major role in industrial food and feed processing. Moulds under favourable humidity and temperature conditions may produce mycotoxins before and after harvesting, handling, storage, and shipments. Aflatoxins (AFs), mainly B1, B2, G1, and G2, as well as fumonisin B1, T-2 toxin, zearalenone, ochratoxins A, and deoxynivalenol are the most important mycotoxins [2].

Among these mycotoxins, AFs are the current concern and have a great deal of attention among the past three decades. Goldblatt *et al.* reported the first research about AFs due to the death of more than 100,000 turkeys on the poultry farm in England, that was found to be related to the consumption of Brazilian peanut meal [3]. AFs are known to be produced by two species of *Aspergillus* genus, especially *Aspergillus flavus* and *Aspergillus parasiticus.* AFs are highly toxic, carcinogenic, mutagenic, teratogenic, compounds that exhibit an immunosuppressive activity, causing both acute and chronic toxicity for humans and animals [4, 5]. Among them, AFB1 is the most potent liver carcinogen in mammals, and it is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) [6].

A. flavus is ubiquitous, favouring the upper part of the plant (leaves, flowers) and produced only B aflatoxins (AFB1 and AFB2). However, *A. Parasiticus* has a more limited distributed, produces B

and G aflatoxins (AFB1, AFB2, AFG1, and AFG2), and they are more adapted to a soil environment. Pittet *et al*. noted that the highest risk of AFs contamination is due to the more frequent growth of *A. flavus* fungi [7]. AFs known as M1 and M2 are the hydroxylated metabolites of AFB1 and AFB2, respectively. These metabolites may be found in the milk and dairy products obtained from the livestock that have consumed AFs contaminated feed [8]. Other well-known *Aspergillus* species such as *A. nomius*, *A. oryzae*, *A. tamarii*, *A. bombycis*, *A. foetidus*, *A. ochraceoroseus* produce AFs, but their toxicity is significantly low [9].

Chemically, these compounds have difurano-coumarin derivatives (Figure 1) which are related to the high toxicity of AFs. Lage dose exposure (>6000 mg) of AFs may cause acute toxicity with lethal effect and also, long term ingestion of diet contaminated with AFB1 has been associated with a high risk of liver cancer [10]. The rank order of the AFs toxicity is B1>G1>B2>G2. Moreover, routine consumption of the AFs contaminated food may happen in developing countries where the populations are suffering from starvation or where regulations are either not enforced or non-existent. Due to this reason, the incidence rate of liver cancer worldwide are 2-10 times higher in developing countries than developed ones [11, 12].

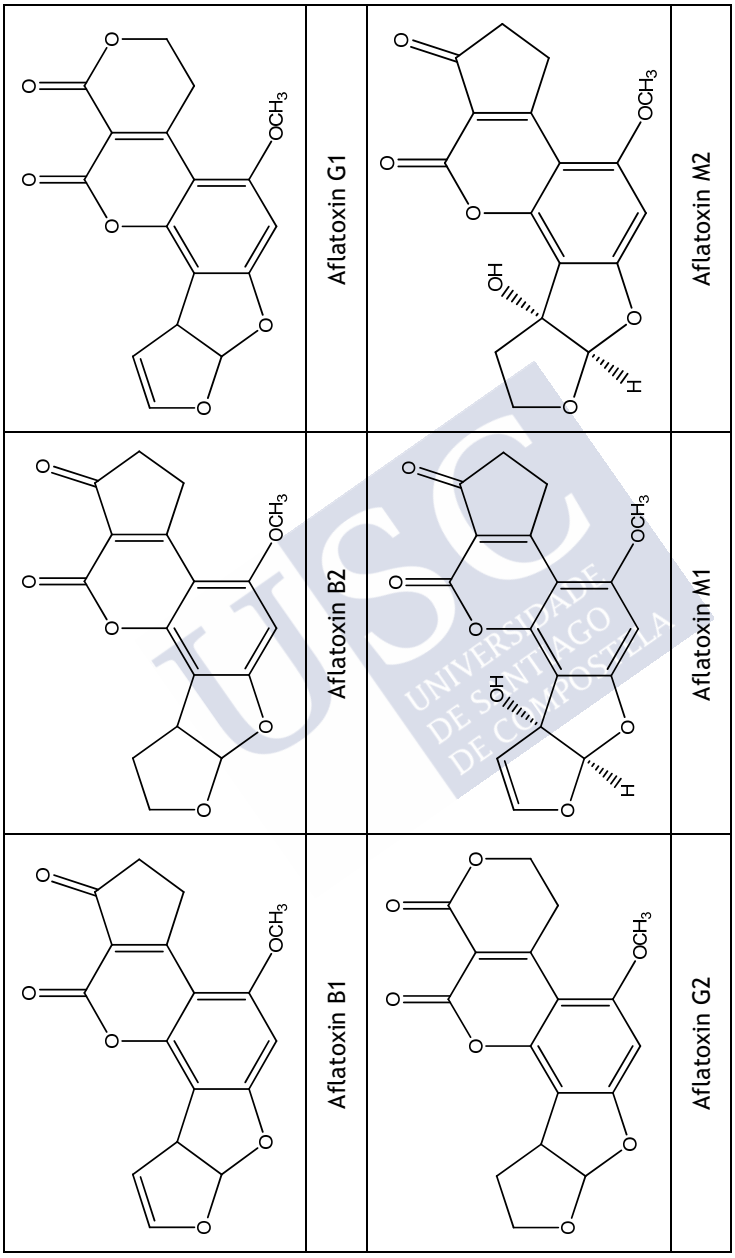

Figure 1: Structures of the aflatoxins

Since the discovery of AFs in 1960, regulation has been focused by many countries to protect the consumers from the harmful effects of these toxins that may contaminate both foodstuff and feedstuffs. The maximum permitted levels (MPLs) of AFs in foods (AFB1 and total AFs level) were reported under the commission regulation (EC) no 1881/2006 amended by the commission regulation no 165/2010. The maximum level of AFB1 and total AFs and AFM1 in food and feedstuff have been published by several Asian countries including China, Singapore, Indonesia, Malaysia, Japan, Korea, and India [13]. From a practical point of view, the best approach is AFs removal from food/feed by preventing the growth of the fungi at the food/feed harvesting, transporting, and storage stages. Otherwise, once AFs contamination has occurred, AFs removal from food and feed is very difficult. Therefore, routine monitoring and analysis of AFs in food and feed are very important to reduce the risk of aflatoxicosis.

For AFs determination, several screening and confirmatory analytical methods have been used. Since AFs are not homogeneously distributed in food and feed, high contaminated hotspots can occur. Thus, sampling, clean-up, and pre-concentration are the most important steps in the analysis. Interfering substances removal is essential during the clean-up stages, while extraction must be quantitative for the analyte.

Several extraction procedures have been proposed for AFs isolation. Liquid-liquid extraction (LLE), a conventional technique based on the analyte distribution in two non-miscible phases, has been used by Maia *et al.* [14] and Chun *et al.* [15] for AFs extraction from cereals and grains in Brazil pet foods and nuts, respectively. The major drawbacks of these methods are the usage of high amounts of organic and chlorinated solvents and the presence of background interferences. As a result, this method is being replaced by solid-phase extraction (SPE) and/or immunoaffinity extraction (IAC). SPE and IAC imply that the analyte interacts with the solid sorbent, and after a washing step, the analyte is eluted with the proper organic solvent. Adsorbents such as C-18, silica gel, phenyl, or amino-propyl bonded phases were commonly used for column/cartridges based SPE [16]. Regarding AFs, SPE procedures have been used when pre-treating

extracts from corn, feed, and milk samples [17-19]. The main drawbacks of the SPE are, however, the low selectivity and the high usage of the solvents. Better selectivity can be achieved when using IAC, which is based on the interactions of analytes and immobilized specific antibodies on the surface of the column [20-24]. Both IAC and/or SPE columns have been used coupled on-line with HPLC. This type of sample preparation has the advantage of the large number of samples that can be purified automatically. However, IAC has been reported to suffer from pressure instability during the pre-concentration stage [25-27].

Other conventional sample pre-treatment methods such as matrix solid-phase dispersion (MSPD) and supercritical fluid extraction (SFE) have been also used for AFs isolation. Nuts have been analysed for AFs after MSPD by using C-18 and sand as solid dispersants under conventional blending/homogenization (use of mortar and pestle) and ultrasound sonication. The same clean-up stage using the carbo-graph-4 SPE column was finally used for both MSPD procedures. However, the authors have pointed out that an extra clean-up procedure is still needed due to the low recoveries obtained and the lack of selectivity [28]. Similarly, Liu et al (2007) have used supercritical fluid extraction (SFE) to extract AFs from Chinese traditional medicinal plants, obtaining recoveries from 28 to 105% [29].These conventional sample pre-treatments have been with analytical techniques such as thin-layer chromatography (TLC), immunochemical tests (ELISA), high-performance liquid chromatography coupled with fluorescence detection (HPLC-FD), tandem mass spectrometry (LC-MS-MS), matrix-assisted laser desorption ionization MS coupled with time of flight (MALDI-TOF) for developing several methods for AFs assessment in food and feed [30-37].

Miniaturized sample preparation techniques are, however, preferred since they require a smaller volume of organic solvents than conventional techniques, and they follow some of the principles of Green Chemistry such as the lower amount of organic toxic solvents, low amount of sample and reagents, low amount of waste products, ease of target isolation/pre-concentration, minimum sample contamination, and short times for sample pre-treatment [38]. The current review is focused on microextraction techniques (solid sorbent based microextraction and liquid phase based microextraction techniques) for AFs extraction from

food and feed. Discussion regarding microextraction techniques for target identification, pre-concentration and/or clean-up purposes, as well as analytical parameters and methodological issues (enrichment factors, LOD and LOQ values, analytical recovery, etc.) of the proposed procedures, have been addressed.

2.2 SORBENT-BASED MICROEXTRACTION TECHNIQUES

New sample pre-treatment methods have been developed in two separate ways to reduce the drawbacks of classical methods and their impacts on the environment. The first direction uses miniaturization as a key factor to reduce reagents/solvents and wastes, yielding microextraction techniques; the second direction is led to find more environmentally friendly solvents for sample pre-treatment. Sorbent-based and liquid-phase microextraction techniques fit to the principles of Green Chemistry [39].

Sorbent-based microextraction methods can be considered as solventless or free-solvent approaches, and the extraction efficiency achieved by using these techniques is highly depend on the extraction solvent used [40]. However, these methods have several advantages such as target stability during the extraction, high affinity though the target molecules, compatibility with conventional chromatography conditions, and easy to use and fastness [41-43]. Microextraction based on solid sorbents became a widely used technique and many new approaches were introduced in Analytical Chemistry. According to the extraction procedure, this technique can be classified as fiber-based solid-phase microextraction (SPME), in-tube microextraction, and stir bar sorptive microextraction (SBSE) [44].

In 1989, Belardi and Pawliszyn developed the solid-phase microextraction (SPME), the technique of great importance in the field of green analytical sample techniques [45]. The fiber should be cleaned and conditioned before using for target pre-concentration. These stages guarantee contaminants removal from the fiber's surface (low influence for high background signals) and provide also special

characteristics to the fiber for enhancing the interaction with the analytes. SPME operation implies the needle pierces the septum of a vial containing the sample, and then the fiber is extended into the sample. Fiber and target interaction can occur in two different ways such as headspace (HS) and direct immersion (DI). In HS-SPME technique, the fiber is exposed only to the vapour phase above a liquid or a solid sample, but in the DI-SPME technique, the fiber is directly immersed in the liquid sample (sample extract) [46].

A new SPME-HPLC technique known as in-tube SPME was recently developed using an open tubular fused silica capillary column as the SPME device. In-tube SPME consists of the capillary column that has an internal coated stationary phase which helps to extract the targets from aqueous samples and desorbs them by introducing the moving mobile phase or a static desorption solvent [47, 48]. The device is suitable for automation and can continuously perform extraction, desorption, and injection using a standard autosampler.

SPME device has enough sensitivity for most applications, but sometimes it is limited by a small amount of coating materials on the needle. It affects low extraction efficiency for some compounds. Novel approaches such as SBSE have been introduced to increase the extraction efficiency [48, 49]. This technique offers a large volume and area of extraction phase and it opens technical innovations in high-throughput and automation for new SPME technologies. SBSE is prepared by removing the Teflon coating of existing Teflon bars, reducing the outer diameter of the magnet, coating the magnet with a glass tube, and covering it with polydimethylsiloxane (PDMS) polymer [50]. The device is placed in the vial containing the liquid sample (sample extract), and extraction is led by magnetic stirring.

SPME technique has lots of advantages over classical sample preparation techniques, but it already has significant drawbacks such as fiber breakage, stripping of coating, bending of the needle, and their expense. Other problems are associated with the relatively low recommended operating temperature and their instability and swelling in organic solvents [51]. To overcome these problems, a series of other micro extraction-based liquid techniques have been introduced,

and these techniques imply that the extraction takes place into a small amount of a water-immiscible solvent (acceptor phase) [42].

2.3 LIQUID-BASED MICROEXTRACTION TECHNIQUES

Liquid phase microextraction (LPME) techniques is a promising tool in Analytical Chemistry that has unique properties of easy sample clean-up, low price, low detection limits, and more environmentally friendly characteristics than other methods [39]. The current trend in sample pre-treatment has led to introducing new types of LPME techniques such as single-drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME), and dispersive liquid-liquid microextraction (DLLME) [52].

SDME is one of the most used and simplest types of LPME technique and it was applied to extract analytes from aqueous samples by forming an acceptor single liquid drop replacing the coated fiber. After extraction, the drop was withdrawn and analyzed by suitable spectroscopic and chromatographic techniques. The extraction efficiency is based on the distribution ratio of the target analytes between a microvolume single drop of the extraction solvent on the tip of either a Teflon rod or the needle tip of a micro-syringe, and the sample solution [53]. SDME provides lots of advantages including the high extraction capacity, low cost, short extraction times, simple operation with no need for special apparatus. According to the mode of usage, SDME can be categorized into three types: direct immersion SDME (DI-SDME), headspace SDME (HS-SDME), and continuous flow microextraction (CFME) [45].

Due to the instability of the drop in SDME, Pedersen-Bjergaard and Rasmussen introduced a new LPME concept referred to as hollow fiber-based liquid-phase microextraction (HF-LPME) [54]. The system consists of a polypropylene hollow fibers (HFs) used as a supporting liquid membrane (SLM) for LPME [42]. Through this procedure, the microvolume of extractant solvent is contained in the HF lumen and it is not directly in contact with the sample solution, and the whole porous HF is sucked in the hydrophobic extraction liquid. As a result, a thin layer is formed within the HF wall [55]. The HF is placed in the sample vial and the sample solution is vigorously

stirred without any loss of extractant solvent in the lumen. Analytes are first extracted to the SLM and then transfer to the extractant solvent inside of the lumen [56]. Basically, HF-LPME can be applied in two different ways such as two-phase and three-phase mode. In the case of three-phase mode, analytes are extracted from an aqueous sample into an organic phase and then back-extracted into a separate aqueous phase. An organic solvent in-between two aqueous phases is immobilized in the wall pores of the HF providing a SLM, and an aqueous acceptor solution was held within its lumen. It is also possible to have two-phase mode, where the analytes are extracted from an aqueous phase to organic phase directly. This mode allows to fill the organic solvent in both wall pores and the HF lumen. HF-LPME has lots of advantages such as effective mass transfer, high extraction efficiency, and potential for automation with several detection systems for on-line extraction and analysis [57].

Razaee *et al.* reported in 2006 the DLLME technique as a new liquid-based microextraction technique that uses extraction solvent volumes in the μL range [58]. The method is rapid, simple, environmental, and economically friendly, and it has a wide range of applications in organic and inorganic analyte assessment in several matrices [59, 60]. The technique is based on the formation of a cloudy solution when injecting the extraction and dispersion solvent into an aqueous sample solution containing the analyte. The key role of the dispersion solvent helps to extraction solvent to form the fine droplets into the aqueous sample and increases the abundant surface contact between the fine droplets and the analyte [61]. Low toxicity and low-cost organic solvents such as acetone, acetonitrile, methanol, and ethanol have been generally used as dispersing solvents because of their miscibility in both water and organic solvents [62]. The extraction solvents can offer several different properties including low solubility in water, ability to form small droplets into the aqueous sample, compatibility with the analytical instruments, and affinity to the analyte. Most popular extraction solvents are chloroform, chlorobenzenes, carbon disulphide, and carbon tetrachloride [63]. Other organic solvents have been proposed as extractants for DLLME such as supramolecular solvents (SUPRAs), ionic liquids (ILs), deep

eutectic solvents (DESs), and switchable solvents (Ss) [64-70]. Although original DLLME consisted of a fast dispersion of the dispersing and extracting solvent into the aqueous sample, several dispersion methods (or several assisted DLLME procedures) have been developed. DLLME assistance can be easily performed by vortex, magnetic and air stirring, ultrasounds, and microwaves, leading to vortex assisted (VA-DLLME), ultrasound-assisted (USA-DLLME), air-assisted (AA-DLLME), magnetic stirring assisted (MAS-DLLME), and microwave-assisted (MWA-DLLME) methods [71-75]. The simplicity of the operation, rapidness, low cost, high enrichment factors, and good recovery inherent to DLLME procedures are the main advantages of the technique which is responsible for the several applications of DLLME for organic and inorganic targets enrichment from simple (water samples) and complex matrices (extracts from environmental, biological, and clinical samples).

2.4 MICROEXTRACTION TECHNIQUES FOR AFLATOXINS EXTRACTION

2.4.1. Application of sorbent-based microextraction techniques

Several researches and review articles on various aspects of the SPME have been reported, and some applications using new sorbents such as graphene oxide polyvinyl poly pyrroline (HF-GO-PVPP) [76], carbon nanotubes [77], silica fiber coating [78], and supel-Q-plot porous polymer type [79] have been proposed for AFs isolation/pre-concentration (Table 1). Among these techniques, the in-tube SPME approach allows convenient automation of the extraction process by reducing the analysis time and providing better accuracy, precision, and high sensitivity. This technique is a manual offline technique which is placed between the injection loop and injection needle of the autosampler. Supel-Q-plot porous polymer sorbent was used in six different capillary columns to evaluate the extraction efficiency dependence on the capillary column type. Methanol (mobile phase) was found to be an efficient solvent for AFs desorption which is advantageous for accomplishing automatically AFs extraction (in-tube SPME) and chromatographic separation within 25 min, and allowing the Analysis of about 50 samples per day [79].

Diaz-Bao *et al*. [80] developed a fast and facile method for the fabrication of magnetic molecularly imprinted stir bars (MMIP-SB) for AFs extraction (M1 from infant formulas, and B1, B2, G1 and G2 from cereal baby foods). The MIP was prepared by bulk polymerization using 5,7 dimethoxycoumarine (DMC) as a dummy template, methacrylic acid (MAA) as a monomer, ethylene glycol dimethacrylate (EGDMA) as a crosslinker, and 2-2 azobis (2-methyl-butyronitril) (AIBN) as an initiator. The pre-polymerization mixture was immediately combined with magnetite to prepare the magnetic molecularly imprinted polymer (MMIP). AFs recovery percentage (60%) is lower when comparing to HF-GO-PVPP sorbent (SPME) but the specificity attributed to the MIP is the main advantage of the method which implies high clean-up performances. Therefore, this technology can be useful for AFs extraction from complex samples with a low consumption of organic solvents. Moreover, the technique was found robust and it can be adapted to several samples sizes (volumes) in accordance to the required legal limits.

Graphene oxide coated SBSE was also applied for AFs isolation from soy milk [81]. In this approach, the surface of a conventional stir bar was modified with dopamine (polydopamine modification) followed by GO modification several time to achieved multilayer GO modified stir bars. After modification, the prepared stir bars showed high tolerance to stirring, and they can be reused at least 10 times without a significant loss of sorption efficiency. In addition, the coating was found to be stable under ultrasonication, and the stir bars preparation is simple, environmentally friendly, and inexpensive.

Table 1: Sorbent based micro extraction technique for AFs

Sample	AFs	MET	Sorbent	DT	LOD	AR %	Ref.
Food sample	B1, B2, G1, G2	SPME	HF-GO-PVPP	HPLC-FLD	0.10-0.37 ng g^{-1}	60.20-108.20	[76]
Cereal	B1, B2	Hollow fiber SPME	Carbon nanotube	HPLC-DAD	0.07 ng mL^{-1} for B1 0.061 ng mL^{-1} for B2	47.43-106.83	[77]
Nuts, cereals, dried fruits, spices	B1, B2, G1, G2	SPME	Supel-Q PLOT	LC-MS	2.1-2.8 pg mL^{-1}	>80	[79]
Cereal flour	B1, B2, G1, G2	SPME	Silica fiber coating	HPLC-FLD	0.035-0.2 ng g^{-1}	55-59 (0.3-1 ng g^{-1}) 49-50 (1-10 ng g^{-1})	[78]
Infant formula and cereal base food	B1, B2, G1, G2, M1	SBSE	MMIP	LC-MS-MS	0.3-1.7 ng g^{-1}	39-60	[80]
Cereal	B1, B2	SBSE	Graphene Oxide	HPLC-laser induced FLD	2.4–8.0 pg mL^{-1}	80.5-102.3	[81]

MET-Micro extraction technique, AFs-Aflatoxin, DT-Detection technique, SPME-Solid phase micro extraction, SBSE- Stir bar sorption extraction, HF-GO-PVPP- hollow fiber graphene oxide polyvinyl poly pyrroline, HPLC-FLD- High-performance liquid chromatography fluorescence detector, LC-MS-MS- Liquid chromatography tandem mass spectrometry, HPLC-DAD-High performance liquid chromatography diode array detector, LOD-Limit of detection, AR%-Analytical recovery percentage

2.4.2 Application of liquid-based microextraction

LPME techniques were developed to overcome the drawbacks of conventional LLE such as the use of large quantities of toxic hazardous and expensive solvents, the long analysis time, simple and multi-stages for extraction [82]. Tables 2 and 3 show LPME procedures for AFs extraction, and from these tables, DLLME is the most common liquid based microextraction technique used for AFs assessment in foodstuff (directly applied to liquid food and food extracts [83-94] or combined with other extractive techniques [95- 109]. Chloroform is mainly used as an extraction solvent for AFs, and other solvents such as ethyl acetate (EtOAc) has been reported to lead lower AFs recoveries [91]. On other cases, such as for daily products, a previous defatting stage by centrifugation is required before DLLME [93].

Recent DLLME developments have been focused on using a drop-on-demand jetting device to increase solvent dispersion (inkjet-based DLLME) [83]. The technique generates a cloudy solution consisting of ultrafine droplets of extraction solvent, which enhances AFs transfer from the acetonitrile/water extract (prepared from wheat) to the organic extractant (chloroform). Other new approaches have been based on combining DLLME with HF-LPME [89, 90]. In this approach toluene (extractant) and acetone (disperisng solvent) are added into the sample (soybean juice) and a polypropylene membrane, previously dipped in octanol, and fixed on stainless steel rods is completely immersed into the sample-toluene-acetone mixture [89]. The extraction was then performed in a closed system with constant magnetic stirring for 60 min. A similar approach but using an U shape polypropylene hollow fiber (6 cm long piece) was applied for AFs extraction by Alsharif *et al.* [90]. The new configuration allows increasing the surface area with the donor phase and extractant dispersion is enhanced. The modified design boasted a better extraction efficiency with shorter extraction times compared with conventional DLLME methods. Moreover, this method provided the additional advantages of lower detection limits, higher recovery for trace AFs level in fruit juices, and good precision.

Regarding HF-LPME, Huang *et al.* [110] have proposed an automated HF-LPME coupled with HPLC-MS/MS for the determination of AFM1 in milk. A simple fabricated HF-LPME was developed by combining pipette tips with hollow fibers based on the prototype [111]. Based on that study, the mass transfer of AFM1 from the sample solution to the extraction phase was greatly affected by the extraction phase. Herein, the use of anti-AFM1 antibody in phosphate buffer solution was found as a promising extraction phase for increasing the affinity of AFM1 [110]. The proposed method was successfully applied for the determination of AFM1 in milk with quantitative recoveries. The method was also applied to edible oil analysis, and performances were quite better than those found by using non-modified polyvinylidene fluoride hollow fibers (PVDF-HFs) since swelling of the hollow fiber is avoided [96]. Mass transfer efficiency was also improved by replacing the hydrophobic PVDF-HFs were modified by polydopamine (PDA) and quaternary polyethyleneimine (QPEI) hydrophilic membranes. The PDA membranes form strong hydrogen bonds with PVDF on the two surfaces of the HFs. Moreover, the PVDF-PDA membranes were grafted with quaternary ammonium compounds to gain surface hydrophilic characteristics. The lumen of the HF was then filled with anti-aflatoxins, and AFs from oil samples were conveniently transferred through the SLM during the shaking process [112].

Table 2: Liquid based microextraction techniques for AFs

Sample Matrix	AFs	MET	Extraction Solvents	DT	LOD	EF	AR%	Ref.
Wheat	B1, B2, G1, G2	IB-DLLME	$CHCl_3$	UHPLC-MS-MS	0.018-0.06 μg kg^{-1}	_a	83.2-93	[83]
Ground nut	B1	DLLME	MeOH:water (80:20)	DEP-MS-MS	0.8 ppb	_a	84.5-99	[84]
Egg and chicken liver	B1	DLLME	ACN:Water (80:20)	HPLC-UV	0.08 μg kg^{-1}	_a	87.5-95	[86]
Milk	M1	DLLME	$CHCl_3$	UHPLC-MS-MS	0.6 ng kg^{-1}	33	61.3-75.3	[87]
Peanut	B1, B2, G1, G2	DLLME	$CHCl_3$	HPLC-FD	0.03-0.10 μg kg^{-1}	_a	83.1-99.3	[88]
Apple and grapes juice	B1, B2, G1, G2	HF-DLLME	Polypropylene HF/octanol	HPLC-MS-MS	0.04-0.06 μg L^{-1}	_a	97.9-107.3	[90]
Tea beverage	B1, B2, G1, G2	DLLME	$CHCl_3$	LC-MS-MS	0.05-0.10 μg L^{-1}	_a	65-127	[91]
Cereal	B1, B2, G1, G2	DLLME	$CHCl_3$	HPLC-FD-UV	0.03-16.2 μg kg^{-1}	_a	90-112	[92]
Yogurt	B1, B2, G1, G2, M1	DLLME	$CHCl_3$	HPLC-FD	1.5-5.5 ng kg^{-1}	_a	69.4-99.7	[93]
Egg	B1	UA-DLLME	$CHCl_3$	HPLC-UV	0.12 μg kg^{-1}	_a	91-94	[95]

Table 2: Liquid based microextraction techniques for AFs (Continued)

Sample Matrix	AFs	MET	Extraction Solvents	DT	LOD	EF	AR%	Ref
Cereal products	B1, B2, G1, G2	DLLME	$CHCl_3$	HPLC-FD	0.01–0.17 μg kg^{-1}	2.5	70-110 50-120	[113]
Milk	M1	HF-LPME	anti-AFM1 in PBS	LC-MS-MS	0.06 μg kg^{-1}	48	61-106.7	[110]
Edible oil	B1, B2, G1, G2	HF-LPME	anti-AFT in PBS	LC-MS-MS	0.02-0.32 ng kg^{-1}	100 - 150	67.78-104.5	[112]
Rice	B1, B2	DLLME	$CHCl_3$	HPLC	0.06-0.5 μg kg^{-1}	125	82.9-112	[114]
Soybean Juice	B1, B2, G1, G2	HF - DLLME	Toluene:Acetone (1:5)	HPLC-FD	0.01-0.03 μg L^{-1}	_a	72-117	[115]

MET- Micro extraction technique, DT-Detection technique, DLLME-Dispersive liquid-liquid microextraction, HF-Hollow fiber, LPME-Liquid phase microextraction, IB-Inkjet based, ACN-Acetonitrile, $CHCl_3$ -Chloroform, PBS-Phosphate buffer saline, $C_4H_8O_4$-Erythrose, HPLC-High performance liquid chromatography, UV-Ultraviolet detection, FD-Fluorescence detection, LC-MS-MS- Liquid chromatography-tandem mass spectroscopy, UHPLC-Ultra-high-performance liquid chromatography, DEP-MS-MS-Direct electrospray probe tandem mass spectroscopy. LOD-Limit of detection, AR-Analytical recovery, EF-Enrichment factor, _a- not given

Table 3: Combined micro-extraction techniques for AFs

Sample Matrix	AFs	MET	Extraction sorbents/solvents	DT	LOD	EF	AR%	Ref.
Egg	B1	UA- DLLME	$CHCl_3$	HPLC-UV	0.12 μg kg^{-1}	_a	91-94	[95]
Herbal tea	B1, B2, G1, G2	VA- DLLME	$CHCl_3$	UPLC-MS-MS	0.001-0.01 μg L^{-1}	_a	70.06-115.65	[96]
Dairy cattle feed	B1, B2, G1, G2	VALDS-ME	1-octanol and toluene	CE-LIF	0.002-0.075 μg L^{-1}	_a	70.97-108.41	[97]
Rice	B1, B2, G1, G2	AA- DLLME	$CHCl_3$	HPLC-FD	0.13-0.68 ng kg^{-1}	_a	76-109.3	[98]
Rice	B1, B2, G1, G2	VALDS-ME	1-octanol and toluene	HPLC-FD	0.0011-0.17 μg kg^{-1}	42-132	70-104	[99]
Milk	M1	LDS-DLLME-VA-D-SPE	hydrophobic oleic acid modified Fe3O4 nanoparticles/1-heptanol	Spectro-fluorimetry	13 ng L^{-1}	_a	91.3-99.5	[100]
Pistachios	B1, B2, G1, G2	DLLME-SPE	$CHCl_3$	HPLC-FD	0.02 μg kg^{-1} 0.04 μg kg^{-1}	_a	85-93	[101]
Animal feed	B1, B2, G1, G2	IL-DLLME-SPE	Fe_3O_4 /1-octyl-3-methylimidazolium hexafluorophosphate	HPLC-FD	0.632- 0.087 ngmL^{-1}	_a	90.3-103.7	[102]
Edible oil	B1, B2, G1, G2	DLLME-IAC	$CHCl_3$	HPLC-FD	1.1×10^{-4}-5.3×10^{-3}ng mL^{-1}	50	96-110	[103]
Milk thistle	B1, B2, G1, G2	QuEChERS - DLLME	QuEChERS/MeCN	UHPLC-MS-MS	0.45- 0.57 μg kg^{-1}	_a	88.4-93.7	[104]
Dairy foods	B1, M1	QuEChERS - DLLME	QuEChERS/$CHCl_3$	HPLC	0.1 μg kg^{-1}-B1, 0.01 μg kg^{-1}M1	_a	51.2-75.7-B1, 52.2-72.2-M1	[105]

Table 3: Combined micro-extraction techniques for AFs (Continued).

Sample Matrix	AFs	MET	Extraction sorbents/solvents	DT	LOD	EF	AR%	Ref.
Cereal and bean food stuff	B1, B2, G1, G2	SPE-DLLME	QuEChERS/C-18 SPE catridge/$CHCl_3$	HPLC	0.03-11 µg kg^{-1}	_a	76.61- 84.13	[106]
Pistachios	B1, B2, G1, G2	LDS-DLLME-VA-D-SPE	1-heptanol/ hydrophobic Fe_3O_4	Spectro-fluorimetry	21 ng L^{-1}	_a	91.6-99.6	[107]
Feed	B1, B2	ISVAEME - SFOD	1-dodecanol	HPLC	16 ng kg^{-1} for B1 120 ng kg^{-1} for B2	_a	97-102	[108]
Soybean	B1, B2, G1, G2	IS-SPE-DLLME	Pu/GO/$CHCl_3$	HPLC-FD	0.09–0.15 µg kg^{-1}	_a	76-101	[109]

VA-DLLME-Vortex assisted dispersive liquid liquid micro extraction, VALDSME- Vortex assisted low density solvent micro extraction, AA-Air assisted, UA-Ultrasound assisted, LDS-DLLME-VA-D-SPE- Low density solvent based dispersive liquid liquid micro extraction followed by vortex assisted dispersive solid phase extraction, IL-Ionic liquid, IAC-Immuno-affinity column, QuEChERS - Quick, easy, cheap, effective, rugged and safe, ISVAEME-SFSD – In-syringe vortex assisted emulsification micro extraction with solidified floating organic drop, IS-In-syringe , HPLC-High performance liquid chromatography, FD-Fluorescence detection, UV-Ultraviolet detection, UHPLC-Ultra high performance liquid chromatography, CE-LIF, Capillary electrophoresis with laser-induced fluorescence detection, MS-MS- Tandem mass spectrometry, LOD-Limit of detection, AR-Analytical recovery, EF-Enrichment factor, _a- not given

2.5 CONCLUSION

Sorbent-based and liquid based microextraction techniques have shown to offer several advantages in good agreement with the principles of the Green Chemistry, and the potential of these techniques has been highlighted for AFs extraction/pre-concentration from food matrices. Practical advantages such as short extraction times, low cost, high enrichment factors (improved sensitivity), high analytical recovery, and high sample throughput, are typically reported. Among all sorbent-based and liquid-based microextraction techniques, DLLME has been found to be one of the most widely used technique for AFs assessment, and the developed methods show enhanced extraction efficiencies. New assistance strategies for increasing dispersion and favouring mass transfer to the organic extract are now under development. In addition, the use of surfactants as dispersing solvents opens new perspectives for further DLLME developments.

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II. OBJECTIVES

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II. OBJECTIVES

The main objective of this thesis is the development of methods for aflatoxins screening and aflatoxins confirmation in fish feed and in fish products, as well as the risk assessment of cultured fish consumption by *in vitro* bioavailability approaches.
The specific objectives are as follows:

- Synthesis of nanoparticles with selective response to aflatoxins such as luminescent quantum dots functionalized with molecularly imprinted polymers (QD-MIPs), and characterization of the prepared nanomaterials using TEM-EDX, FT-IR and XRD

- Development of room temperature phosphorescent screening methods based on QD-MIPs for a fast and low-cost determination of aflatoxins

- Synthesis of molecularly imprinted polymers as new selective absorbents in micro-solid phase extraction procedures for the isolation/pre-concentration of aflatoxins from cultured fish and fish feed extracts.

- Development of micro-solid phase extraction procedures (porous membrane-protected molecularly imprinted polymer micro-solid-phase extraction and molecularly imprinted polymer dispersive micro-solid phase extraction) combined with HPLC-MS/MS for aflatoxins assessment in fish feed and cultured fish. The developed methods will be compared with other solvent-based micro-extraction techniques such as dispersive liquid liquid micro-extraction.

- Selection of the best pre-concentration method for trace amounts of aflatoxins in dialysates from *in vitro* bioavailability (dialysability) approaches, and the assessment of the bioavailability of aflatoxins in cultured fish. Possible aflatoxins transformations/degradation will be studied by high resolution mass spectrometry.

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III. RESULTS AND DISCUSSION

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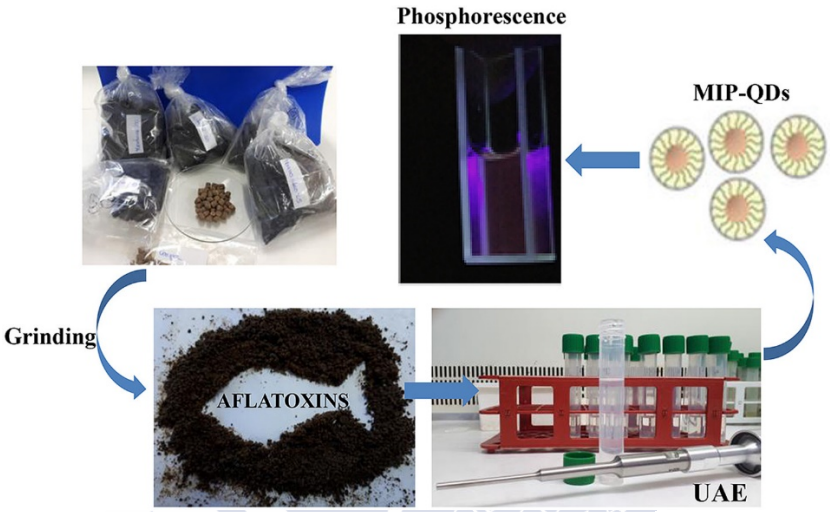

CHAPTER 1

ROOM TEMPERATURE PHOSPHORESCENT DETERMINATION OF AFLATOXINS IN FISH FEED BASED ON MOLECULARLY IMPRINTED POLYMER@Mn-DOPED ZnS QUANTUM DOTS

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ANALYTICA CHIMICA ACTA (2020), 1103: 183-191

DOI : 10.1016/j.aca.2019.12.060

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Room temperature phosphorescent determination of aflatoxins in fish feed based on molecularly imprinted polymer@Mn-doped ZnS quantum dots

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Abstract

Possibilities of room temperature spectrometry based on Mn-doped ZnS quantum dots coated with a molecularly imprinted polymer based nanosensor have been explored for the sensitive and selective determination of aflatoxins. Synthesized polymeric nanoparticles exhibit intense room temperature phosphorescence (total decay time of 0.004 s) and aflatoxins quench the room temperature phosphorescence when interact with the recognition cavities of the molecularly imprinted polymer attached to the phosphorescent quantum dots. Room temperature was recorded by scanning from 520 nm to 720 nm (maximum peak intensity at 594 nm) after excitation at 290 nm. The prepared imprinted material was found to have higher adsorption capacity than those based non-imprinted quantum dots, demonstrating high adsorption uptake for aflatoxins. In addition, selectivity studies have demonstrated that the material offers a specific recognition for aflatoxins. Room temperature phosphorescence quenching by aflatoxins was found to be linear within the 2-20 μg L^{-1} range, and a limit of detection of 3.56 μg kg^{-1} was obtained. This value was lower than the maximum acceptable/residual level (aflatoxins in feeds) published by the European Commission. The results indicate a simple room temperature phosphorescence nanosensor for aflatoxins detection in fish feed as a versatile tool having excellent sensitivity and selectivity.

Keywords: Room temperature phosphorescence detection, Aflatoxins, Quantum dots, Molecular Imprinting, Nanosensor

1.1 INTRODUCTION

Moulds belonging to the *Aspergillus* genus are common contaminants of commodities which generated toxic compounds, refrerred as mycotoxins, that can be present in foodstuff and feedstuff [1,2]. Based on their biological effect, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, teratogens, mutagens, carcinogens, and allergens [3]. Aflatoxins (AFs) are a group of mycotoxins produced mostly by the filamentous fungi known as *Aspergillus flavus* and *Aspergillus parasiticus* [4]. Animal feeds are highly susceptible to contaminated with AFs [5], and their presence in feeds used in aquaculture is needed. AFs have been found in tree nuts, peanuts, and oilseeds (including corn and cotton). The major AFs of concern are B1, B2, G1 and G2 [6]. These toxins are usually found together in the feeds in various proportions [7]. The maximum limit of AFB1 at 20 μg kg^{-1} has been established for all feed materials and feedstuffs for cattle, sheep and goats by the European Commission (Commission Directive 2003/100/EC) [8]. The Directive, however, makes no mention about AFs content in fish feed.

Aflatoxins have proved to be carcinogenic, mutagenic, hepatotoxic and immunosuppressive for all living beings. Therefore, the development of a screening analytical methodology for sensitive and rapid detection of AFs is of great interest. Screening methods are widely based on immunoassays. They guarantee the avoidance of false-negative results, are reliable, offer better sensitivity, and require low or no sample pre-treatment [9]. Molecularly imprinted polymer with Mn-doped ZnS quantum dots (QDs) is a low-cost and rapid artificial immunoassay methodology for assessing total AFs in fish feeds.

Quantum dots or semiconductor nanocrystals (NCs) are spherical shaped, rod-like, branched nanocrystals closely related with technological and industrial applications in optoelectronic devices [10]. Due to their luminescent properties, QDs are considered simple

and inexpensive fluorescent and room temperature phosphorescent sensor probes for various applications. Usually, QDs exhibit strongly size dependent optical and electrical properties such as size tunability, narrow emission spectra, broad excitation spectra, strong signal intensity, and high photostability with biocompatibility [11,12]. Although CdSe QDs were first proposed for analytical applications, these materials have potential toxicity due to their inherent chemical composition and as a consequence of their nano structure properties such as inhalation of the particles [13]. The toxicity of Cd has led to the development of non-cadmium based QDs such as ZnSe and ZnS [9]. In recent years, a range of ZnS nanocrystals doped with different transition metals or rare-earth metal ions have been reported. Bol *et al.* reported that Cu and Mn-doped ZnS are two major well studied doped QDs due to their technological suitability for fluorescent properties [14]. Unfortunately, although those direct sensing approaches are highly sensitive, they are lacking enough selectivity. Thus, improvements in selectivity of the QDs based probes are necessary.

Molecularly imprinted polymers (MIPs) have good recognition cavities for target molecules and have attracted attention due to their outstanding advantages such as stability, relative ease and low-cost preparation, and potential application to a wide range of target molecules [15,16]. Due to their high selectivity, the combination of MIPs with the QDs provides a new tool for analyte recognition. Although fuorescent MIP@QDs (Mn-doped ZnS QDs included) have been applied for developing several sensor probes, these composite materials also exhibit long-lived room temperature phosphorescence (RTP) emission [17,18], and some applications for pollutants [17-22] assessment have been proposed. RTP offers higher sensitivity than that obtained by fluorescence spectrometry, and it is therefore an appealing technique to assess very low target concentrations. This is the case of AFs in foodstuffs and feedstuffs for which fluorescent measurements based on QDs-nanosensors do not allow assess the target near or below the maximum acceptable/residue level (MRL) [23].

To the best of our knowledge, the RTP detection of AFs based on MIP@Mn doped ZnS QDs has not been reported. The aim of the

current work has been to explore the possibilities of using this phosphorescence MIP@QDs based probe for developing simple and low-cost analytical methods for AFs assessment in a complex sample matrix such as fish feed. To date, the assessment of AFs in feed has been carried out mainly by chromatographic-based techniques [24]. These methodologies are quite expensive, and although HPLC methods allow the identification and determination of single AFs, reliable and low-cost methodologies for AFs screening are required. Fast AFs screening in fish feed using MIP@QDs based RTP measurements have been developed in a novel way in the current work. This sensor has good sensitivity with selectivity and an economical advantage over chromatographic methods.

1.2 MATERIALS AND METHODS

1.2.1 Instrumentation

RTP analysis was performed with a Cary Eclipse fluorescence spectrophotometer (Varian, Victoria, Australia) equipped with a xenon lamp and 10 mm quartz cell and phosphorescence working mode. Confirmation of the template removal was done with 3200 Q-TRAP LC/MS/MS system (ABSciex, Concord, Canada) equipped with a Flexar FX-15 UHPLC binary chromatographic pump (Perkin Elmer, Waltham, MA, USA) and a Flexar UHPLC autosampler (Perkin Elmer, Waltham, MA, USA). Separations were performed with a Zorbax Eclipse C18 reverse phase column (100 mm length x 4.6 mm i.d, 3.5 μm particle diameter) from Agilent (Santa Clara, CA, USA), connected to a C18 guard column (4 mm length, 3.0 mm i.d) from Phenomenex (Torrance, CA, USA). An UCI-150 ultrasonic cleaner bath (power of 325 W and frequency of 37 kHZ) from Raypa (Barcelona, Spain) was used for synthesizing the MIP@QDs nanocrystals. AFs extraction from fish feed was performed with a VibraCell VCx 130 ultrasound probe from Sonics (Newtown, CT, USA). QDs-MIP characterization was performed by transmission electron microscopy coupled with X-ray (EDX) microanalysis (Libra 200FE OMEGA, Zeiss, Oberkochem, Germany), Fourier transform infrared spectrometry (FT-IR) (Spectrum two FT-IR, Perkin Elmer, Waltham, USA), and X-ray diffraction spectrometry (XRD)

(EMPYREAM, PaNalytical, Almelo, Netherlands). Other laboratory devices were as follows: Basic20 pH-meter (glass-calomel electrode) from Crison (Barcelona, Spain), Centromix centrifuge (Selecta, Barcelona, Spain), a vibrating ball mill with 15mL zircon oxide cups and 7mm diameter zircon balls (Retsch, Haan, Germany), and VLM EC1 metal block thermostat and N_2 sample concentrator from VLM (Leopoidshohe-Greste, Germany).

Data from the analysis were collected and subjected to statistical treatment by using STATGRAPHICS Centurion XVI.I (Manugistics Inc., Rockville, MD, USA).

1.2.2 Reagents

Aflatoxins stock standard solutions (1000 mgL^{-1}) were prepared from solid AFB1, AFB2, AFG1, and AFG2 aflatoxins from Sigma-Aldrich (Steinheim, Germany) by dissolving in methanol (LC-MS grade) purchased from Merck (Darmstadt, Germany). Mn-doped ZnS QDs were synthesized using heptahydrate zinc sulfate (Panreac, Barcelona, Spain), sodium sulfide (Fluka, Buchs, Switzerland), and manganese dichloride (Merck). Polyethylene glycol (PEG 5000-7000) and dimethyl sulfoxide (DMSO) were from Panreac (Barcelona, Spain). Sodium hydroxide and dipotassium hydrogen phosphate were from Merck. MIP was synthesized by using 5,7-dimethoxycoumarin (DMC), divinylbenzene (DVB) and methacrylic acid (MA) obtained from Sigma-Aldrich, and by using 2,2' azobisisobutyronitrile (AIBN) from Fluka. Acetonitrile and methanol (supragradient HPLC grade) were from Merck, toluene (HPLC grade) was from Scharlau (Barcelona, Spain), and formic acid from Panreac. Retinol, 7-dehydrocholesterol, and β-carotene stock solutions (1000 mg L^{-1}) were prepared in methanol from solid reagents (Sigma-Aldrich). Other compounds used in cross-reactivity studies, such as carrageenan (kappa and lambda) were from CEAMSA (Porriño, Spain), agar-agar were from Algamar (Redondela, Spain), and carboxymethyl cellulose (CMC) from Scharlau. These reagents were dissolved in hot water to prepare 1000 mg L^{-1} stock standard solutions. Ultrapure water (18.2 MΩcm resistivity) was obtained from a Milli-Q purification device (Millipore, Bedford, MA, USA). Other consumables were as follows:

ACCUREL PP membrane (Membrana, Wuppertal, Germany), Durapore 0.20 µm membrane filters (Millipore), and 0.22 µm cellulose acetate syringe filters (LLG, Meckenheim, Germany).

1.2.3 Synthesis of MIP-PEG-Mn-doped ZnS QDs

PEG-Mn-doped ZnS QDs were synthesized as described elsewhere [23,25]. Surface modification [26,27] of prepared Mn-doped ZnS QDs was achieved with PEG, reagent which modify the QDs' surface with hydroxyl groups, favouring bonds between the modifier-nanoparticle assemblies through hydroxyl groups and the vinyl-groups of reagent involved in MIP synthesis [28]. The nanomaterial was isolated by centrifugation (3000 rpm, 20 min), rinsed three times with 5 mL of methanol, and finally dried at room temperature inside a desiccator before storing in amber vials at 4 °C.

Synthesis of MIP coated PEG-Mn-doped ZnS QDs was performed using the emulsion polymerization technique [29]. The template (DMC, 0.0699 g, 0.3392 mmol) and the monomer (MA, 115 µL, 1.356 mmol) were dissolved in the 4 mL of DMSO sparged with N_2 and kept at room temperature in the dark for 12 h for self-assembly. Synthesized QDs (0.2015 g) were dispersed in 25 mL of ultrapure water and then mixed with the pre-polymerization mixture (DMC and MA in DMSO). The cross-linker [1.25 mL (6.98 mmol) of DVB] and initiator [0.0997 g (0.607 mmol) of AIBN] were then added. Ultrasounds favour DVB and AIBN transfer to the DMSO phase as well as dispersion of DMSO droplets in water and over the dispersed nanoparticles. Based on previous studies [23,25], polymerization was performed at room temperature and at 37 kHz (325 W) for 4h. To avoid excessive temperature increase, water was renewed each 30-40 min. Once polymerization was finished the synthesized material was centrifuged (3000 rpm, 20 min) and washed three times with methanol. Finally, synthesized MIP coated PEG-Mn doped ZnS QDs were dried at room temperature inside a desiccator for 24 h and kept at 4 °C in the dark. Non-imprinted polymers (NIPs) coated PEG-Mn doped ZnS QDs were synthesized as above but without using the template.

The preparation of Mn-doped ZnS QDs @ MIP is presented schematically in Figure 1.

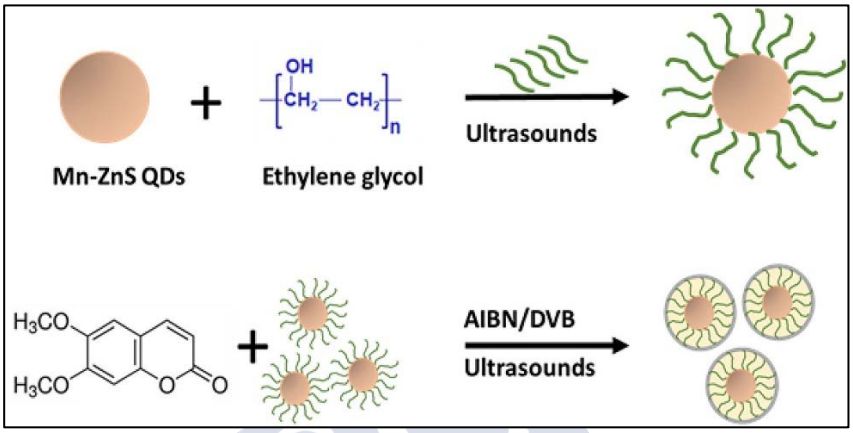

Figure 1: Schematic diagram for the preparation of MIP@Mn-doped ZnS QDs

1.2.4 Template removal procedure

The template was removed from the MIP-PEG-Mn-doped ZnS QDs by subjecting 200 mg of dried MIP coated PEG-Mn doped ZnS to an ultrasound assisted extraction using acetonitrile/formic acid (95:5) as an extraction mixture (20 ultrasounds cycles at 37 kHz for 30 min with 20 mL of fresh extracting solution each cycle). The DMC content in the washing solution was analysed using HPLC-MS/MS, and the negligible DMC concentrations were found in the twentieth washing solution. The template-free MIP-PEG-Mn-doped ZnS QDs was rinsed three times with methanol and ultrapure water and dried at room temperature inside a desiccator and kept at 4 °C in the dark.

1.2.5 AFs isolation from fish feed samples

Fish feed samples were from local fish feed manufacturers in Santiago de Compostela, Spain. All fish feed samples were ground (vibrating ball mill) and stored at -20 °C until further analysis. The samples were subjected to the following extraction procedure: 0.250 g of homogenised fish feed was weighted into a 30 mL centrifuge tube and extracted with 10mL of 60:40 acetonitrile/0.1 M/0.1 M KH_2PO_4-NaOH buffer (pH 6.0) by sonication with ultrasound probe (40% amplitude, continuous mode, 7 min). The final pH of the extract was 7. The extract solution was isolated by centrifugation (3000 rpm, room temperature, 10 min). AFs in the extracts were then analysed by the MIP@Mn-doped ZnS QDs and NIP@Mn-doped ZnS QDs phosphorescence probe.

1.2.6 Room temperature phosphorescence measurement

RTP spectra of the MIP@Mn-doped ZnS QDs and NIP@Mn-doped ZnS QDs were recorded at excitation and emission wavelengths of 290 nm and 594 nm (scanning of the wavelength range from 520 nm to 720 nm) under operating conditions listed in Table S1 (ESI section). In all experiments, a constant mass of MIP@Mn-doped ZnS QDs or Mn-doped ZnS QDs was dispersed in a constant volume of 0.1 M KH_2PO_4 (pH 5.0 adjusted with formic acid) to fix a 10 mg L^{-1} concentration, and the phosphorescence intensities were recorded. The determination of AFs was based on the changes in the phosphorescence intensity (quenching effect). All the measurements were carried out under ambient temperature (20-25 °C). Calibration range was fixed within the 0-20 µg L^{-1}, and Figure 2 shows the overlaid phosphorescence spectra when increasing AFB1 concentrations.

1.2.7 Liquid chromatography-tandem mass spectrometry measurement

DMC contents in the washing solutions were analysed by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) using gradient mode (0.1% formic acid in methanol and 0.1% formic acid in ultrapure water as mobile phases, flow rate

250 µL min^{-1}) under the optimum acquisition setting listed in Table S2 (ESI section). The same chromatographic conditions (Table S2, ESI section) were also used for assessing each AF in selected fish feed samples (Application section) for comparison purposes.

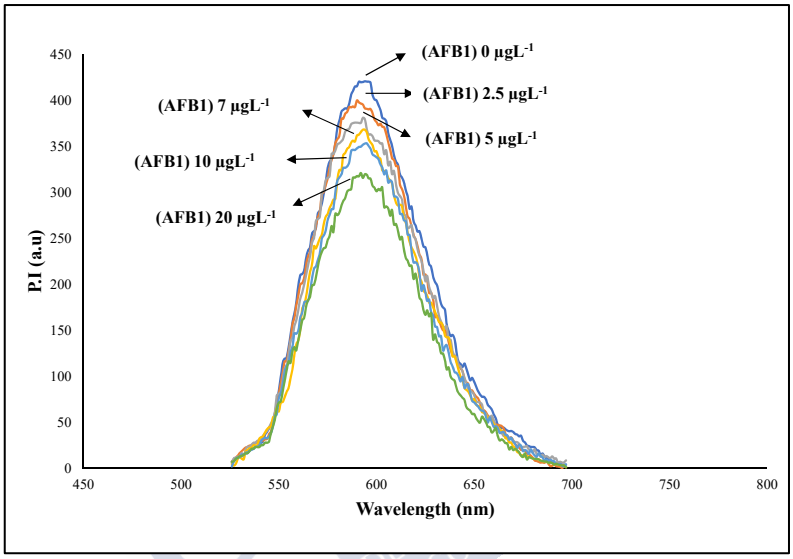

Figure 2: Overlaid RTP spectra of MIP-PEG-Mn-doped ZnS QDs at several AFB1 concentrations

1.3 RESULTS AND DISCUSSION

1.3.1 Characterization

FT-IR spectra of the -PEG-Mn-doped ZnS QDs, MIP-PEG-Mn-doped ZnS QDs, and NIP-PEG-Mn-doped ZnS QDs are shown in Figure S1 (electronic supplementary material, ESI) where characteristic peaks at 612, 985, 1080, 1650 cm^{-1} were observed in PEG-QDs spectra (Figure S1(a)). The peak at 610 cm^{-1} belongs to the ZnS band and it is assigned to sulphides [25]. In addition, the peaks at 1113 and 3424 cm^{-1} were assigned to strong and broad asymmetrical bands of stretching vibrations of C-H and C-OH, respectively. FT-IR for MIP-

PEG-Mn-doped ZnS QDs and NIP-QDs (Figure S1(c) and Figure S1(b), ESI section) show the presence of the bands at 1113 and 1350 cm^{-1} (C-H stretching and C-H bending), and 3424 cm^{-1} (C-OH stretching) in both MIP and NIP-QDs. In addition, the characteristic band at 610 cm^{-1} (characteristic band from inorganic ions) was not observed (was weak) in MIP and NIP QDs. This finding proved that the MIP/NIP layer was efficiently anchored onto the surface of the PEG-Mn doped ZnS QDs.

The transmission electron microscopy (TEM) images of PEG-Mn-doped ZnS QDs (a), MIP-PEG-Mn-doped ZnS QDs (b) and NIP-PEG-Mn-doped ZnS QDs (c) are shown in Figure 3. The prepared material consists of agglomerated nanoparticles covered with a thin MIP layer. The presence of Zn, S, C and Mn in the prepared composites was confirmed by EDX (Figure 4).

The X-ray diffraction pattern (XRD) of PEG-Mn-doped ZnS QDs, MIP-PEG-Mn-doped ZnS QDs, and NIP-PEG-Mn-doped ZnS QDs had the same diffraction peaks corresponding to a lattice plane of (111), (220) and (311) of cubic zinc blender. The size of the prepared materials was calculated using the Debye Scherrer method, and the average size of -PEG-Mn-doped ZnS QDs was 1.8±0.37 nm; whereas, 2.3±0.88 nm and 1.9±0.74 nm average sizes were calculated for MIP-PEG-Mn-doped ZnS QDs and NIP-PEG-Mn-doped ZnS QDs, respectively. These findings show that the calculated mean size of PEG-Mn-doped ZnS QDs, MIP-PEG-Mn-doped ZnS QDs, and NIP-PEG-Mn-doped ZnS QDs are quite similar. Despite the thin MIP layer over the agglomerated PEG-Mn-doped ZnS QDs, the composite material offers defined MIP cavities and a target recognition by imprinting because of the excellent results after selectivity and imprinting studies (please see next sections).

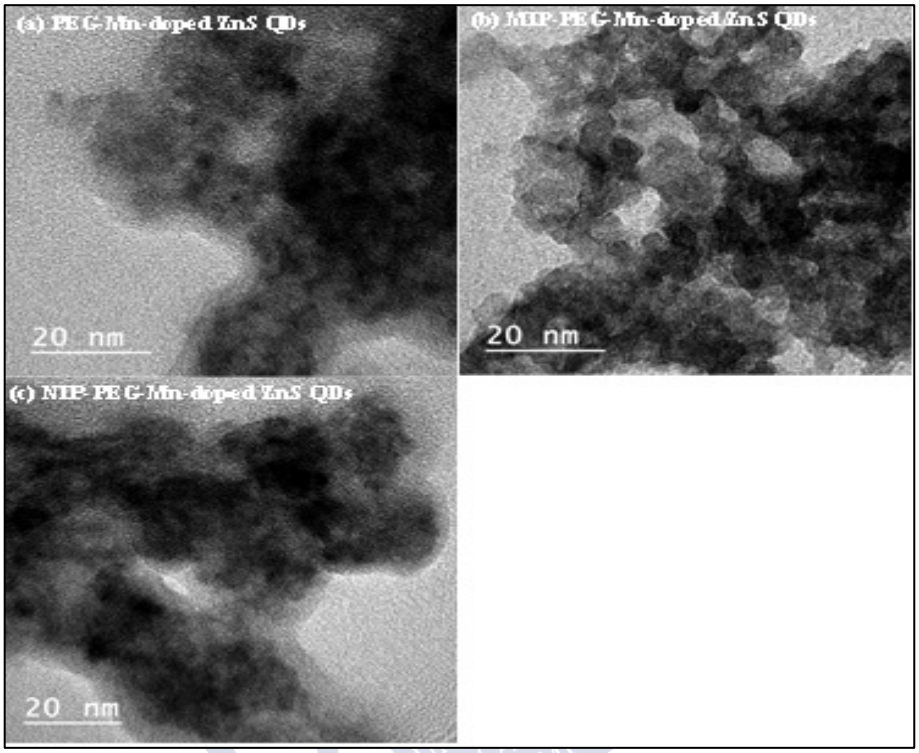

Figure 3: Transmission electron microscope images for PEG-Mn-doped ZnSQDs (a), MIP-PEG-Mn-doped ZnSQDs (b) and NIP-PEG-Mn-doped ZnSQDs

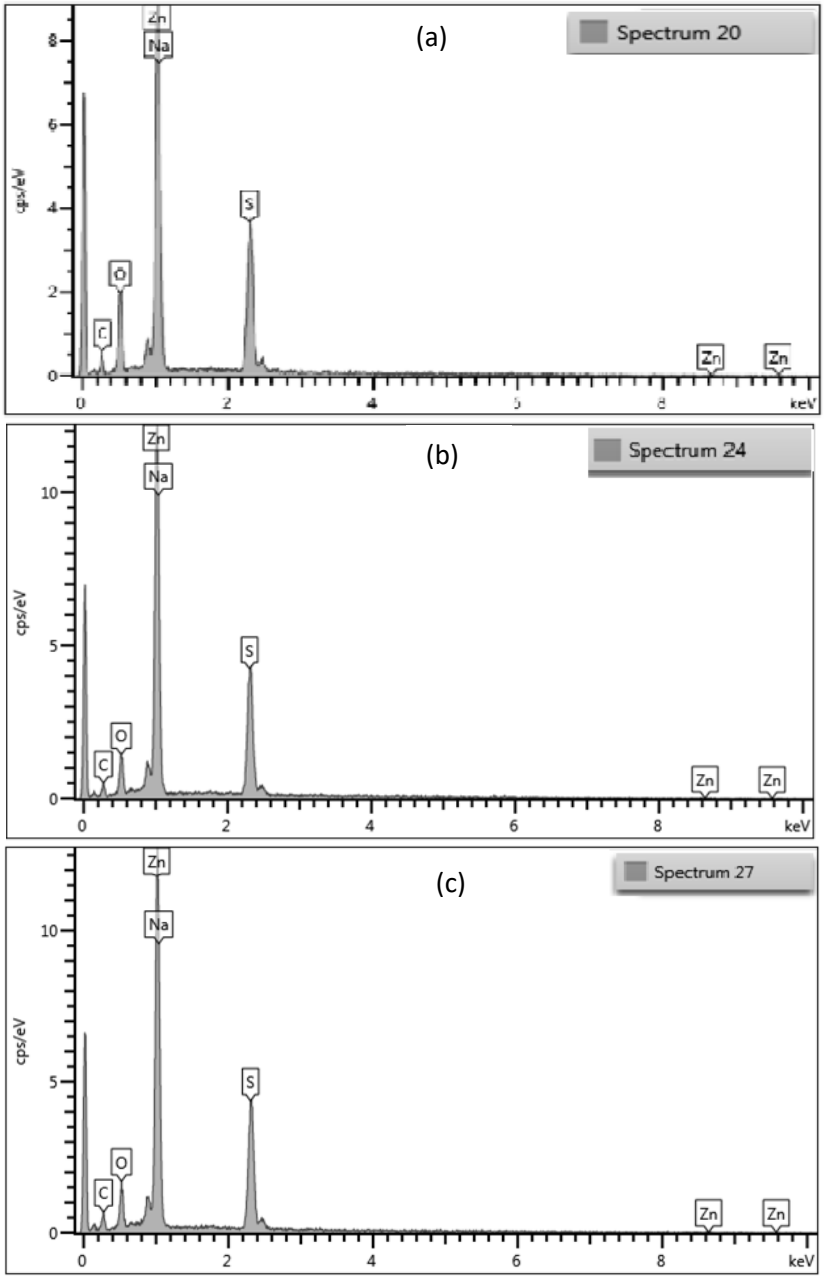

Figure 4: TEM-EDX images for -PEG-Mn-doped ZnS QDs (a), MIP-PEG-Mn-doped ZnS QDs (b) and NIP-PEG-Mn-doped ZnS QDs (c)

1.3.2 Phosphorescence study

The RTP spectra had only one shape emission peak at 594 nm which was attribute to the triplet state transition of the Mn^{2+}. The RTP intensity (without saturation) varied with the MIP/NIP-PEG-Mn-doped ZnS QDs concentration; and thermal stability of the QDs and the shape of the RTP intensity also varied with the QD particle size [30]. Excitation and emission wavelengths were the same when using MIP/NIP-PEG-Mn-doped ZnS QDs. Prepared MIP/NIP-PEG-Mn-doped ZnS QDs were tested to be stable for more than one month without obvious RTP intensity losses. Additionally, Figure 5 shows the RTP spectra for the synthesized -PEG-Mn-doped ZnS QDs, NIP-PEG-Mn-doped ZnS QDs, and MIP-PEG-Mn-doped ZnS QDs before and after template removal. It can be seen that RTP emitted by the prepared PEG-Mn-doped ZnS QDs diminishes when they are covered with the MIP and NIP layer, although MIP-PEG-Mn-doped ZnS QDs RTP is higher after template removal. That means that the interaction of template (DMC) and/or AFs with the recognition cavities of the MIP layer will quench the QDs' RTP.

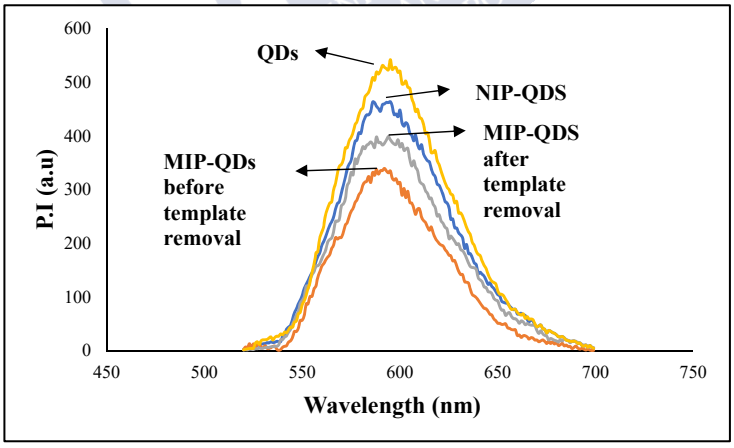

Figure 5. Overlaid RTP spectra of -PEG-Mn-doped ZnS QDs, NIP-PEG-Mn-doped ZnS QDs and MIP-PEG-Mn-doped ZnS QDs before and after template (DMC) removal

1.3.3 Optimization of the response of MIP-PEG-Mn-doped ZnS QDs

Parameters affecting the adsorption of AFs onto MIP-PEG-Mn-doped ZnS QDs nanoparticles such as the pH of the MIP-PEG-Mn-doped ZnS QDs sample extract mixture, MIP-PEG-Mn-doped ZnS QDs concentration, and AFs-MIP-PEG-Mn-doped ZnS QDs interaction time were fully evaluated. Aqueous 0.1 M/0.1 M KH_2PO_4/NaOH buffer at fixed pHs 7.0 and 6.0 (0.1 M KH_2PO_4 adjusted at 5.0 with formic acid) were used to suspend the MIP-PEG-Mn-doped ZnS QDs before mixing with a certain volume of sample extract inside the cuvette. The highest RTP intensities were observed at room temperature, thus refrigerated MIP-PEG-Mn-doped ZnS QDs dispersions were allowed to reach room temperature, and dispersions were shaken just before use.

1.3.3.1. Effect of the pH

Several pH values were tested to obtain the best interaction between the AFs and the MIP-PEG-Mn-doped ZnS QDs' recognition cavities. Imprinted cavities of the MIP-PEG-Mn-doped ZnS QDs were similar to the coumarin ring of the DMC, AFB1, AFB2, AFG1, and AFG2. Hence the pH effect was optimized using AFB1. The MIP-PEG-Mn-doped ZnS QDs solutions were prepared using 0.1 M/0.1 M KH_2PO_4/NaOH buffers at pHs 6.0 and 7.0. A pH of 5.0 was also tested but in this case a 0.1 M KH_2PO_4 solution was mixed with some drops of formic acid until obtaining the desired pH. Several volumes of the AFB1 (100 µg L^{-1}) within the 0-0.4 mL range (prepared in KH_2PO_4/NaOH buffer and KH_2PO_4/formic acid at the tested pHs) were mixed with a fixed volume of 1.5 mL of 10 mg L^{-1} MIP-PEG-Mn-doped ZnS QDs at the selected pH. The mixtures were then made up to 2.0 mL with the buffer solution (0.5-0.1 mL) at the selected pH. After a delay time of 15 min for allowing an efficient interaction between AFB1 and MIP-PEG-Mn-doped ZnS QDs nanoparticles, the RTP intensity was recorded. Experiments in triplicate have shown good linearity between RTP quenching and AFB1 concentration, although a better linear relationship was obtained when using a pH 5.0. In addition, the highest graph's slope was also observed when

working at pH 5.0 (– 3.9530) and (– 3.8464) and (– 2.9735) for pHs of 7 and 6, respectively. Therefore, pH 5.0 was selected as the best pH for further experiments.

1.3.3.2 Effect of the MIP-PEG-Mn-doped ZnS QDs concentration

Several MIP-PEG-Mn-doped ZnS QDs concentrations (10, 20, 50, 100, and 200 mg L^{-1}) were prepared using 0.1 M KH_2PO_4 (pH 5.0 adjusted with formic acid). A fixed volume of 1.5 mL of MIP-PEG-Mn-doped ZnS QDs dispersions were then mixed with several volumes of AFB1 (0-0.4 mL), also prepared in 0.1 M KH_2PO_4 (pH 5.0 adjusted with formic acid) before making up to 2.0 mL with 0.1 M KH_2PO_4 (pH 5.0 adjusted with formic acid). After a delay time of 15 min, RTP quenching was recorded in triplicate for each AFB1 concentration level (results plotted in Figure 6). The best linear regression (regression coefficient of 0.9947), highest slope (– 6.8655), and repeatability of measurements were obtained when using the smallest MIP-PEG-Mn-doped ZnS QDs concentration. Therefore, MIP-PEG-Mn-doped ZnS QDs concentrations of 10 mg L^{-1} were selected for further experiments. Additionally, experiments by increasing the AFB1 concentration up to 20 µg L^{-1} showed that RTP quenching was slowed down due to self-absorption quenching.

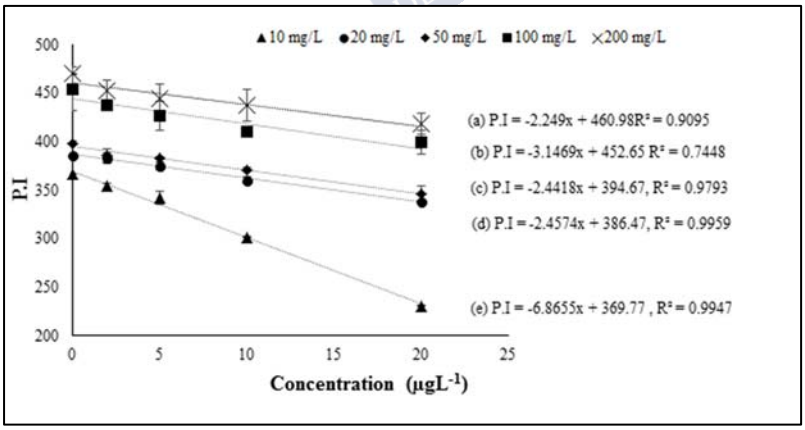

Figure 6: Effect of MIP-PEG-Mn-doped ZnS QDs concentration on the RTP quenching by AFB1

1.3.3.3 Effect of the interaction between AFB1 and MIP-PEG-Mn-doped ZnS QDs

The RTP quenching was not so good when the measurement was performed just after mixing the AFs and the MIP-PEG-Mn-doped ZnS QDs solutions/dispersions, and a certain time was needed for allowing an efficient interaction between the AFs and composite MIP-PEG-Mn-doped ZnS QDs. Hence, several experiments were performed (1.5 mL of 10 mg L^{-1} MIP-PEG-Mn-doped ZnS QDs, 10 µL of 100 µg L^{-1} AFB1 standard solution (concentration 10 µg L^{-1}), and 0.1 M KH_2PO_4 (pH 5.0 adjusted with formic acid) by recording the RTP quenching at the interaction time of 0 min (just after mixing), and after every 1 min. The highest interaction time tested was 20 min. Results (RTP measurements three replicates) have shown instability within the first 10 min; whereas, RTP intensity remains constant within the 10-20 min range. An interaction time (delay time) of 15 min was therefore selected.

1.3.4 Imprinting effect with MIP-PEG-Mn-doped ZnS QDs and NIP-PEG-Mn-doped ZnS QDs for AFs

This experiment was performed to establish the responses of MIP/NIP-PEG-Mn-doped ZnS QDs for four types of AFs and DMC (template for MIP synthesis) under optimum operating conditions. Results (Figure S2 (a-e), ESI section) show that the RTP intensity of the MIP-PEG-Mn-doped ZnS QDs was quenched linearly with increasing concentrations of AFs and DMC. The quenching effect depends on the recognition capacity of the imprinting cavities of the MIP. In this case, MIP-PEG-Mn-doped ZnS QDs showed a similar affinity for AFs, which are structurally similar to the template molecule (DMC). Stern-Volmer constants (Ksv) were calculated as the slope of the lineal graph when plotting the P_0/P ratio versus the quencher concentration (being P_0 the initial RTP) as shown in Figure S3 (f-j), ESI section. Ksv values obtained for AFB1 and AFB2 are quite similar to those obtained when using DMC. However, Ksv values for AFG1 and AFG2 were found to be lower. The ratios between Ksv for DMC ($Ksv_{(DMC)}$) and the Ksv values for AFs ($Ksv_{(DMC)}/Ksv_{(AFB1)}$, $Ksv_{(DMC)}/Ksv_{(AFB2)}$, $Ksv_{(DMC)}/Ksv_{(AFG1)}$, and

$K_{SV(DMC)}/K_{SV(AFG2)}$) were used to establish AFs affinity to the prepared MIP-PEG-Mn-doped ZnS QDs material. Ratios of 1.4, 1.4, 2.0, and 2.4 (Table 1) were calculated for AFB1, AFB2, AFG1, and AFG2, respectively, small ratios which show that the prepared MIP-PEG-Mn-doped ZnS QDs offers good recognition capacity for AFs as well as for DMC (template, ratio of 1.0).

Similar experiments were performed using NIP-PEG-Mn-doped ZnS QDs instead of MIP-PEG-Mn-doped ZnS QDs to prove if the interactions between DMC and AFs with MIP-PEG-Mn-doped ZnS QDs occur through the imprinted recognition cavities. Results (Figure S3 (a-e), ESI section) show that RTP quenching is not observed when using NIP-PEG-Mn-doped ZnS QDs at several DMC and AFs concentrations. These findings could prove that the MIP layer has good recognition cavities for DMC and AFs. However, it must be mentioned that the low degree of porosity, typically found in NIP, can also contribute to the absence of quenching due to a more efficient shield of the luminescent QDs. According to the Stern-Volmer equation analysis of both MIP-PEG-Mn-doped ZnS QDs and NIP-PEG-Mn-doped ZnS QDs, the imprinting effect of the MIP-PEG-Mn-doped ZnS QDs composite material was calculated as the $K_{SV(MIP)}/K_{SV(NIP)}$ ratios for each analyte (Table 1). High ratios were observed for DMC (13.8), but also for AFB1 (11.1), AFG2 (10.5), AFB2 (8.2), and AFG1 (5.4), which proves that the MIP-layer has recogonition cavities for DMC and four types of AFs.

Table 1: Stern-Volmer constants (MIP/NIP-PEG-Mn-doped ZnS QDs) for DMC, AFB1, AFB2, AFG1 and AFG2 and other compounds, and imprinting and selectivity factor

	Stern-Volmer constants		Imprinting Effect	Selectivity Factor	
	$K_{sv,MIP}$	$K_{sv,NIP}$	$K_{sv,MIP}/K_{sv,NIP}$	$K_{sv,MIP(DMC)}/K_{sv,MIP(X)}$	$K_{sv,MIP(DMC)}/K_{sv,NIP(X)}$
DMC	0.014	0.0010	14	-	14
AFB1	0.010	$9.3\ 10^{-4}$	11	1.4	15
AFB2	0.010	0.0012	8.2	1.4	12
AFG1	0.0072	0.0013	5.4	2.0	11
AFG2	0.0059	$5.6\ 10^{-4}$	11	2.4	25
Vit. A	$6.0\ 10^{-5}$	$1.4\ 10^{-4}$	0.434	237	102
Vit. D	$4.0\ 10^{-5}$	$2.0\ 10^{-4}$	0.196	361	71
ß-Carotein	$1.3\ 10^{-5}$	$2.2\ 10^{-4}$	0.059	1108	65
CMC	$2.2\ 10^{-4}$	$2.6\ 10^{-4}$	0.864	63	55
Agar	$2.8\ 10^{-5}$	$1.2\ 10^{-4}$	0.234	511	120
Kappa	$5.4\ 10^{-5}$	$1.6\ 10^{-4}$	0.336	263	88

(X) = AFB1, AFB2, AFG1, AFG2, Vit. A, Vit. D, ß-Carotein, CMC, Agar, Kappa

1.3.5 Selectivity study with MIP-PEG-Mn-doped ZnS QDs and NIP-PEG-Mn-doped ZnS QDs

A selectivity study was done by calculating the Ksv values for constant for other compounds present in fish feed such as vitamin A (retinol), 7-dehydrocholesterol, β-carotene, 16-carboxymethyl cellulose (CMC), agar and carrageenan (Kappa). These substances were tested as potential phosphorescence quenchers within the 0-1 mg L^{-1} range. Table 1 lists the Ksv constants for experiments using MIP-PEG-Mn-doped ZnS QDs and NIP-PEG-Mn-doped ZnS QDs for all quenchers, and also the imprinting factors (defined as above). Very small Ksv constants were calculated when using MIP/NIP-PEG-Mn-doped ZnS QDs; hence, small imprinting factors (within the 0.059 for β-carotene and 0.864 for CMC) were obtained. These findings imply that the prepared MIP-PEG-Mn-doped ZnS QDs material is quite selective for AFs.

Selectivity factors expressed as the ratios of Ksv obtained from the MIP-PEG-Mn-doped ZnS QDs using DMC (template) as a quencher and Ksv obtained from MIP-PEG-Mn-doped ZnS QDs using each compound are listed in Table 1. Low ratios (from 1.4 to 2.4) were found for four types of AFs; whereas, higher ratios (from 63.4 to 1108) were found for the other compounds. Again, we can confirm that prepared MIP-PEG-Mn-doped ZnS QDs material is highly selective for DMC and four types of AFs.

1.3.6 Calibration and matrix effect

Several independent calibration plots were prepared using DMC and four other AFs at concentration levels coving the 0-20 μg L^{-1} range (total volume of 2.0 mL using 1.5 mL of 10 mg L^{-1} MIP-PEG-Mn-doped ZnS QDs). Each concentration level was measured in triplicate, and three calibration experiments for each analyte as a calibrant were performed in three different days. The mean slopes of the external calibration graphs were 0.0278±0.0058 for AFB1, 0.0225±0.0060 for AFB2, 0.0275±0.0045 for AFG1, 0.0227±0.0047 for AFG2 and 0.0268±0.0063 for DMC, respectively, showing there were no significant differences ($p>0.05$) between the slopes of external calibrations when using DMC and the four AFs. Hence, AFB1 was

selected for further experiments (standard addition calibrations), using several volumes of an extract from a fish feed sample at the dilution ratios of 1:40, 1:20, 1:10 and 1:5 (total volume of 2.0 mL) using 1.5 mL of 10 mg L^{-1} MIP-PEG-Mn-doped ZnS QDs solutions, and experiments performed in three different days. The mean slope of the external calibration graph was 0.0253±0.012; whereas, the mean slopes of the standard addition graphs were as follows: 0.0276±0.007 (1:40 dilution factor, 50 µL of extract), 0.0297±0.0038 (1:20 dilution factor, 100 µL of extract), 0.0209±0.0025 (1:10 dilution factor, 200 µL of extract), and 0.0301±0.0075 (1:5 dilution factor, 400 µL of extract). There was no significant difference (p>0.05) between the external calibration graphs and standard addition graph when using any of the dilution ratios tested. These findings imply that the matrix effect from the fish feed extract is neglegible, even when using small dilution factors such as 1:5. The absence of matrix effect is a clear advantage over similar nanosensors based on fluorescent measurements which have been reported to require calibrations based on the standard addition technique [23] and/or additional steps for matrix removal [9].

1.3.7 Limit of detection and quantification

In the present study, the LOD and LOQ were established using the following equations:

$$LOD = 3.3\ X\ \frac{S}{b} \qquad LOQ = 10\ X\ \frac{s}{b}$$

where b is calibration graph slope, and s is the standard deviation of the appropriate number of the blank samples (n=11) or residual standard deviation of the calibration line in LOD region or standard deviation of the intercept [31]. A 1.5 mL of MIP-PEG-Mn-doped ZnS QDs (10 mg L^{-1}) solution and 0.5 mL of 0.1 M KH_2PO_4 (pH 5.0 adjusted with formic acid) was used as a blank. It was measured 11 times for assessing s. Calculated LOD and LOQ (taking into account dilutions and fish feed sample mass) were 3.56 µg $kg^{-1}$and 10.65 µg kg^{-1}, respectively. The European Commission (EC) [8] has introduced the maximum acceptable/residue level (MRL) for AFs in animal feeds as 0.02 mg kg^{-1} (20 µg kg^{-1}) The LOD and LOQ offered by the

proposed RTP analysis are lower than the EC cut off value for confirmation analysis.

1.3.8 Precision and accuracy

Intraday-precision and inter-day precision of the method were assessed by performing external standard solutions (negligible matrix effect). The intraday assay consisted of preparing three external calibration graphs in three different days. The first calibration was obtained by replicating the lowest AFB1 concentration (2 µg L^{-1}) seven times; whereas, the other concentration levels were replicated twice. Likewise, the second and third standard calibrations were obtained by replicating an intermediate (10 µgL^{-1}) concentration level, and the highest (20 µg L^{-1}) AFB1 concentration, respectively, also seven times each (remaining concentrations levels were performed in duplicate). The inter-day precision was obtained by preparing seven external calibrations in seven different days, replicating each AFB1 concentration twice. All obtained results are shown in Table2, which shows RSD values lower than ≤ 20%. Good intraday and interday precision is therefore achieved since values obtained are in good agreement with European guideline for validation pesticide residue analysis in food and feed (SANCO) [32]. Table 2 also lists the analytical recovery of intraday and inter-day assays. Thus, the values were closer to 100% for all cases, implying good intraday and inter-day accuracy.

In addition to the analytical recovery, accuracy was also assessed by determining the total AFs concentration in the certified reference material (CRM) ERM-BE 376 (compound in feeding stuff). The CRM sample was thoroughly mixed and homogenised by rolling it on a low-profile roller for 30 min before use. AFs extraction (n=5) was carried out as previously detailed in the Experimental Section, and each extract was analysed in duplicate for total AFs content. The actual (certified) total AF level in the CRM was 18.78±2.70 µg kg^{-1}, and the concentration found after applying the proposed method was 17.52±2.19 µg kg^{-1} (recovery of 93%). Therefore, good agreement between found concentration and the certified total AFs concentration was observed, which implies good accuracy.

Table 2: Intraday and inter-day precision (RSD%) and intraday and inter-day analytical recovery (AR%) of the method

[AFB1] (μg L^{-1})	RSD%[a]	RSD%[b]	AR%[a]	AR%[b]
2	13	14	84±11	88±14
5	NE	11	NE	108±6
7	12	6	103±12	107±10
10	NE	6	NE	109±6
20	3	4	93±3	98±5

(a) Intraday assay (n=7); (b) inter-day assay (n=7); NE = not evaluated

1.3.9 Application

Six fish feed samples from local fish feed manufacturers were analysed in triplicate with the proposed MIP-PEG-Mn-doped ZnS QDs based RTP method for total AFs. In addition, samples were also analysed using a MIP-based SPE method with HPLC-MS-MS for AFB1, AFB2, AFG1, and AFG2. Good agreement between total AFs levels after applying the proposed phosphorescent probe (screening analysis) and the HPLC-based method (confirmative analysis) can be seen in Table 4. These findings have been statistically verified by applying a t-paired test since the calculated t-value has found to be lower than the tabulated t-value regarding 95% confidence level and five degrees of freedom of ($t_{tabulated\ (95\%,5)} = 2.57$) as shown in Table 4. Findings show the presence of AFs in some fish feed used in aquaculture facilities, and total concentration levels are higher than the MRL for AFs in animal feeds established by the European Commission, a concentration of 20 μg kg^{-1}.

Table 3: Total AFs concentrations in selected fish feed samples by MIP-PEG-Mn-doped ZnS QDs RTP probe and HPLC-MS/MS

	[Total AFs] μg kg^{-1}	
Sample code	RTP MIP-PEG-Mn-doped ZnS QDs	HPLC-MS/MS[a]
1	48 ± 11	55 ± 2
2	90 ± 10	91 ± 1
3	32 ± 1	34 ± 4
4	93 ± 14	99 ± 4
5	82 ± 16	106 ± 6
6	47 ± 8	50 ± 1
	$t_{calculated}$	1.95
	$t_{tabulated\ (95\%,\ 5)}$	2.57

(a) Sum of single AFB1, AFB2, AFG1, and AFG2 concentrations

1.4 CONCLUSIONS

RTP based on MIP-PEG-Mn-doped ZnS QDs has been found to offer high sensitivity for assessing pollutants and contaminants such as AFs in complex materials, which is a clear advantage over those nanosensors based on fluorescence measurements. In addition, repeatability of RTP determinations was good, and quite similar slopes for the calibration plots were obtained in several days. The good repeatability of measurements is advantageous over fluorescence measurements, which has been reported to be affected by un-controlled lab conditions. RTP determinations which MIP-PEG-Mn-doped ZnS QDs are not affected by matrix effect, and determinations can be carried out using aqueous calibrations. Reported nanosensor-based fluorescence measurements usually required an effective sample matrix removal and/or the use of the standard addition technique for calibration, additional steps that are avoided when using nanosensors with RTP properties. Determinations based on MIP-PEG-Mn-doped

ZnS QDs RTP imply the use of low-cost analytical instruments, and its applicability can be therefore worldwide for screening purposes. Measured AFs concentrations have been found to be similar to those obtained after using expensive instrumentation such as HPLC based techniques.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Acknowledgments

This work was supported by the *Dirección Xeral de I+D – Xunta de Galicia Grupos de Referencia Competitiva* (project number 6RC2014/2016 and ED431C2018/19), and Development of a Strategic Grouping in Materials - AEMAT (grant ED431E2018/08). The authors also wish to thank Dr. Bruno Dacuña-Mariño (*Unidade de Raios X*) and to Dr. M. José Pazos-Guldrís (*Unidade de Microscopía*) at *Rede de Infraestruturas de Apoio á Investigación e ao Desenvolvemento Tecnolóxico* – University of Santiago de Compostela) for XRD and TEM technical support.

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1.6 ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Table S1. Phosphoresce spectrometry operating conditions

Total decay time (s)	0.004
Number of flashes	1
Delay time (ms)	0.100
Gate time (ms)	3.00
Excitation wavelength (nm)	290
Emission wavelength (nm)	594
Start (nm)	520
Stop (nm)	720
Excitation slit (nm)	20
Emission slit (nm)	20

Table S1: Instrument parameter for HPLC-MS-MS

Compound[a]	Precursor ion (m/z)	Product ion (m/z)	DP(V)	EP(V)	CE(V)	CXP(V)
DMC	206.800	121.100	46.590	7.630	34.150	3.000
B1	313.000	241.000	70.390	4.800	52.340	7.230
B2	315.000	259.200	84.900	2.820	39.320	2.240
G1	329.000	200.120	59.940	4.180	55.140	4.500
G2	331.000	213.200	68.780	4.110	35.000	3.000

HPLC			
Column	Zorbax Eclipse C18 reverse phase column (100 mm length x 4.6 mm i.d, 3.5 µm particle diameter)		
Injection volume	20 µL		
Flow rate	250 µL/min		
Mobile phase composition	0.1% formic acid in ultrapure water (A) and 0.1% formic acid in methanol (B)		
	Total time (min)	A%	B%
	0.1	50	50
	3.0	50	50
	8.0	0	100
	12.0	0	100
	13.0	50	50
	15.0	50	50

DP-Declustering potential, EP-Entrance potential, CE-Collision energy, CXP-Collision cell exit potential

[a] Electron spray operation conditions are: Ions spray voltage (IS)-5500 kV, Ion source temperature – 300 °C, nebulizer gas and curtain gas (N_2, 40 psi), collision gas (N_2, high)

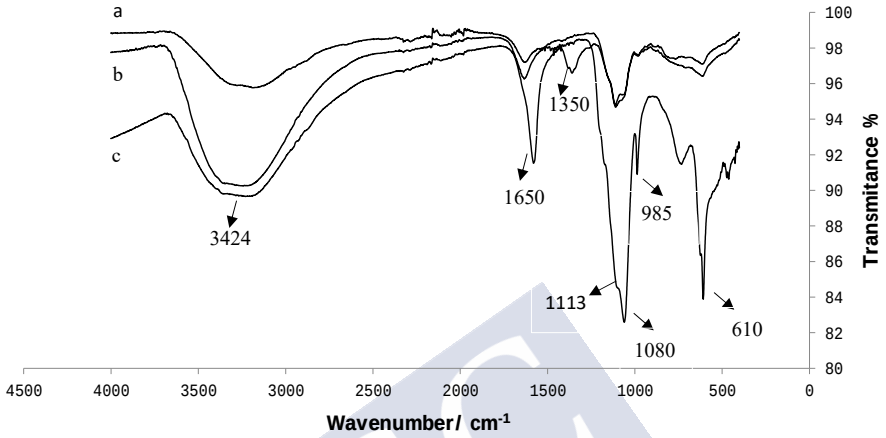

Figure S1: Fourier transform infra-red spectra for PEG-QDs (a), MIP-QDs (c) and NIP-QDs (b)

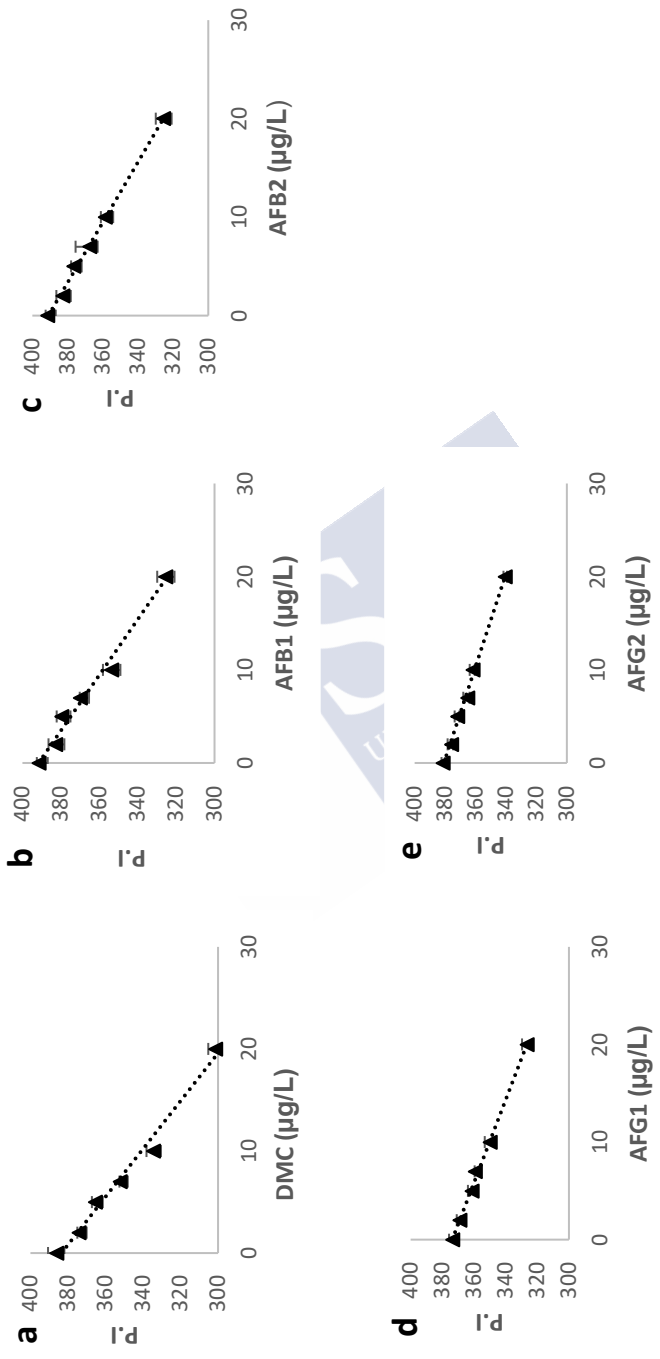

Figure S2: Effect of concentration of DMC (a), AFB1 (b), AFB2 (c), AFG1 (d) and AFG2 (e) on the RTP quenching of MIP-coated PEG-QDs

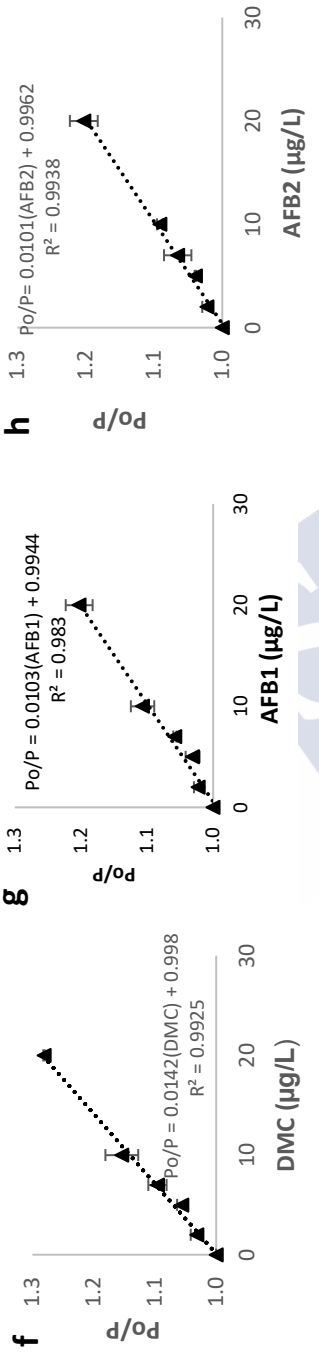

Figure S3. Stem-Volmer equations for DMC (f), AFB1 (g), AFB2 (h), AFG1 (i) and AFG2 (j) on the RTP quenching of MIP-coated PEG-QDs

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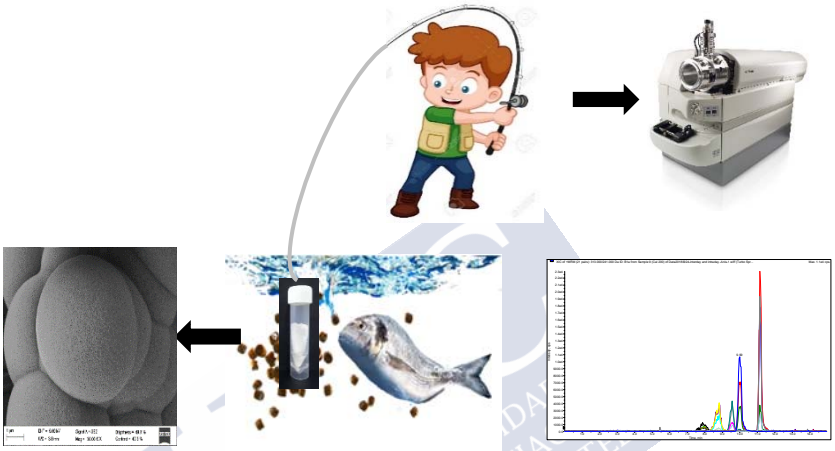

CHAPTER 2

COMBINED ULTRASOUND ASSISTED EXTRACTION AND MOLECULARLY IMPRINTED POLYMER – MICRO-SOLID PHASE FOR AFLATOXINS DETERMINATION IN FISH FEED USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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JOURNAL OF CHROMATOGRAPHY A 1609 (2020) 460431

DOI : 10.1016/j.chroma.2019.460431

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Combined ultrasound assisted extraction and molecularly imprinted polymer – micro-solid phase for aflatoxins determination in fish feed using liquid chromatography-tandem mass spectrometry

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Abstract

A combined procedure based on using ultrasounds for target isolation followed by porous membrane-protected micro solid phase extraction using a molecularly imprinted polymer as an adsorbent has been developed as a highly selective extraction and clean-up procedure for isolating aflatoxins B1, B2, G1, and G2 from fish feed before ultra-high-performance liquid chromatography tandem mass spectrometry determination. Polymeric adsorbent beads have been synthesized by the precipitation polymerization method which guarantees a homogeneous particles size distribution and the integrity of the generated imprinted cavities. In addition, polymerization was performed using a higher proportion of organic solvent (toluene) in the porogen mixture, which generates MIP particles adequate for interacting with targets dissolved in organic (hydro-organic) mixtures (extracts from fish feed). These approaches led to a selective and high efficient pre-concentration method for AFs. Ultrasound-assisted extraction (10 mL of 60:40 acetonitrile/0.1 M KH_2PO_4 pH 6.0, 40% amplitude, continuous sonication for 7.0 min) allowed an efficient aflatoxins isolation from fish feed. In addition, the resulting pH of the extract (pH 7.0) has been found to be the optimum for performing clean-up/pre-concentration (enrichment factor of 33.3) by molecularly imprinted polymer based micro-solid phase extraction (orbital horizontal shaking speed at 150

rpm for 10 min for loading, and 5 mL of 95:5 acetonitrile/formic acid as eluting solution using ultrasounds 35 kHz for 15 min). The current proposal was shown to be an accurate and precise method through relative standard deviation of intraday and inter-day tests below 20% and analytical recoveries in the range of 80-100%. The limits of detection were within the 0.42-1.15 μg kg^{-1} range, quite lower than those established by European Commission guidelines for aflatoxins in animal feeds.

Keywords: Molecularly imprinted polymer, Porous membrane protected, Aflatoxins, Fish feed, Tandem mass spectrometry, Ultrahigh-performance liquid chromatography

2.1 INTRODUCTION

Besides facing the complexity of many food/feed and environmental materials, developed analytical methods must to be sensitive enough to detect/determine pollutants at the low levels (maximum acceptable/ residue level, MRLs) proposed by the international organisations. A previous sample pre-treatment stage is therefore needed before analysis by modern instrumental techniques. Sample preparation techniques should be fast and repeatable procedures which avoid analytes losses and guarantee analyte integrity. Modern trends in sample pre-treatment methods imply the minimization of organic solvents and/or highly toxic or ecotoxic reagents, and the prevention of waste generation. Sorbent-, liquid phase- and membrane-based extraction/microextraction procedures have shown to be appealing techniques for pre-treating liquid samples [1-3]; whereas, assisted extraction procedures based on ultrasounds, microwaves, pressurization, and matrix solid phase dispersion (MSPD) can be successfully applied for target isolation from solid matrices. Advanced assisted procedures for solid materials lead to high analyte extraction efficiencies, but co-extraction of other matrix components is also important and further clean-up stages are required [4]. Solid phase extraction (SPE) is commonly used for clean-up and pre-concentration

purposes [5], and recent trends are focused on miniaturizing the extractive process (micro solid phase extraction, μ-SPE), increasing the sample throughput, and also developing adequate interfaces for online coupling with the detector systems [6]. In addition, the use of new adsorbents for SPE such as carbon nanotubes (CNTs), molecularly imprinted polymers (MIPs) and (magnetic) nanoparticles ((M)NPs) combined with μ-SPE have been reported to increase selectivity and hence, applicability for treating complex samples [4].

Aflatoxins (AFs) are a class of structure related mycotoxins produced as secondary metabolites by fungi belonging to several *Aspergillus species*, and AFs referred as AFB1, AFB2, AFG1 and AFG2, have been identified as the most dangerous and most frequently AFs found in food/feed. AFs extraction from solid materials requires aqueous mixtures of polar organic solvents (methanol, acetonitrile or acetone) [7], and most of developed procedures imply mechanical shaking (extraction times from 3 min to 72 h) [8-11]. Ultrasound assisted extraction (UAE) procedures with ultrasounds water-bath have been also developed to speed-up AFs extraction (times within the 5–10 min range) from rice [12] and soy-based products [13].In addition, microwave assisted extraction (MAE) has been also reported for AFs extraction from peach seed, milk powder and corn flour (extraction time of 10 min) [14]. Although liquid-phase based extraction and microextraction (homogenous liquid-liquid extraction HLLE; and dispersive liquid-liquid microextraction, DLLME) have been proposed for clean-up and/or pre-concentration before AFs isolation [8,11], most of developments are based on solid phase extraction (SPE) [9,10,12,13] and also on dispersive solid phase extraction/microextraction (D-SPE/D-μ-SPE) [14]. Novel magnetic nanocomposites sorbents are currently being used to induce dispersion in D-SPE/D-μ-SPE [15],and magnetite-graphene and nano zirconia have been proposed for AFs extraction [12-14].

Other group of μ-SPE procedures imply the adsorbent enclosing inside a polypropylene (PP) membrane which allows freely analytes diffusion before adsorption onto the enclosed solid adsorbent [16]. As recently reviewed by Sajid [17], conventional and new adsorbents such as carbon based sorbents, zeolites, metal-organic frameworks (MOFs),

mesoporous silica based materials, and molecularly imprinted polymers (MIPs), have been proposed in µ-SPE. MIP-based adsorbents offer excellent selectivity and they can be used for targets pre-concentration purposes and also decontamination of food and pharma samples [18,19]. The use of molecularly imprinted polymers (MIPs) as adsorbents in µ-SPE (molecularly imprinted micro-SPE, MIMSPE [20]) has been also reported for several pollutants in environmental samples [20,21], foodstuff [22], and clinical/forensic materials [23-28].

Regarding AFs, magnetic-MIP composites [29] and magnetic molecularly imprinted stir bars [30] have been recently developed for corn and infant cereal-based food and infant formula analysis [29-31]. In addition, MIP technology was also used for preparing monolithic molecularly imprinted polymeric capillary columns for AFB1 chromatographic separations [32] and MIP@quantum dot composites for fluorescent screening [33,34]. As the best of our knowledge, MIMSPE for AFs pre-concentration has not been reported. MIMSPE (use of single cone-shape PP devices as a µ-SPE system) is therefore a novel development for AFs pre-concentration. Improvements based on MIP particles synthesis by the precipitation polymerization method in presence of large amount of organic solvent (toluene) led to homogeneous MIP particles size distributions and to effective MIP particle-analyte interactions when performing AFs pre-concentration from organic solutions (fish feed extracts).

2.2 MATERIALS AND METHODS

2.2.1 Instrumentation

Aflatoxins (AFB1, AFB2, AFG1, AFG2) determination was performed with a 3200 Q TRAP LC/MS/MS system from ABSciex (Concord, Canada) equipped with electrospray ionization source, with a Flexar FX-15 UHPLC binary pump with integrated vacuum degasser (Perkin Elmer, Waltham, MA, USA) and with a Flexar UHPLC autosampler (Perkin Elmer). Chromatographic separations were achieved on a Zorbax C_{18} reverse phase column (100 mm length, 4.6 mm i.d, 3.5 µm particle diameter) from Agilent (Santa Clara, CA, USA) connected to a C_{18} guard column (4 mm length, 3.0 mm i.d) from Phenomenex (Torrance, CA, USA) under controlled temperature (40°C) using a

GECKO 2000 column heater from Amchro GmbH (Hattersheim, Germany). A low-profile roller (Stovall, Greensboro, NC, USA), placed inside a Boxcult temperature-controlled chamber (Stuart Scientific, Surrey, UK), was used for MIP synthesis. The same Boxcult chamber equipped with a Rotabit orbital rocking platform shaker (J.P. Selecta, Barcelona, Spain) was used for performing the loading step of the MIP-μ-SPE procedure. Elution stage was assisted by using a Raypa UCI-150 ultrasonic cleaner water bath (ultrasound frequencies of 17 and 35 kHz, 325 W) from R. Espinar S.L (Barcelona, Spain). A VibraCell VCx 130 ultrasound probe from Sonics (Newtown, CT, USA) was used for AFs isolation from fish feed. A Laborcentrifugen 2K15 centrifuge (Sigma, Osterode, Germany) was used for extract isolation from fish feed. A VLM EC1 metal block thermostat and nitrogen sample concentrator from VLM (Leopoldshohe-Greste, Germany) was used for extract solvent removal. MIP/NIP characterization was performed by using a Spectrum two FT-IR Fourier transform infrared spectrometer (FT-IR) from Perkin Elmer, an Ultra Plus field emission scanning electron microscope from Zeiss (Oberkochem, Germany) for SEM images, and a Micromeritics ASAP 2000 (Norcross, GA, USA) for BET and porosity measurements. Soxhlet extraction systems consisted of a 200 mL glass still pot attached to a glass distillation path, and a glass condenser, and heated with a Pilz WHG2 laboratory heating mantle from Winkler (Heidelberg, Garmany). Other devices were: a vibrating ball mill with 15 mL zircon oxide cups and 7 mm diameter zircon balls (Retsch, Haan, Germany), a Basic20 pH-meter with a glass-calomel electrode (Crison, Barcelona, Spain), a Classic ML analytical balance (Mettler Toledo, Columbus, OH, USA), a Selecta 207 oven (Barcelona, Spain), and a Lauson heat-sealer (Barcelona, Spain). UHPLCMS/MS data processing was performed with MultiQuant 2.1 software (ABSciex).

2.2.2 Reagents

Aflatoxins stock standards solutions (1000 mg L^{-1}) were prepared from solid AFs (AFB1, AFB2, AFG1 and AFG2) from Sigma-Aldrich (Steinheim, Germany) dissolved in methanol (LC-MS grade) purchased from Merck (Darmstadt, Germany). Similarly, 5,7-demethoxycoumarin (DMC) from Sigma-Aldrich was also prepared in methanol (stock standard solution of 1000 mg L^{-1}) and it was used as an internal standard for sample pre-treatment optimization. U-[$^{13}C_{17}$]-AFB1 in ACN (certified concentration of 0.501±0.0008 µg mL^{-1}) was purchased from LGC Standards (Wesel, Germany), and it was also used as an internal standard for method validation. All standard solutions were stored at -20°C in the dark. Retinol, 7-dehydrocholesterol, and β-carotene stock solutions (1000 mg L^{-1}) were prepared in methanol from solid reagents (Sigma-Aldrich). Other compounds used in cross-reactivity studies, such as carrageenan (kappa and lambda) were from CEAMSA (Porriño, Spain), agar-agar were from Algamar (Redondela, Spain), and carboxymethyl cellulose (CMC) from Scharlau (Barcelona, Spain). These reagents were dissolved in hot water to prepare 1000 mg L^{-1} stock standard solutions. MIP synthesis required methacrylic acid (MAA) and divinylbenzene (DVB) from Sigma-Aldrich, and 2,2'-azobisisobutyronitrile (AIBN) from Fluka (Buchs, Switzerland). Solvents such as acetonitrile, methanol (HPLC grade), and reagents such as ammonium acetate, neutral alumina, potassium dihydrogen phosphate, and sodium hydroxide were purchased from Merck. Toluene (HPLC grade), acetic acid (glacial) were from Panreac (Barcelona, Spain). Certified reference material ERM-BE376 (compound feeding stuff) was purchased from European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (Geel, Belgium). Ultrapure water, 18 MΩ cm of resistivity, was obtained from a Milli–Q purification device (Millipore Co., Bedford, MA, USA). Other used consumables were: ACCUREL® PP membrane (Membrana, Wuppertal, Germany), cellulose thimbles for Soxhlet extraction (33 mm i.d, 37 mm e.d., 118 mm height) from Prat-Dumas (Couze-et-Saint-Front, France), Durapore 0.20 µm membrane filters (Millipore), nonsterile MCE syringe filters (0.45 µm) from Sterlitech (Kent, WA, USA), and

disposable syringe (sterile, 2mL) from Dispomed (Gelnhausen, Germany).

2.2.3 Fish feed samples

Fish feed samples were from local fish feed manufacturers in Santiago de Compostela, Spain. All fish feed samples were ground by vibrating ball mill, and they were stored at -20 °C until further analysis.

2.2.4 Synthesis of MIP particles

Because of the high toxicity of AFs, a dummy template is preferred for the synthesis of the imprinted polymers [30]. However, dummy templates must be chemical structurally similar to analytes under assessment so that the generated recognition cavities can be complementary to those analytes. Since AFs are difuranocoumarin derivatives, AFs are chemical structurally similar to DMC (5,7-dimethoxycoumarin), and they exhibit the same methoxycoumarin structure (Fig. S1, electronic supplementary information ESI). DMC is therefore a dummy template of choice when preparing MIPs for AFs recognition [29,30,32–34].

DMC (0.0699 g) was mixed with 115 µL of MAA and 25 mL of porogen (1:3 acetonitrile/toluene) into 30 mL glass tube. The mixture was stirred for 5 min and covered with aluminium foil and kept in the dark overnight to allow self-assembly of the template and monomer. A large porogen volume (25 mL) leads to further MIP synthesis by precipitation polymerization, which allows homogeneous and dispersed MIP particles and guarantees the integrity of the generated imprinted cavities. In addition, the high proportion of toluene in the porogen mixture allows MIP particles with recognition cavities more available for target retention in organic solvents (acetonitrile/water extracts from fish feed samples).

After template-monomer self-assembly, 1.25 mL of previously purified DVB and 0.1 g of AIBN were added into the pre-polymerization mixture and stirring for 1 min. The mixture was purged with nitrogen for 5 min and immediately sealed and placed in a low-profile roller (33 rpm on its long axis) inside a temperature-controllable

chamber (the temperature was ramped from room temperature to 60 °C for 2 hours, and then maintained at 60 °C for 24 hours).

DVB was purified by passing a few milliliters of the reagent through a previously prepared mini column containing neutral alumina (approximately 0.5g). Similarly, AIBN was purified by crystallization at -20 °C after dissolving the reagent in methanol at 50-60 °C.

After finished the polymerization, the synthesized material was vacuum filtered and washed with acetonitrile (20 mL, 3 times) and oven dried overnight at 40 °C.

Non-Imprinted polymers (NIPs) were also prepared by following the same method as MIPs, but without adding the template (DMC). Synthesized NIPs were then subjected to the same filtering and washing steps described above.

2.2.5 Template removal procedure

DMC template was removed from the synthesized MIP by Soxhlet extraction. Approximately, 300 mg of dried MIP beads were placed into a cellulose thimble and were treated with 200 mL of 85:10:5 methanol/water/acetic acid mixture until template was not detected in the washing solutions (UHPLC-MS-MS analysis). After Soxhlet extraction (total template removal), the material inside the cellulose thimble was fully rinsed with ultrapure water and then oven-dried at 40 °C for 12 hours before use.

2.2.6 Preparation of the MIP-µ SPE device

MIP-µ-SPE device was prepared by cutting the PP membrane in circle shape (12 cm diameter), and by folding 3 times to obtain a quarter circle (cone) as shown in Figure 1. MIP particles (approximately 50 mg) were placed into either one of the closed-end folds of the cone, and the upper part of the device was heat-sealed. Before use, all prepared MIP-µ-SPE devices were conditioned by sonication with 5 mL of 0.1 M KH_2PO_4/NaOH buffer solution (pH 6) for 10 min. The MIP-µ-SPE devices are soaked in buffer solution meanwhile they were stored. Control NIP-µ-SPE devices were prepared using the same procedure as described above. Several MIP-µ-SPE devices (at least 20) can be arranged inside the temperature-controlled chamber (Figure 1).

2.2.7 AFs isolation from fish feed by ultrasound assisted extraction
Dried/homogenized fish feed (0.250g) was mixed into a 30 mL centrifuge tube with 10 mL of 60:40 acetonitrile/0.1 M KH_2PO_4 buffer pH 6 (pH adjusted by using 0.1 M NaOH). The pH of the final extracting solution is 7.0. The tube containing the sample and the extractant was placed in an ice-bath, and the mixture was then ultrasonicated for 7.0 min at 40% amplitude (40% of the ultrasonicator power/frequency (130 W/20kHz)) using continuous sonication. Ultrasound dissipation in the solid sample-extractant mixture leads to an efficient AFs extraction in short times and also a temperature increase which is minimized by placing the test tube in an ice-bath. Finally, the extract was isolated by centrifugation (3000 rpm, 4°C, 10 min).

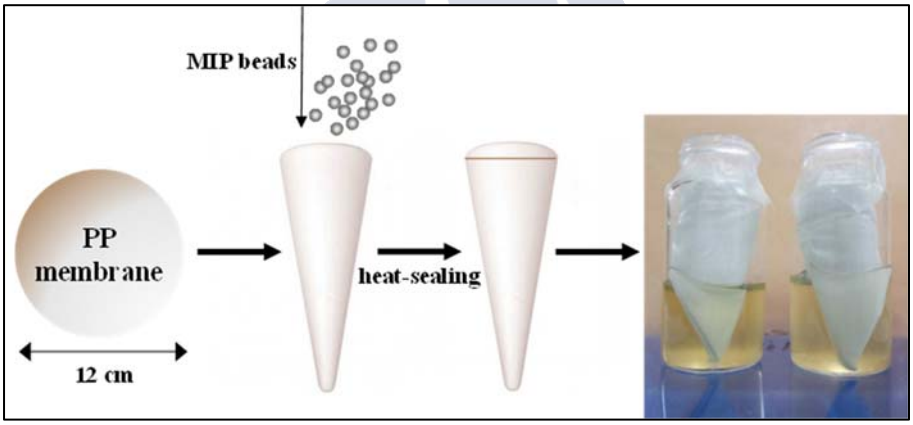

Figure 1. Schematic of preparation of MIP-μ-SPE devices

2.2.8 AFs pre-concentration by MIMSPE
The isolated extract (10 mL) was directly subjected to MIMSPE by mechanical orbital-horizontal shaking at 150 rpm and at room temperature for 10 min. The MIP-μ SPE devices was then separated with tweezer and rinsed with 5mL of 0.1M/0.1M KH_2PO_4/NaOH (pH 6.0). Elution stage was performed by sonication with 5 mL of 95:5

acetonitrile/formic acid for 15 min (water-bath sonication, 35 kHz, 325 W). Finally, the eluate was dried under N_2 gas and it was again dissolved in 300 µL of methanol. Taking into account the volume of the fish feed extract (10 mL) and the volume of the re-dissolved eluate after MIMSPE and drying (300 µL), the enrichment factor was 33.3. Retention properties of MIP beads remained constant at least after 20 uses.

Figure 2 shows a workflow summarising the steps involved in the UAE-MIMSPE procedure.

2.2.9 UHPLC-MS/MS measurement

Chromatographic separation was performed under gradient elution [0.1% formic acid in ultrapure water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B)] at a flow rate of 250 µL min^{-1}. MS/MS acquisition settings are listed in Table 1. Multi reaction monitoring mode (MRM) was used for data acquisition, and *m/z* (precursor ions) → *m/z* (product ions) transitions were acquired using positive electrospray ionization under optimized ion source potentials and collision energies listed in Table 1. Figure 3 shows a chromatogram for a mixture of AFs (standard) and also for a fish feed the sample under optimized sample pre-treatment conditions.

Standard addition graphs were prepared in duplicate (10 mL of a fish feed extract) by spiking with AFs standards covering concentrations of 0.075, 0.15, 0.30, 0.60, 1.5, and 3.0 µg L^{-1}. Taking into account a pre-concentration factor of 33.3 AFs concentrations were 2.5, 5.0, 10, 20, 50, and 100 µg L^{-1} in the reconstituted extract. DMC (1.5 µg L^{-1}) or U-[$^{13}C_{17}$]-AFB1 (1.5 µg L^{-1}) were used as internal standards for sample pre-treatment optimization and for validation, respectively. By assuming a pre-concentration factor of 33.3, the concentration of internal standards in the reconstituted solution was 50 µg L^{-1} DMC or U-[$^{13}C_{17}$]-AFB1.

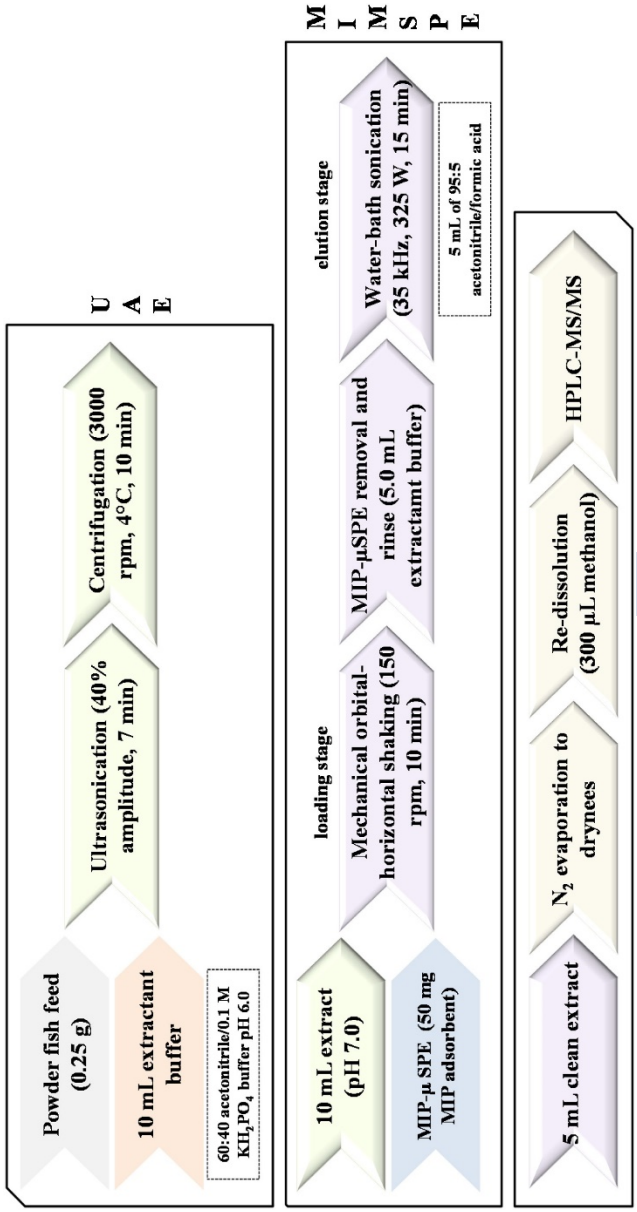

Figure 2: UAE-MIMSPE workflow for AFs extraction from fish feed

2.2.10 Statistical analysis

Statgraphics Centurion XVI v16.1.15 (Manugistics Inc., Rockville, MD, USA) software was used for statistical (Cochran C-test and ANOVA) evaluation for comparing standard deviation and mean values, respectively.

Table 1: Instrument parameters for UHPLC-MS/MS

MS-MS Compound[a]	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)[b]	EP (V)[b]	CE (V)[b]	CXP (V)[b]
DMC	206.800	121.100	46.590	7.630	34.150	3.000
AFB1	313.000	241.000	70.390	4.800	52.340	7.230
AFB2	315.000	259.200	84.900	2.820	39.320	2.240
AFG1	329.000	200.120	59.940	4.180	55.140	4.500
AFG2	331.000	213.200	68.780	4.110	35.000	3.000
U-[$^{13}C_{17}$]-AFB1	312.800	268.100	41.700	3.370	20.070	3.870
HPLC						
Column		Zorbax Eclipse C_{18} reverse phase column (100 mm length × 4.6 mm i.d, 3.5 µm particle diameter)				
Injection volume		20 µL				
Flow rate		250 µL min^{-1}				
Mobile phase composition		0.1% formic acid in ultrapure water (A) and 0.1% formic acid in methanol (B)				

Total time (min)	A%	B%
0.1	50	50
3.0	50	50
8.0	0	100
12.0	0	100
13.0	50	50
15.0	50	50

[a] Electrospray operation conditions are: Ion spray voltage (IS), 5500 kV; Ion source temperature, 300 °C; nebulizer gas and curtain gas (N_2), 40 psi; collision gas (N_2), high

[b] DP-Declustering potential, EP-Entrance potential, CE-Collision energy, CXP-Collision cell exit potential

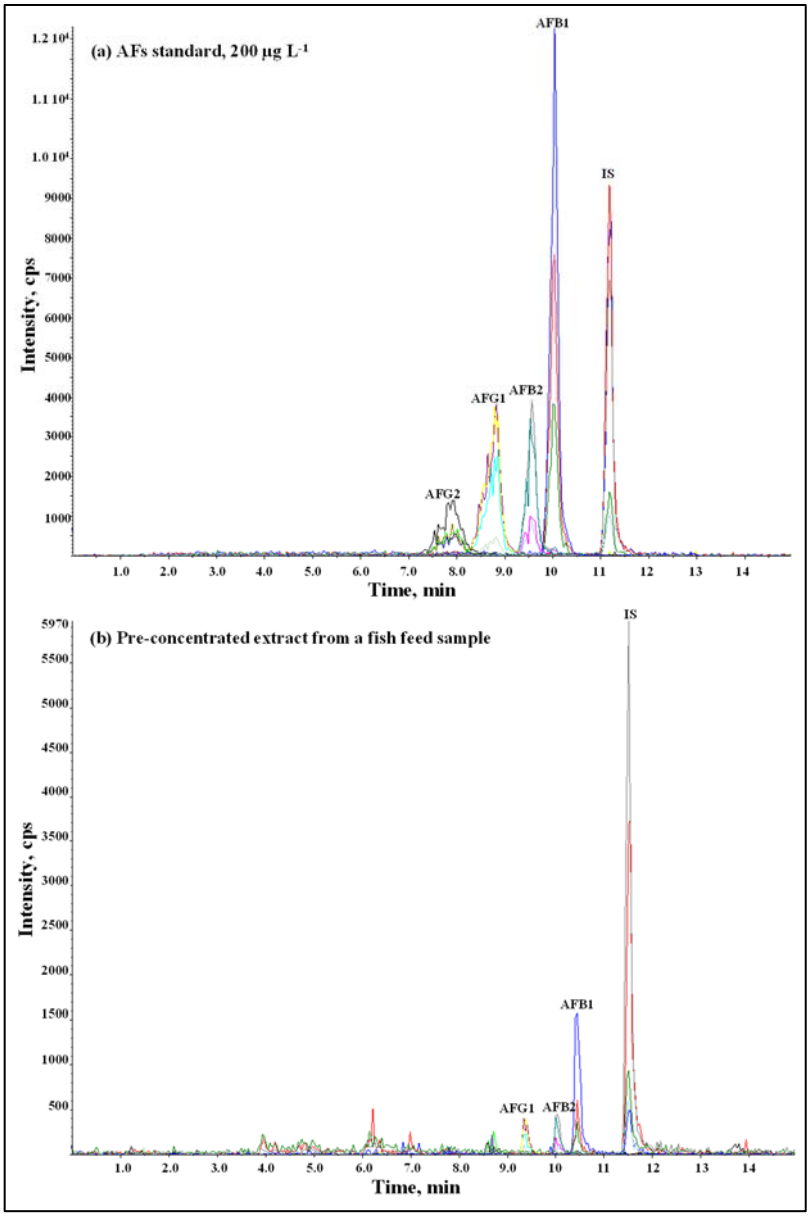

Figure 3: MRM chromatogram of a 200 µg L^{-1} AFB1, AFB2, AFG1 and AFG2 standard solution (a) and a fish feed sample (b)

2.3 RESULTS AND DISCUSSION

2.3.1 Characterization

Synthesized materials (MIP and NIP) have been characterized by SEM and FT-IR. High degree of agglomeration of MIP/NIP spherical particles was observed in SEM images (Figure S1(a,b), electronic supplementary information ESI), and beads of approximately 5µm and 3µm in diameter were obtained for MIP and NIP, respectively. FT-IR spectra (Figure S2, ESI) provided similar information concerning the functioning groups present in MIP and NIP beads [bands at 1350 and 2900 cm^{-1} (C-H stretching) and at 1650 cm^{-1} (C=O stretching)]. These findings demonstrate that MIP (after template removal) and NIP have similar characteristic bonds, which implies a successful template removal without MIP damage.

BET and porosity studies (Table S1, ESI) show that MIP (before and after template removal) and NIP can be classified as mesopores (pores higher than 0.2 Å). Surface area and pore volume of MIP before and after template removal are quite similar and higher than those values found in NIP. These findings suggest that the template removal procedure does not change surface are and porosity of the prepared materials. In addition, higher pore diameters have been observed in MIP than in NIP which agrees to the imprinting effect of templates in MIPs.

2.3.2 Optimization of MIMSPE conditions

MIMSPE operating conditions have been optimized by using fish feed extracts obtained from un-spiked and spiked (1.5 µg L^{-1} of each AFs and DMC as an internal standard) fish feed sample under non-optimized UAE conditions: 60:40 acetonitrile/aqueous 0.1 M KH_2PO_4 (mixture pH of 6.0), 60% amplitude, for 10 min (continuous sonication). Un-spiked samples were always analysed in order to subtract the AFs naturally occurring in the fish feed sample and to evaluate properly the recovery of the spikes. All parameters affecting the MIMSPE loading and elution conditions, as well as those affecting UAE (Section 3.3) have been studied by using a univariate optimization approach.

2.3.2.1 Loading conditions

Variables affecting AFs adsorption through the MIP's cavities such as pH of the extract, loading time and orbital horizontal stirring speed were evaluated using fish feed sample (0.250 g) spiked with 1.5 µg L^{-1} of each AF and DMC (50 µg L^{-1} after pre-concentration). Elution was performed under non-optimized conditions using sonication and 5 mL of 95:5 acetonitrile/formic acid (eluting solution) for 10 min (water-bath sonication, 35 kHz, 325 W). All experiments were performed in triplicate and recoveries were calculated using a matched calibration graphs.

Several pH values were tested to attain the best interaction between AFs and MIP recognition cavities. Since the pH of extract is dependent on the final pH of the extracting solution used in UAE, the pH of the extracting solution for UAE and the best pH of the extract for an efficient MIMSPE pre-concentration were studied at the same time. Several extracting solutions consisting of 60:40 acetonitrile/aqueous 0.1M KH_2PO_4 at four pHs 5.0, 6.0, 7.0 and 8.0 were tested. As shown in Figure 4(a), the highest recoveries for all AFs were obtained when the fish feed extract exhibits a pH of 7.0. Oxygen atoms in AFs are responsible of hydrogen acceptor properties of AFs, and a low protonation degree is expected when working at neutral pHs. Since the pH of the polymerization mixture was close to 7.0, interactions between analytes and recognition cavities in MIP particles are favoured at neutral pHs. It must be said that a 60:40 acetonitrile/aqueous 0.1M KH_2PO_4 (pH 7.0) extract is obtained when mixing acetonitrile (60%) and aqueous 0.1 M KH_2PO_4 at pH 6.0 (40%). In addition, strong acidic or basic conditions enhance AFs decomposition [35], which also leads to lower recoveries. Our findings agree with those reported by other authors that applied extraction techniques such as hollow fiber-solid phase microextraction (HF-SPME) [35] and DLLME [36] and that have reported a neutral environment as the best conditions for enhancing AFs extraction. Therefore, an extracting solution consisted of 60:40 acetonitrile/aqueous 0.1M KH_2PO_4, pH 6.0 was finally selected, which gives an extract of pH 7.0 (optimum pH for MIMSPE).

After selecting the optimum pH of the extract several experiments were performed at different orbital-horizontal stirring speeds (Figure

4(b)). High recoveries for AFB2 and AFG2 were obtained for high speeds (within 100 and 200 rpm). However, AFB1 and AFG1 recoveries were gradually increased until 150 rpm, and they were slightly lower at 200 rpm. Recovery impairment at high speeds agree with previous data regarding MIMSPE [24,28] and other μ-SPE procedures [37,38], and it is attributed to back-diffusion phenomena when using high shaking speed and also when using long loading times. Therefore, 150 rpm was selected as best orbital-horizontal shaking speed for further experiments.

As shown in Figure 4(c), back-diffusion phenomena were observed when using large loading times, independently of the elution (sonication) time used. In accordance to these findings, a loading time of 10 min was finally selected.

2.3.2.2 Elution conditions

Experiments regarding elution were tested by using several elution mixtures based on acetonitrile or methanol at acid pHs (pH given by the small proportion of formic acid) and under sonication (water-bath sonication, 35 kHz, 325 W). Moderate acid pHs promote AFs protonation and hence AFs desorption from MIP particles. After preliminary studies, acetonitrile (95%) and formic acid (5%) was the selected mixture since it offered the higher elution efficiency for all AFs. Regarding the elution (sonication) time, Figure 4(c) shows that AFs are conveniently eluted when using the highest sonication time tested (15 min) independently of the loading time fixed for AFs loading. Therefore, an elution time of 15 min was finally selected.

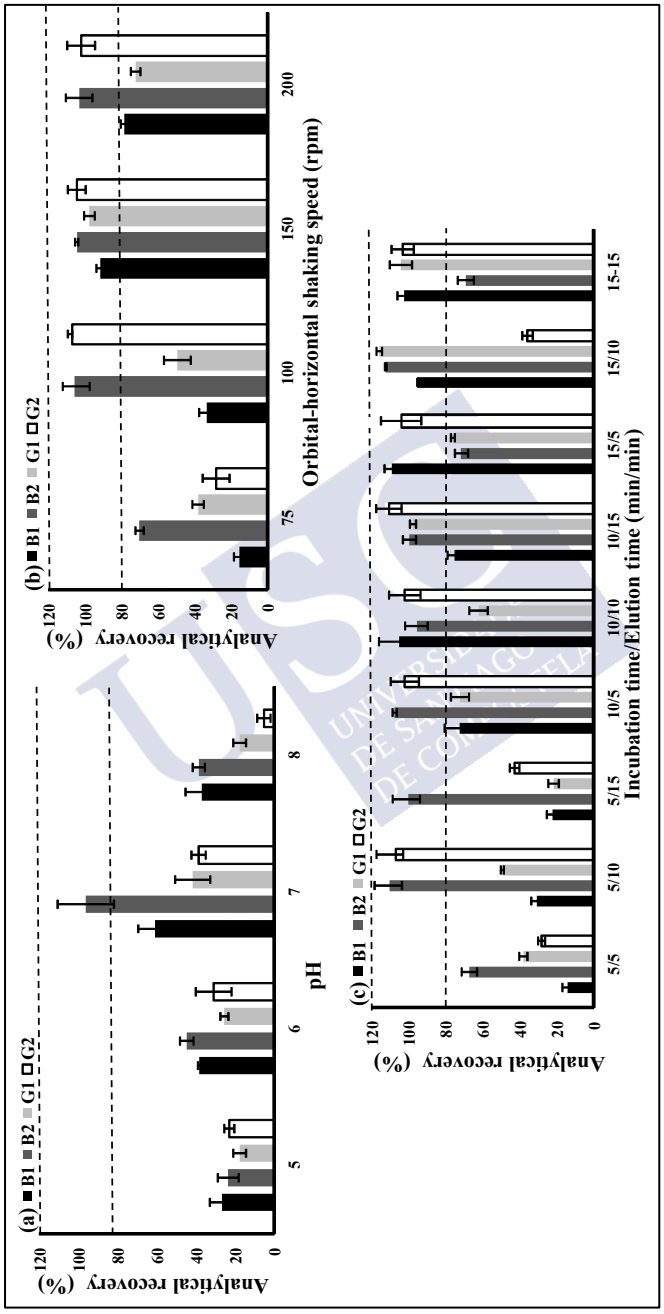

Figure 4: Effect of the pH of extracting solution (a), the orbital horizontal shaking speed (b), and the loading time/ elution time (c) on the AFs recovery

2.3.3 Optimization of the UAE conditions

AFs are low molecular mass polar compounds, and hence extraction is favoured when polar organic solvents (also water/polar organic solvents mixtures) such as methanol, acetonitrile or acetone are used [7]. Sample matrix also conditions the selection of the extracting solution. Previous reports have stated that a little amount of water enhances AFs extraction when pre-treating dried solid samples as a consequence of sample wetting which leads a further extractive effect by organic solvents [7, 39]. As reported by Rodríguez-Cervantes et al., 40% is an adequate water proportion when combining with the organic solvent for AFs extraction from dried materials [39], and an extracting solution consisting of 60:40 acetonitrile/phosphate aqueous was selected in the current study. The pH of the mixture was varied and AFs extractions from fish feed sample was successful when using slightly acid pHs. These findings are explained taking into account the hydrogen acceptor capacity of AFs. However, the pH of the extracting solution was fixed at 7.0 because this pH was the optimum pH for achieving a further quantitative pre-concentration by MIMSPE. Moreover, neutral pHs guarantee AFs integrity [35]. Therefore, a 60:40 acetonitrile/0.1 M KH_2PO_4 (pH 6.0), which leads to an extracting mixture of pH 7.0 was selected for UAE.

Figures 5 (a,b) shows effect of UAE conditions (ultrasound amplitude, sonication time) on AFs extraction from fish feed samples. MIMSPE operating conditions were fixed at the optimum values, and AFs recoveries were assessed by using matched calibrations and after performing the experiment in triplicate. Regarding ultrasound amplitude (Figure 5(a)), the recoveries for all AFs were gradually increased until ultrasonicating at 40%; whereas, recoveries decreased when sonicating at high amplitudes. In addition, high recoveries were obtained when sonicating for 7 min and it was decreased when increasing the sonication time (Figure 5(b)). Although higher extraction yield could be expected when using high ultrasound amplitudes and extraction times, high ultrasound amplitudes and sonication times affect the integrity of the extracted targets. In addition, high ultrasound amplitude and sonication time increase the temperature of the mixture, which also affect AFs integrity.

Finally, continuous (ultrasonication for 7.0 min) and discontinuous (seven cycles of ultrasonication for 59 and six cycles of relaxing in between each ultrasonication cycle) ultrasonication modes were tested. Results (Figure 5 (c)) showed higher extraction efficiencies when using a continuous ultrasonication (AFs recoveries close to 100%) compared to discontinuous ultrasonication (AFs recoveries within the 60-80% range). Discontinuous sonication is not able to promote AFs releasing because of the short ultrasound amplitude selected. Better extraction can be obtained by continuous ultrasonication at short ultrasonication amplitudes and short times.

2.3.4 Cross-reactivity and imprinting effect

Parameters such as extraction efficiency (analytical recovery), distribution ratio (D) and selectivity coefficient ($S_{DMC/D}$) were calculated to study the selectivity (imprinting effect) of the synthesized material for AFs. The experiment was performed using solutions containing 0.6 μg L^{-1} of each AF and DMC (template molecule) prepared in an acetonitrile/aqueous phosphate mixture. Similarly, other compounds present in fish feed such as vitamin A and D, β-carotein, CMC, agar-agar, and carrageenan (kappa and lambda) were investigated for cross-reactivity (acetonitrile/aqueous phosphate mixtures containing 1.0 mg L^{-1} of each compound). Results in triplicate for MIMSPE experiments using MIP and NIP and after UHPLC-MS-MS assessment showed extraction efficiencies closed to 100% for DMC (template) and AFs when using MIP-μ-SPE (Table 2). Extraction efficiencies for AFs were however lower than 30% for experiments involving NIP-μ-SPE. These findings show that AFs (and DMC) adsorption occurs through the generated recognition cavities in the synthesized MIP. The extraction efficiencies for other fish feed ingredients were within the 12–48% range for MIP-μ-SPE, and from 14 to 50% for NIP-μ-SPE. These results show that retention of these compounds in MIP is not attributed to the selective imprinting cavities but is attributed to nonspecific adsorption (surface adsorption).

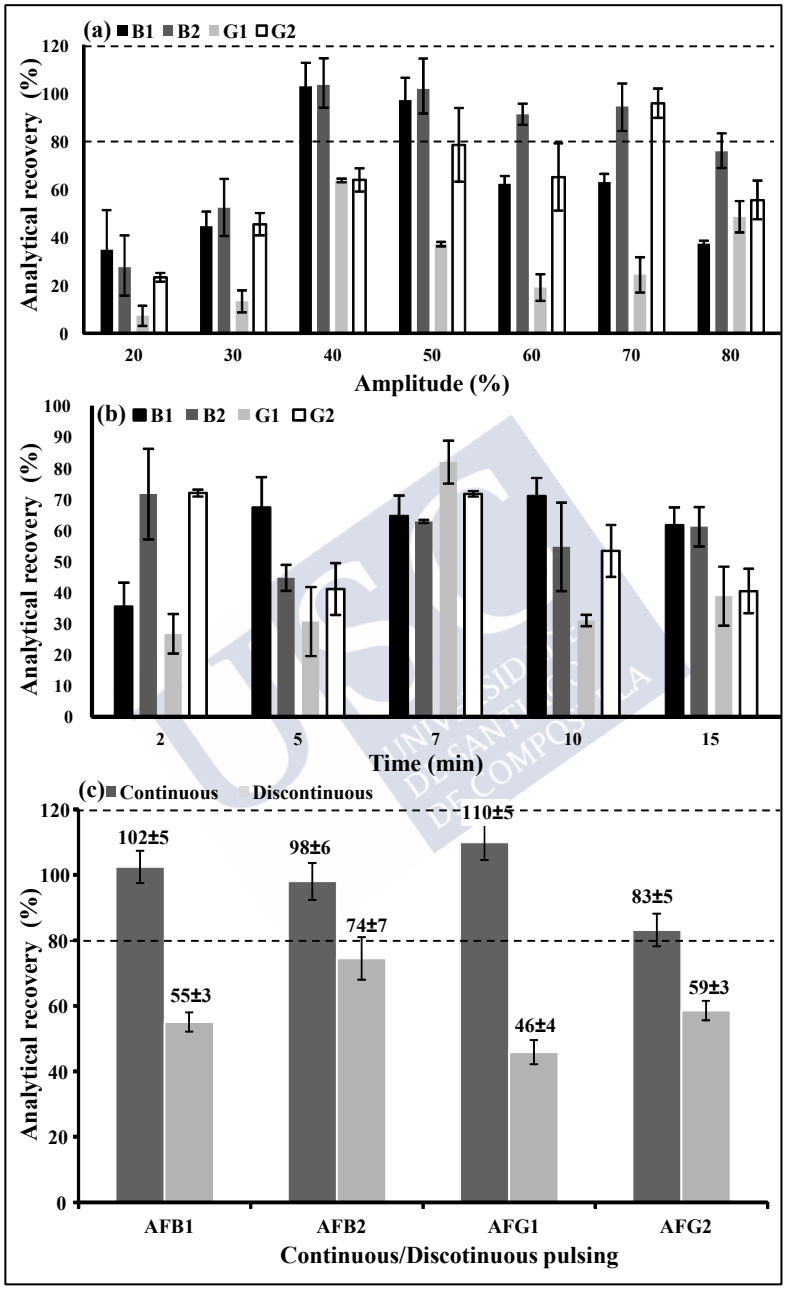

Figure 5: Effect of the ultrasound amplitude (a), the ultrasonication time (b), and the continuous/discontinuous ultrasonication mode (c) on the AFs recovery

Table 2: Extraction efficiency (%), distribution ratio (D) and selectivity coefficient for MIP-μ-SPE and NIP-μ-SPE

	Extraction efficiency (%)[a]	Distribution ratio (D)[b]	Selectivity coefficient ($S_{DMC/X}$)[c]
		MIP	
DMC	98	41	---
AFB1	95	18	2
AFB2	93	14	3
AFG1	93	13	3
AFG2	90	9	5
Vit A	12	0.14	293
Vit D	48	0.94	44
ß-Carotein	10	0.11	373
CMC	10	0.12	342
Agar-agar	37	0.59	69
Lambda	40	0.66	62
Kappa	23	0.29	141
		NIP	
DMC	13	2	21
AFB1	26	0.35	117
AFB2	25	0.34	121
AFG1	23	0.31	132
AFG2	22	0.28	146
Vit A	14	0.17	241
Vit D	50	1	41
ß-Carotein	8	0.09	456
CMC	11	0.12	342
Agar-agar	37	0.58	71
Lambda	44	0.79	52
Kappa	29	0.42	98

A_1=Amount of analyte in aqueous solution at equilibrium

A_2=Amount of analyte enriched by MIP/NIP at equilibrium

A_T =Total amount of analyte used in extraction

D_{DMC}= Distribution ratio of dimethoxycoumarine (template)

D_X=Distribution ratio of other compounds (X = vitamin A, vitamin D, ß-carotein, carboxymethyl cellulose (CMC), agar-agar, carrageenan Lambda, carrageenan Kappa); [a] % =$(A_2/A_T) \times 100$, [b] D =(A_2/A_1); [c] $S_{DMC/X}=D_{DMC}/D_X$

As listed in Table 2, high distribution ratios and low selectivity coefficients were obtained for DMC and AFs. Regarding the other fish feed ingredients, low distribution ratios and high selectivity coefficients were obtained. In general, high distribution ratios and low selectivity coefficients prove that MIP offers imprinting properties and high selectivity for DMC and AFs.

2.3.5 Analytical performance

2.3.5.1 Calibration and matrix effect

Matrix effect has been estimated by comparing the slopes of methanol calibration curves and standard addition curves covering concentrations of 2.5, 5, 10, 20, 50, 100 µg L^{-1} for each AF and using 50 µg L^{-1} of U-[$^{13}C_{17}$]-AFB1 as an internal standard. Standard additions were prepared by spiking the fish feed sample (0.250g) mixed with 10 mL of the extractant with increasing AFs concentrations of 0.075, 0.15, 0.3, 0.6, 1.5, 3.0 µg L^{-1} (concentrations within the 2.5 – 100 µg L^{-1} range after MIMSPE pre-concentration).

Mean slopes (also the standard deviations) for seven methanol calibrations and seven standard addition curves obtained in different days are listed in Table 3. Matrix effect expressed as the ratio (percentage) between calibration and standard addition slopes was higher than 20% for all AFs (28% for AFB1, 21% for AFB2, 34% of AFG1 and 49% for AFG2). A statistical evaluation was performed by comparing the standard deviation and average values of the slopes of methanol calibration and standard addition at a confidence interval of 95%. The application of the Cochran'C test (comparison of the standard deviation of slopes) led to conclude that there were not statistically significant differences between the standard deviation of the average slope for calibration and standard addition for each AFs. Therefore, ANOVA was applied for comparing the average slopes (95% confidence interval), and statistically significant differences were not found between the slopes of calibration and standard addition for AFB1 and AFB2 (p-values of 0.0999 and 0.5854, respectively, values > 0.050 at 95% significance level). However, ANOVA showed that slopes for calibration and standard addition of AFG1 and AFG2 were statistically significant different at a 95 % significance level (p-values of 0.0308

and 0.0318 for AFG1 and AFG2, respectively, < 0.050). Therefore, matrix effect is important when assessing AFG1 and AFG2, and accurate results are obtained when performing the standard addition technique for determinations. Matrix effect was not evaluated in previous reported methods based on MIP-based SPE and HPLC using MS/MS [29,30] and fluorescent [31] detection.

Finally, the regression coefficients were higher than 0.9979 for AFB1, 0.9988 for AFB2, 0.9986 for AFG1 and 0.9959 for AFG2, which shows good linearity for all cases.

2.3.5.2 Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were established by subjecting eleven blank samples (blanks obtained after applying UAE and MIP-μ-SPE) to HPLC-MS-MS and integrating the noise at the retention times and m/z (precursor ion) → m/z (product ion) for each AF. Then, LOD/LOQ values were established by using the following equations:

$$LOD = 3\,X\,\frac{S}{b} \qquad LOQ = 10\,X\,\frac{s}{b}$$

where b is the mean slope of the standard addition calibrations, and s is the standard deviation of the measurements of the blanks. LOD/LOQ values, expressed as μg kg^{-1} and assuming a pre-concentration factor of 33.3, are listed in Table 3. These values are lower than the EU regulation limit for animal feed (20 μg/kg) [40], although slighter higher than those previously reported (some of them within the ng kg^{-1} range) [29-31].

2.3.5.3 Precision and accuracy

Intraday and inter-day precision and analytical recovery were established by spiking several aliquots of the same fish feed sample with AFs concentrations at several concentration levels (0.075, 0.15, 0.3, 0.6, 1.5, 3.0 μg L^{-1}), which implies 2.5, 5.0, 10, 20, 50 and 100 μg L^{-1} after extract re-dissolution. Intraday assays consisted of preparing three standard addition in three different days by replicating the lowest concentration of all four AFs (0.075 μg L^{-1}) seven times; whereas, the other concentration levels were replicated twice. Likewise, the second

and third standard additions were obtained by replicating the intermediate (0.6 µg L^{-1}) and the highest (3.0 µg L^{-1}) concentration levels also seven times.

Table 3 Mean slopes of calibration and standard addition, and LOD and LOQ values

	Slope (mean ± SD)			
	Calibration[a]	Standard addition[a,b]	LOD (µg kg^{-1}) [b]	LOQ (µg kg^{-1}) [b]
AFB1	0.0088±0.0015	0.011±0.0029	0.42	1.3
AFB2	0.0033±0.0019	0.0039±0.0021	0.88	2.7
AFG1	0.0049±0.0013	0.0066±0.0013	1.1	3.3
AFG2	0.0013±0.0009	0.0019±0.0024	1.2	3.5

[a] n=7; [b] preconcentration factor 33.3

Similarly, inter-day assays (precision and analytical recovery) were obtained by preparing six standard additions in seven different days but replicating each concentration level twice. Results (Table 4) show RSD values lower than 15% for AFs concentrations close to the LOQ of the method, and lower than 7% for intermediate and high AFs levels. Precision is quite similar than those reported by other authors using MIT (lower than 10%) [29-31]. Good intraday and inter-day precision in the measurements are therefore demonstrated. Table 4 also lists the analytical recoveries of the intraday and inter-day assays, which were within the 80-100% range for all cases. The proposed method offers quantitative analytical recoveries, which is a clear advantage over other reported method which showed analytical recoveries lower than 60% [30] or lower than 80% for low AFs concentration [29]. Uncorrected matrix effect could be the reason of the non-quantitative analytical recoveries in these proposals.

In addition, accuracy was also assessed by determining the AFs concentrations (AFB1, AFB2, and AFG1) in the ERM-BE 376 (compounds in feeding stuff) CRM. The material was thoroughly mixed and homogenized by rolling it on a low-profile roller for 30 min before use. AFs extraction and pre-concentration (n=6) was carried out as previously detailed in the experimental section, and extracts were

analyzed by UHPLC-MS-MS. As shown in Table 5, sensitivity of the method was high enough for assessing AFB1 and AFG1 (12.05±0.35 and 4.90±0.54 μg kg^{-1} for AFB1 and AFG1, respectively); however, sensitivity was not good enough to assess AFB2, which certified concentration was lower than the LOD of the method for this AF.

Table 4: Intraday and inter-day precision (RSD%), and intraday and inter-day analytical recovery (AR%) of the method

	Intra-day[a]		Inter-day[a]	
	RSD	AR%	RSD	AR%
AFB1				
2.5	7	103±1	9	95±2
5	---[b]	---[b]	8	89±2
10	---[b]	---[b]	11	90±5
20	8	100±2	9	93±5
50	---[b]	---[b]	8	90±4
100	7	101±7	7	98±7
AFB2				
2.5	3	100±1	12	96±4
5	---[b]	---[b]	6	92±3
10	---[b]	---[b]	13	84±4
20	14	97±3	12	97±2
50	---[b]	---[b]	11	90±5
100	8	83±7	7	95±7
AFG1				
2.5	14	88±5	9	82±2
5	---[b]	---[b]	9	88±3
10	---[b]	---[b]	13	88±4
20	15	92±3	9	90±5
50	---[b]	---[b]	10	86±5
100	13	98±8	9	92±8
AFG2				
2.5	11	90±11	10	88±2
5	---[b]	---[b]	5	89±6
10	---[b]	---[b]	11	86±6
20	11	95±3	10	88±2
50	---[b]	---[b]	12	93±5
100	3	100±3	7	98±6

[a] n=7; [b] Not evaluated

2.3.5.4 Reusability of MIMSPE devices

Reusability of the MIMSPE devices was studied by using three independent MIMSPE devices for pre-concentrating several aliquots of an extract spiked with AFB1 at 3.0 µg L^{-1}. After each loading/elution cycle, MIP beads were sonicated with 5 mL of 0.1M/0.1M KH_2PO_4/ NaOH (pH 6.0) for 5 min, and then soaked in a clean 0.1M/0.1M KH_2PO_4/NaOH (pH 6.0) solution until a new use (section 2.8). Results (AFB1 analytical recovery) after each successive loading/ elution cycle showed good retention properties since analytical recoveries were within the 80-120 % range after twenty successive loading/elution cycles (Figure S3, ESI). Reusability of MIMSPE devices can be therefore established at twenty times.

2.3.6 Application

Applicability of the proposed UAE-MIP-µ-SPE and HPLC-MS-MS was demonstrated by analyzing six fish feed samples commercially available in the Spanish market. Each sample was subjected to the optimized procedure in triplicate. Results listed in Table 6 show the presence of AFB1, AFB2 and AFG1 in all analysed fish feed samples; whereas, AFG2 was quantified in four samples. Fish feed samples have shown AFB1 and AFB2 concentrations higher than the European Commission (EC) regulation value, although AFG1 and AFG2 were lower than EU MRL (20 µg kg^{-1}) in most fish feed samples.

Table 5: Found AFs concentrations in ERM-BE376 (compound feeding stuff) CRM

	Certified value (µg/kg)	Found value (µg/kg)	Recovery %
AFB1	12.9±1.8	12.1±0.35	93
AFB2	0.68±0.10	< 0.88	---
AFG1	5.2±0.8	4.9±0.5	94

Table 6: Concentrations (expressed as µg kg^{-1}) of AFB1, AFB2, AFG1 and AFG2 in fish feed samples

	[AFB1] (µg kg^{-1})	[AFB2] (µg kg^{-1})	[AFG1] (µg kg^{-1})	[AFG2] (µg kg^{-1})
S1	27.0±1.4	16.9±4.9	7.7±0.1	1.5±0.04
S2	23.1±0.4	27.8±0.4	13.9±1.0	28.1±1.4
S3	23.9±4.0	4.6±1.1	3.7±0.04	---[a]
S4	22.9±1.4	70.7±5.8	4.9±1.0	---[a]
S5	26.7±2.6	42.4±1.9	9.1±0.2	28.2±0.9
S6	24.6±0.3	18.4±9.4	2.2±0.2	4.5±0.9

[a] < 1.2 µg kg^{-1}

2.4 CONCLUSIONS

Ultrasonication combined with porous membrane protected MIMSPE has been shown to be a convenient and cost-effective sample pre-treatment for AFs isolation and selective pre-concentration from fish feed samples. Selected ultrasonication conditions allowed an efficient AFs extraction and guaranteed the stability (integrity) of the targets. In addition, the selective MIMSPE procedure allows effective pre-concentration of AFs (an enrichment factor of 33.3) and sensitive AFs assessment (LODs within the 0.42 – 1.2 µg kg^{-1} range, values lower than EU regulation limits for AFs in animal feed). Matrix effect was found also to be negligible for some AFs (AFB1 and AFB2), which implies that clean extracts are obtained. The high clean-up efficiency is mainly attributed to the high MIP's selectivity through AFs and also to the PP membrane that acts as the barrier and avoids the interaction of large biomolecules from the fish feed extract with the MIP's particles. The procedure was found to be precise (RSD values lower than 20%) and accurate (analytical recoveries within the 80-100% range, and good accordance between certified and found AFs concentrations in the ERM-BE 376 CRM). MIP-µ-SPE devices can be re-used 20 times (20 loading/eluting cycles) which offers a practical advantage over commercially available SPE cartridges which are single-use devices. The proposed material can be useful for AFs enrichment from foodstuff after targets extraction. The application of the method to fish feed

shows the presence of AFs in this feedstuff. Fish feed is scarcely studied for AFs and these findings open new insights for the control of these substances in aquaculture products.

Acknowledgments
This work was supported by the *Dirección Xeral de I+D – Xunta de Galicia Grupos de Referencia Competitiva* (project number 6RC2014/2016 and ED431C2018/19), and Development of a Strategic Grouping in Materials - AEMAT (grant ED431E2018/08). The authors also wish to thank Dr. M. José Pazos-Guldrís (*Unidade de Microscopía)* at *Rede de Infraestruturas de Apoio á Investigación e ao Desenvolvemento Tecnolóxico* – Universidade de Santiago de Compostela) for SEM technical support, and Dr. Carmen Álvarez-Lorenzo (I+D Farma – Universidade de Santiago de Compostela) for BET and porosity measurements.

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2.6 ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Table S1. Surface are and porosity analysis in NIP and MIP

	BET surface area (m^2 g^{-1})	Pore volume (cc g^{-1})[a]	Pore diameter (Å)[b]	Pore diameter (Å)[c]
NIP	607.7	0.1803	29.38	26.81
MIP[d]	804.2	0.01063	89.17	95.31
MIP[e]	706.7	0.03061	80.49	83.97

[a] BJH method cumulative desorption pore volume
[b] BJH method adsorption pore diameter
[c] BJH method desorption pore diameter
[d] MIP before template removal
[e] MIP after template removal

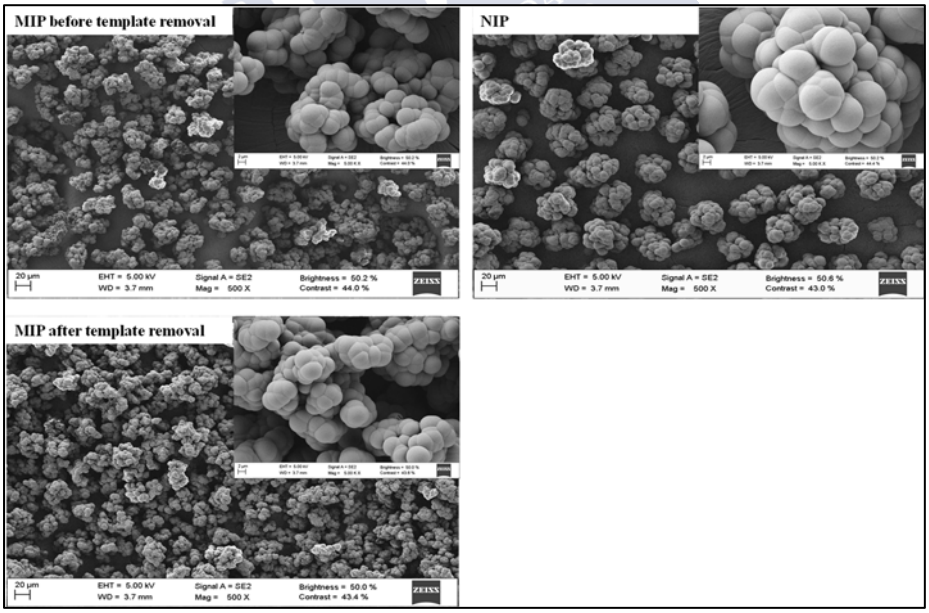

Figure S1: Scanning Electron Microscope (SEM) images of MIP after template removal and before template removal, and NIP

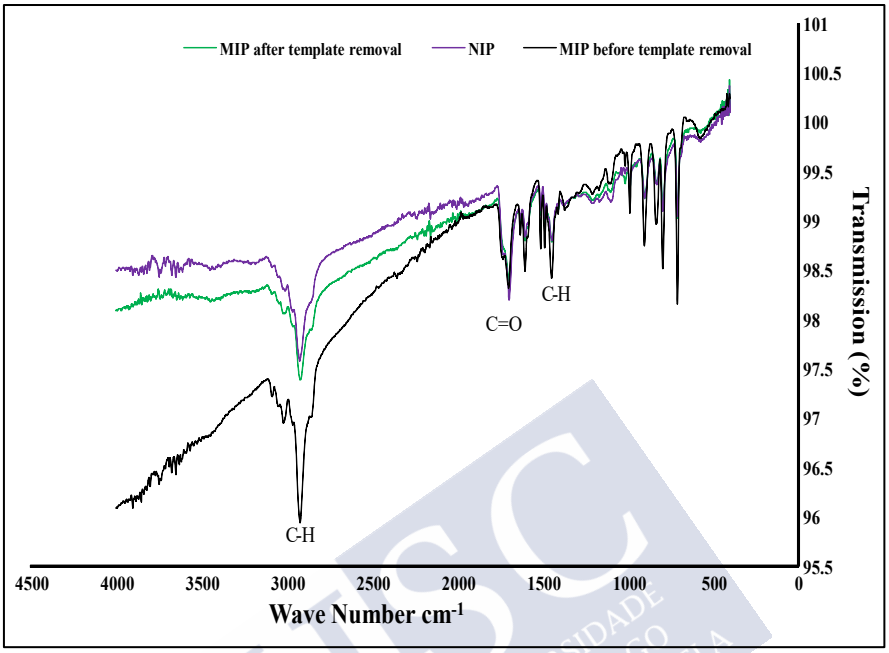

Figure S2: Fourier Transform Infrared Spectroscopy (FT-IR) images of MIP after template removal and before template removal, and NIP.

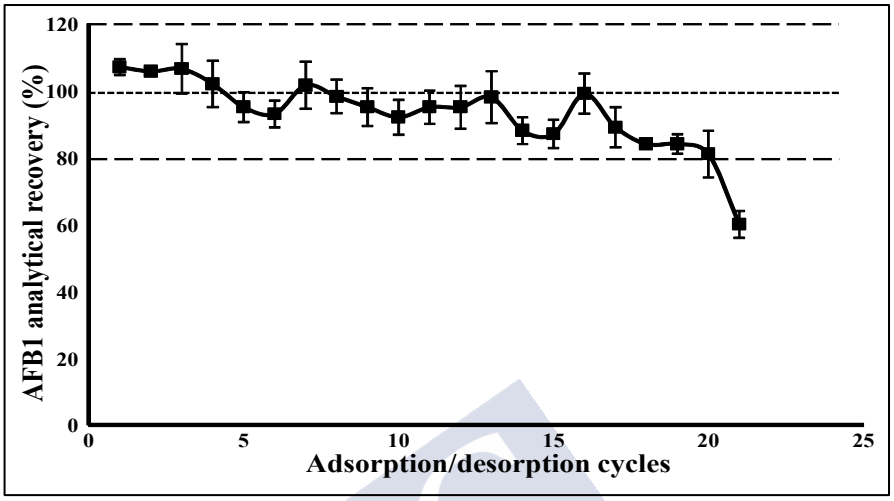

Figure S3. Effect of the number of loading/elution cycles on the adsorption capacity of the MIMSPE devices (AFB1 concentration of 3.0 µg L^{-1})

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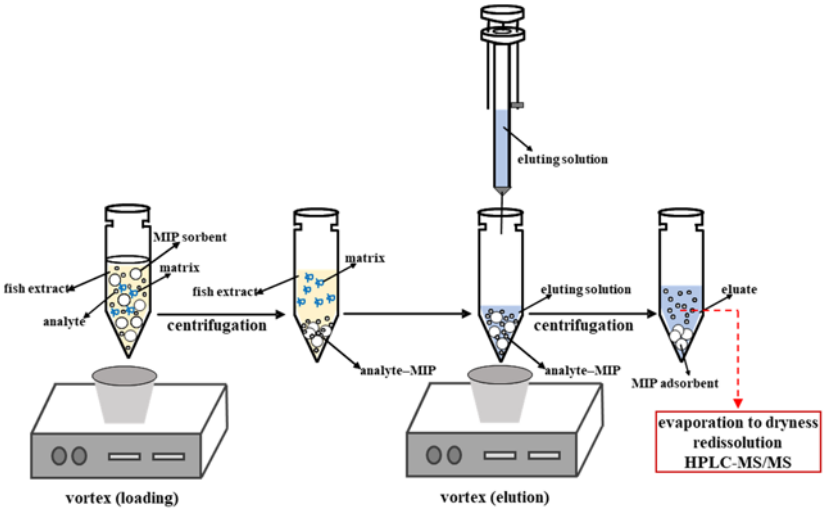

CHAPTER 3

VORTEX ASSISTED – DISPERSIVE MOLECULARLY IMPRINTED POLYMER MICRO-SOLID PHASE EXTRACTION FOR AFLATOXINS ISOLATION FROM CULTURED FISH BEFORE UHPLC-MS/MS ASSESSMENT

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Vortex assisted – dispersive molecularly imprinted polymer micro-solid phase extraction for aflatoxins isolation from cultured fish before UHPLC-MS/MS assessment

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Abstract

A dispersive micro solid phase extraction approach using a molecularly imprinted polymer as an adsorbent has been developed for pre-concentrating aflatoxins from cultured fish. Aflatoxins were first isolated from fish muscle and liver by an ultrasound assisted extraction procedure using a 60:40 acetonitrile/0.1M KH_2PO_4 aqueous buffer (pH 6.0) mixture. Polymeric adsorbent beads were synthesized using 5,7-demethoxycoumarin as a dummy template, methacrylic acid as a functional monomer, divinylbenzene as a crosslinker, and 2,2'-azobisisobutyronitrile as an initiator. Parameters affecting the steps of extraction procedure including the sample (fish extract) pH, adsorption stirring speed and time, desorption stirring speed and time, elution solvent ratio, and polymer capacity, were investigated and optimized. The limit of detection was found to vary from 0.29 to 0.61 $\mu g\ kg^{-1}$ for the several aflatoxins. The proposed method was shown to be accurate and precise. Intraday and inter-day relative standard deviation were lower than 20%, and intraday and inter-day analytical recoveries were within the 80-100% range. The prepared adsorbent in the dispersive micro solid phase extraction format was re-usable, and the pre-concentration procedure was found to be simple, rapid and highly selective and sensitive to identify/quantify AFs in fish.

Keywords: Molecularly imprinted polymer, Dispersive micro solid-phase extraction, Aflatoxins, Fish, High-performance liquid chromatography tandem mass spectroscopy.

3.1. INTRODUCTION

The accurate determination of compounds at trace levels without any sample pre-treatment, even when using sensitive analytical techniques, is a very difficult task because the instrumental limits of detection are usually close to the target concentration in the samples. In addition, compatibilities between the sample matrix and the instrumental techniques is also an important issue, and some matrix components must be removed (or their presence must be minimised) for proper operation of the instrumental technique. Sample pre-treatment emerges as the answer to these shortcomings; thus, development of simple, rapid, sensitive, and low-cost sample treatment methods is a subject of great interest. Solid phase extraction (SPE) using a large variety of adsorbents (silica, activated carbon, cellulose, chelating resins, and polyurethane foam) is a good sample pre-treatment option for organic and inorganic targets [1]. Advantages of SPE procedures regarding the versatility of the technique, and off-line batch and column (cartridges) approaches, and also on-line SPE with separation techniques (chromatography) and spectrometric methods, have been fully described [2]. The extent of the target molecule-adsorbent interaction in cartridge-based SPE procedures depends on the selected flow rate in the loading stage.

Dispersive solid phase extraction (dSPE) is an alternative approach for SPE in which the adsorbent is dispersed into the sample (extract) [3]. The dispersion facilitates a close contact between the adsorbent and the target, thus enhancing kinetic adsorption and leading to high extraction efficiencies [3, 4]. The performance of the dispersive technique when adding amounts of the solid adsorbent within the mg range (typically 50 mg) leads to a new extractive technique referred to as dispersive micro solid phase extraction (D-μ-SPE) [5, 6]. Both dSPE and D-μ-SPE can be used for clean-up purposes and for pre-concentrating trace amounts of targets.

The dispersion of the sorbent plays an essential role in D-μ-SPE during the loading (analyte isolation) and elution stages. Dispersion can be enhanced by applying a supplementary energy source such as ultrasound and mechanical shaking (vortex), which favours particle size reduction (adsorbent dis-aggregation) and maximizes the surface area of the adsorbent particles. In addition, ultrasounds change the process kinetics, which is dependent on the ultrasound exposure time [7-9]. Vortex stirring is a soft and low-cost shaking technique that can be used to enhance mass transference in micro-extraction processes. Dispersion assistance is more repeatable when comparing with ultrasounds (water-bath ultrasounds) because ultrasounds assistance depends on the position of the water-bath tank since ultrasound fluency is not the same in all the positions of the tank [10]. Other advantages of vortex assistance are the avoidance of analyte degradation and adsorbent aggregation, and the lower extraction kinetics when compared to ultrasounds assistance processes [11-13].

Adsorbents used in D-μ-SPE play a critical role in the overall extraction procedure. Nanostructure adsorbents (high surface area) have been found to increase the absorption capacity [14,15]. Adsorbents used in D-μ-SPE consist of micro-materials (typically SPE adsorbents) [16-18], nanostructured adsorbents [19,20], and hybrid materials [21]. Selectivity is one of the main disadvantages of these materials, and molecularly imprinted polymers (MIPs) have emerged as appealing selective adsorbents for SPE [22]. Molecularly imprinted technology (MIT) generates selective template (target molecule) binding sites in a synthetic polymer and MIPs result appealing adsorbents for miniaturized sample preparation techniques [22-24]. MIP synthesis around a magnetic nanoparticle (magnetic-molecularly imprinted polymers, M-MIPs) leads to composites with magnetic and/or luminescent properties which can be used in SPE procedures and for chemosensing [25]. M-MIPs offer as an advantage that tedious filtration/centrifugation stages are avoided (adsorbent separation from the bulk sample and from the extract is achieved by a magnet) [25], and several applications for batch-based SPE have been reported [26]. In addition, M-MIPs are excellent adsorbents for dSPE and D-μ-SPE since

dispersion is achieved by magnetic stirring. Therefore, M-MIPs as adsorbents for dSPE have been fully applied [27].

More than 16 types of aflatoxins (AFs) have been identified, and AFB1, AFB2, AFG1 and AFG2 have been categorized in group 1 carcinogen by International Agency for Research on Cancer (IARC) [28]. Feeds are highly susceptible to be contaminated with AFs, and the presence of these contaminants has been reported in feeds used in aquaculture facilities [29, 30]. Some studies have therefore proved that cultured fish can be affected by AFs through contaminated feedstuff [31, 32]. Aquaculture is a fastest-growing food-producing sector, thus the risk exists that AFs may be transferred to humans through the consumption of contaminated cultured fish. An accurate monitoring and determination of AFs in cultured fish is therefore needed because of the irreversible AFs effects on humans and animals. The aim of the current work has been to explore the possibilities of D-µ-SPE using a selective MIP adsorbent (dispersive molecularly imprinted polymer micro-solid phase extraction, D-µ-MISPE) and vortex stirring assistance. The used MIP has been previously shown excellent pre-concentration capabilities for AFs from fish feedstuff in a porous membrane-protected micro solid phase extraction format [30], and the higher pre-concentration factor achieved in a D-µ-MISPE format has allowed to assess trace levels of AFs (B1, B2, G1, G2, and metabolite M1) in aquaculture fish.

3.2 MATERIALS AND METHODS

3.2.1 Instrumentation

A 3200 Q TRAP LC/MS/MS (ABSciex, Concord, Canada) with an electrospray ionization source, a Flexar FX-15 UHPLC binary pump with integrated vacuum degasser (Perkin Elmer, Waltham, MA, USA), and a Flexar UHPLC autosampler (Perkin Elmer), was used. A reverse-phase Zorbax C-18 column (100 mm length, 4.6 mm i.d, 3.5 µm particle diameter) from Agilent (Santa Clara, CA, USA) connected to a C-18 guard column (4 mm length, 3.0 mm i.d) from Phenomenex (Torrance, CA, USA) was used for chromatographic separation under controlled temperature (40°C) using a GECKO 2000 column heater (Amchro GmbH, Hattersheim, Germany). UHPLC-MS/MS data processing was

performed with MultiQuant 2.1 software (ABSciex). MIPs were prepared by using a low-profile roller (Stovall, Greensboro, NC, USA) placed inside a Boxcult temperature-controlled chamber (Stuart Scientific, Surrey, UK). An ASE 150 pressurized liquid extraction (PLE) device (Dionex. Co, Sunnyvale, CA, USA) with 10 mL of stainless steel cells, 60 mL collection vial and cellulose filters (Dionex), was used for template removal from the polymer. A VibraCell VCx 130 ultrasound probe from Sonics (Newtown, CT, USA) was used for AFs isolation from fish, and a Laborcentrifugen 2K15 centrifuge (Sigma, Osterode, Germany) was used for separating the extract and to recover the adsorbent. A Reax top shaker and mixer (Heidolph, Schwabach, Germany) was used for D-μ-MISPE assistance. A VLM EC1 metal block thermostat and nitrogen sample concentrator from VLM (Leopoldshohe-Greste, Germany) was used for evaporating the extract solvent. A Spectrum-Two Fourier transform infrared spectrometer with attenuated total reflection (ATR) sampling mode (Perkin Elmer, MA, USA), and a ZEISS EVO LS 15 scanning electron microscope (Carl Zeiss, Oberkochen, Germany) were used for MIP characterization. Other devices were a Crison Basic20 pH-meter (Barcelona, Spain), a Classic ML analytical balance (Mettler Toledo, Columbus, OH, USA), and a domestic blender (Taurus 850, Barcelona, Spain).

3.2.2 Reagents

Aflatoxins stock standards solutions (1000 mg L^{-1}) were prepared from solid AFs (B1, B2, G1, and G2) from Sigma-Aldrich (Steinheim, Germany) dissolved in methanol (LC-MS grade) purchased from Merck (Darmstadt, Germany). Aflatoxin M1 reference material solution (506 mg L^{-1} in acetonitrile) was purchased from LGC standards (Middlesex, UK). Dummy template (5,7-dimethoxycoumarin, DMC) was also from Sigma-Aldrich. Certified reference material ERM-BE376 (compound feeding stuff) was from the European Commission, Joint Research Centre Institute for Reference Materials and Measurements (Geel, Belgium). Methacrylic acid (MAA) and divinylbenzene (DVB) were from Sigma Aldrich, and 2,2'-azobisisobutyronitrile (AIBN) from Fluka (Buchs, Switzerland). DVB and AIBN were purified before use as shown elsewhere. Solvents such

as acetonitrile, methanol, neutral alumina, potassium dihydrogen phosphate, and sodium hydroxide were from Merck. Toluene (HPLC grade) was purchased from Panreac (Barcelona, Spain). Ultrapure water, 18 MΩ cm of resistivity, was obtained from a Milli–Q purification device (Millipore Co., Bedford, MA, USA). Other consumables included were Durapore 0.22 µm membrane filters (Millipore), PVDF syringe filters (4.0 mm diameter and 0.22 µm pore size) from Perkin Elmer, disposable syringes (sterile, 2 mL) from Dispomed (Gelnhausen, Germany), and 2 mL polypropylene Eppendorf tubes (Labbox, Barcelona, Spain).

3.2.3 MIPs preparation

MIP synthesis (precipitation polymerization procedure) has been described elsewhere (MIP-based porous membrane-protected micro solid phase extraction) [29] and implies the use of DMC as a dummy template (0.0699 g), MMA (115 µL) as a monomer, DVB (1.25 mL) as a crosslinker, AIBN (0.1 g) as an initiator (0.1 g), and 25 mL of 1:3 acetonitrile/toluene mixture as a porogen. Non-imprinted polymers (NIPs) were prepared following the same method as MIPs, but without adding the template. DMC was removed from the synthesized MIP (500 mg) by PLE (80°C, 103 bars, and 60% flush volume for 20 min using 8:2 methanol/ultrapure water). The PLE procedure was repeated six times (negligible AFs signals in the extracts). Finally, MIP beads were oven-dried at 40 °C for 12 h and stored at room temperature. Synthesized MIP and NIP characterization by SEM and ATR-FTIR can be found elsewhere [29].

3.2.4 AFs extraction from fish by ultrasound-assisted extraction (UAE)

Cultivated fish, gilt-head bream (*Sparus aurata*), Japanese sea bass (*Lateolabrax japonicus*), Brown trout (*Salmo trutta*), and Turbot (*Scophthalmus maximus*), were bought in local fish markets. All samples were washed with clean tap water before removing the gut, head, bones, fins and scales. The flesh (all samples) and the liver (gilt-head beam and Japanese sea bass) were homogenized separately by using a domestic blender and were stored at -20°C in sealed plastic

bottles. Gilt-head beam (flesh) was used for method optimization and validation.

AFs were isolated from homogenized fish samples (1.000 g) with 10 mL of 60:40 acetonitrile/0.1M KH_2PO_4 buffer, pH 6 (pH adjusted using 0.1 M NaOH) by UAE at 40% amplitude (40% of the sonicator power, 130 W/20 kHz frequency) for 7.0 min. The mixtures were then filtered, and the supernatant (extract, pH within the 6.8-7.0 range) was recovered.

3.2.5 Dispersive micro solid phase extraction procedure (D-µ-MISPE)

Optimized D-µ-MISPE conditions consisted of 40 mg of MIP adsorbent dispersed in 1.5 mL of 0.1M KH_2PO_4 buffer (pH 6) inside a 2 mL Eppendorf tube and subjected to vortex stirring for 1.0 min (adsorbent conditioning stage), followed by centrifugation (15000 rpm, 10 min). Loading step was performed by adding 1.5 mL of standards or fish extracts (spiked extracts at 100 µg L^{-1} for AFB1, AFB2, AFG1 and AFG2, 20 µg L^{-1} for AFM1, and 50 µg L^{-1} for the internal standard (DMC)) to the conditioned MIP beads, and stirring at 1500 rpm for 3.0 min (creamy colour solution through mixed/dispersed MIP particles). The mixtures were then centrifuged (15000 rpm, 10 min), and the recovered MIP adsorbent was mixed with 0.5 mL of 97.5:2.5 acetonitrile/formic acid for elution (elution stage by vortex stirring at 2000 rpm for 4.0 min). After centrifugation (15000 rpm, 10 min) the supernatants were collected and fully dried under N_2 flow, re-dissolved in 100 µL of methanol, and finally filtered (0.22 µm).

Preliminary experiments (non-optimized D-µ-SPE conditions) were performed with 20 mg of MIP, loading with fish extracts adjusted to pH of 7.0 (2500 rpm vortex stirring for 5.0 min), and elution with 0.5 mL 95:5 acetonitrile/formic acid.

Table 1: Operating conditions for UHPLC-MS/MS

MS/MS						
Compound[b]	Precursor ion (m/z)	Product ion (m/z)	DP(V)[a]	EP(V)[a]	CE(V)[a]	CXP(V)[a]
DMC	206.800	121.100	46.590	7.630	34.150	3.000
AFB1	313.000	241.000	70.390	4.800	52.340	7.230
AFB2	315.000	259.200	84.900	2.820	39.320	2.240
AFG1	329.000	200.120	59.940	4.180	55.140	4.500
AFG2	331.000	213.200	68.780	4.110	35.000	3.000
AFM1	329.200	273.300	35.000	10.000	20.100	9.000

UHPLC			
Column	Zorbax Eclipse C18 reverse phase column (100 mm length × 4.6 mm i.d, 3.5 µm particle diameter)		
Injection volume	20 µL		
Flow rate	250 µL min^{-1}		
Mobile phase	0.1% formic acid in ultrapure water (A) and 0.1% formic acid in methanol (B)		
	Total time(min)	A (%)	B (%)
	0.1	50	50
	3.0	50	50
	8.0	0	100
	12	0	100
	13	50	50
	15	50	50

(a) DP, Declustering potential; EP, Entrance potential; CE, Collision energy; CXP, Collision cell exit potential

(b) Electron spray operation conditions are: Ions spray voltage (IS), 5500 kV; Ion source temperature, 300 °C; nebulizer gas and curtain gas (N_2), 40 psi; collision gas (N_2), high

3.2.6 Liquid chromatography-tandem mass spectrometry measurement

Chromatographic separation as well as MS/MS acquisition setting (positive mode) are shown in Table 1 (five AFs and DMC structures are given in Table S1, electronic supplementary information, ESI). Working standards at 2.5, 5, 10, 20, 50, 100 and 200 µg L^{-1} for AFB1,

AFB2, AFG1 and AFG2, and 2.5, 5, 10, 20, 30, 40, and 50 $\mu g L^{-1}$ for AFM1 were used for method optimization (DMC at 50 $\mu g\ L^{-1}$ as an internal standard). After verifying the existence of the matrix effect, the standard addition method was used for method validation and sample analysis. Therefore, spiked fish extracts (within the 0.167-13.33 $\mu g\ L^{-1}$ range for AFB1, AFB2, AFG1 and AFG2, and within the 0.167-3.33 $\mu g\ L^{-1}$ range for AFM1) were used for preparing the standard additions (DMC was spiked at 3.33 $\mu g\ L^{-1}$). Since the proposed pre-concentration is an off-line process, the pre-concentration factor was established as the sample volume (1.5 mL) to eluate volume (0.1 mL) ratio [33,34], which results in a pre-concentration factor of 15. Further experiments will demonstrate that the developed D-µ-MISPE pre-concentration method is robust even when loading 5.0 mL of fish extract, which leads to a pre-concentration factor of 50.

Figure 1 shows a chromatogram obtained for a standard solution [100 $\mu g\ L^{-1}$ for all AFs, except AFM1 (20 $\mu g\ L^{-1}$)] and for pre-concentrated fish extracts (flesh from a gilt head beam and a liver from a Japanese sea bass) by D-µ-MISPE (pre-concentration factor of 15).

3.3 RESULTS AND DISCUSSION

3.3.1 Optimization of D-µ-MISPE parameters

To obtain the best D-µ-SPE results several parameters including loading and elution operating conditions have been investigated and optimized. All experiments (each set of conditions) have been run in triplicate using spiked homogenate fish flesh (several homogenates from gilt-head beam) with 6.0 $\mu g\ L^{-1}$ (AFB1, AFB2, AFG1, AFG2), 1.0 $\mu g\ L^{-1}$ (AFM1) and 3.0 $\mu g\ L^{-1}$ (DMC). Assuming a pre-concentration factor of 15, these concentrations are within the 0-100 $\mu g\ L^{-1}$ calibration range used for calculations (DMC as an internal standard at 50 $\mu g\ L^{-1}$). Un-spiked fish extracts were also treated and the naturally occurring AFs concentrations were subtracted from the concentrations obtained in spiked extracts.

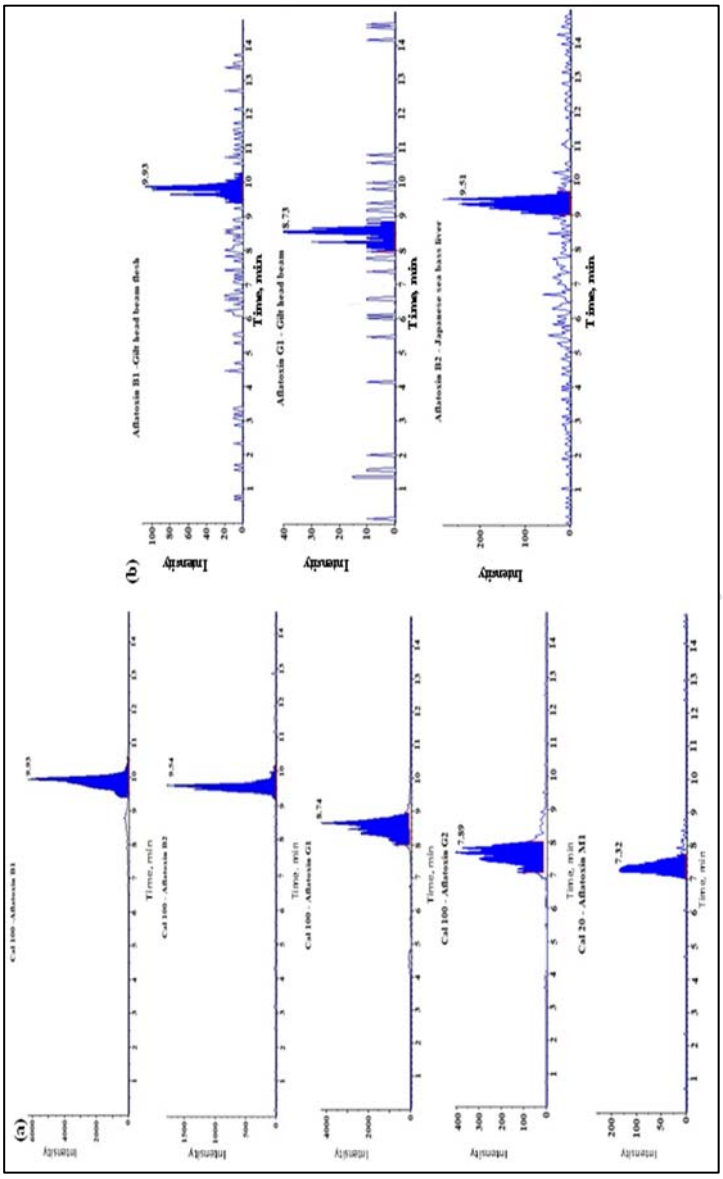

Figure 1: MRM chromatogram of a 100 µg L^{-1} AFB1, AFB2, AFG1 and AFG2, and 20 µg L^{-1} AFM1 standard solution (a) and a flesh fish and a liver fish sample (b)

3.3.1.1. Effect of the pH

The pH of a sample extract can play an important role in extraction efficiency. The effect of the pH was studied in the 5.0-9.0 range. The pH of the fish extract after UAE was found to be within the 6.8-7.0 range (60:40 acetonitrile/0.1M KH_2PO_4 buffer). Therefore, pHs lower than 7.0 were obtained by adding small volumes of 0.1M formic acid, and pHs higher than 7.0 were obtained by adding small volumes of 0.1 M NaOH. As illustrated in Figure 2(a) the highest analytical recoveries were obtained when using a pH of 7.0. Acid and alkaline pHs resulted in poor extraction efficiency. These findings agree with those reported by other authors who have reported low AFs extraction efficiencies attributed to AFs decomposition at highly acidic or alkaline pH values [35,36], and also with Sereshti *et al*. [37] who have recently reported the convenience of a pH 7.0 when pre-concentrating AFs from bread extracts with magnetic three-dimensional graphene as an adsorbent. In addition to AFs stability at a neutral pH, the polymerization mixture when synthesizing the MIP particles exhibited a pH close to 7.0, and the imprinted recognition cavities involved a low protonation degree in oxygen atoms in AFs (DMC) at a neutral pH; hence, better MIP-AFs interaction is expected when targets exhibit a similar ionization degree [29]. Therefore, pH 7.0 was selected, a pH which offers the operating advantage that pH adjustment before D-μ-MISPE is not required (the pH of the fish extract after UAE is within the 6.8–7.0 range).

3.3.1.2 Effect of the vortex speed and time for loading

From the literature regarding DSPE, both the stirring time and the stirring speed are known to affect the efficiency of target adsorption (loading stage) and elution (elution stage) [3]. The influence of the vortex time and speed was investigated in the range of 1-5 min and 500-2500 rpm, respectively. By fixing a vortex speed of 2000 rpm, the effect of the vortex time for loading (Figure 2(b)) resulted in a gradual increase on AFs pre-concentration up to 3.0 min, and the pre-concentration yields worsened when loading at more than 3.0 min. Regarding the vortex speed for loading (fixed vortex time for loading time of 3.0 min), the extraction efficiency was found to increase when loading at high vortex speeds (from 500 to 1500 rpm). However, the

extraction yields decrease when using vortex speeds higher than 1500 rpm (Figure 2(c)). Low vortex speed and/or times for loading imply a less efficient contact between the dispersed MIP particles and the dissolved targets. Analytical recovery impairment at high vortex speeds and times during the loading stage may be explained through back diffusion at high shaking speed and long shaking times, phenomena that has been reported for many micro-extraction techniques [38,39], even for micro-extraction procedures that use adsorbents for pre-concentration [40,41]. Since the maximum efficiency was observed when loading at 1500 rpm for 3.0 min, these values were selected and fixed for further experiments.

3.3.1.3. Effect of the desorption solvent

The selection of the elution solvent is mainly based on the solubility of the analyte. Thus, the AFs are fully soluble in organic solvents such as acetonitrile, methanol, toluene, among others. Preliminary experiments were performed using a 95:5 acetonitrile/formic acid as an eluting solvent, a mixture found to be a convenient solution for AFs desorption using MIPs as an adsorbent [29]. A small proportion of formic acid leads to an acid pH of the eluting solution which promotes protonation in retained AFs and favours desorption. Several acetonitrile/formic acid ratios (97.5/2.5, 95/5, 92.5/7.5 and 90/10) were studied using a volume of 500 μL of eluting solvent. As shown in Figure 3(a), slightly better analytical recoveries have been obtained when using an eluting mixture containing the lowest proportion of formic acid, and 97.5:2.5 acetonitrile/formic acid was therefore chosen for elution. Analytical recovery worsening when using more acidic elution solutions could be related to AFs degradation after desorption.

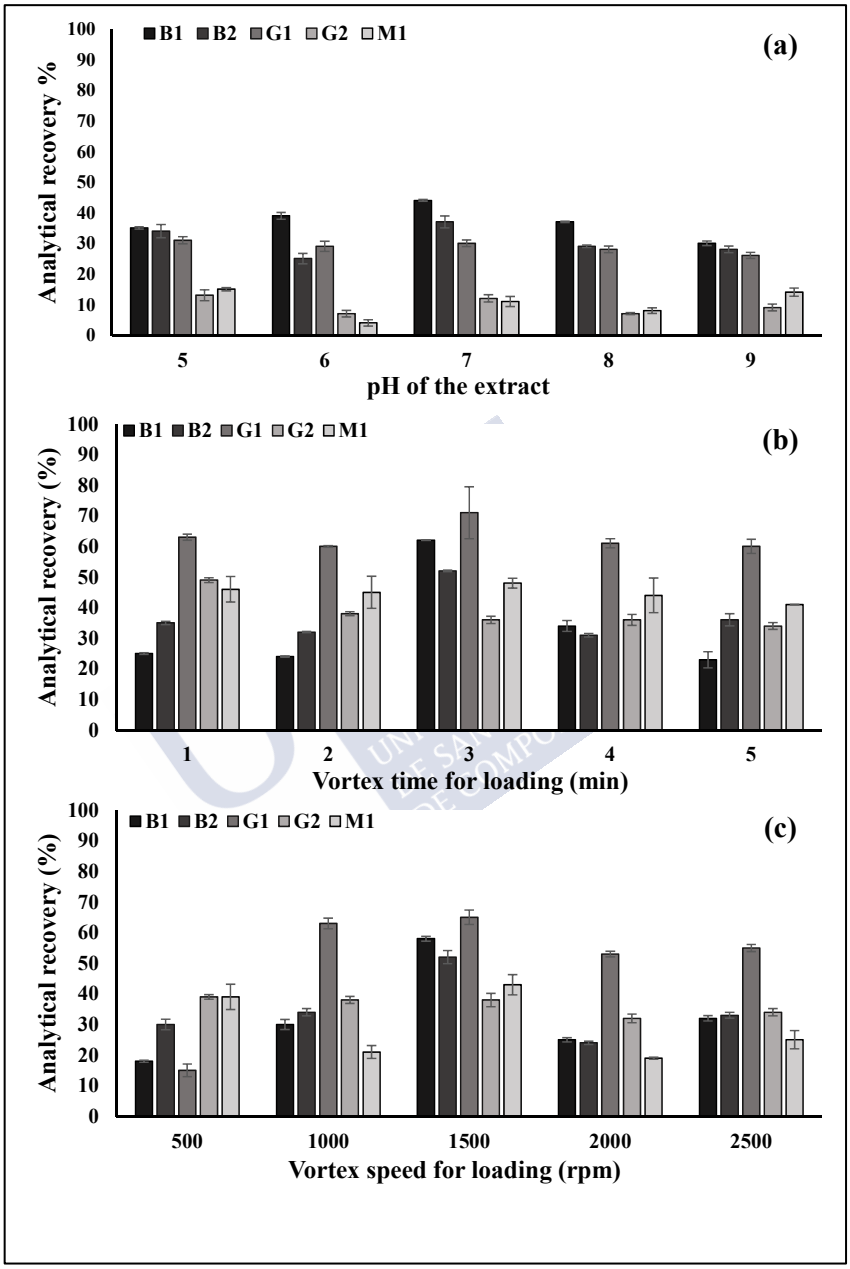

Figure 2: Effect of the pH of extraction solution (a), the loading vortex time (b), and the loading vortex speed (c) on the analytical recovery of AFs

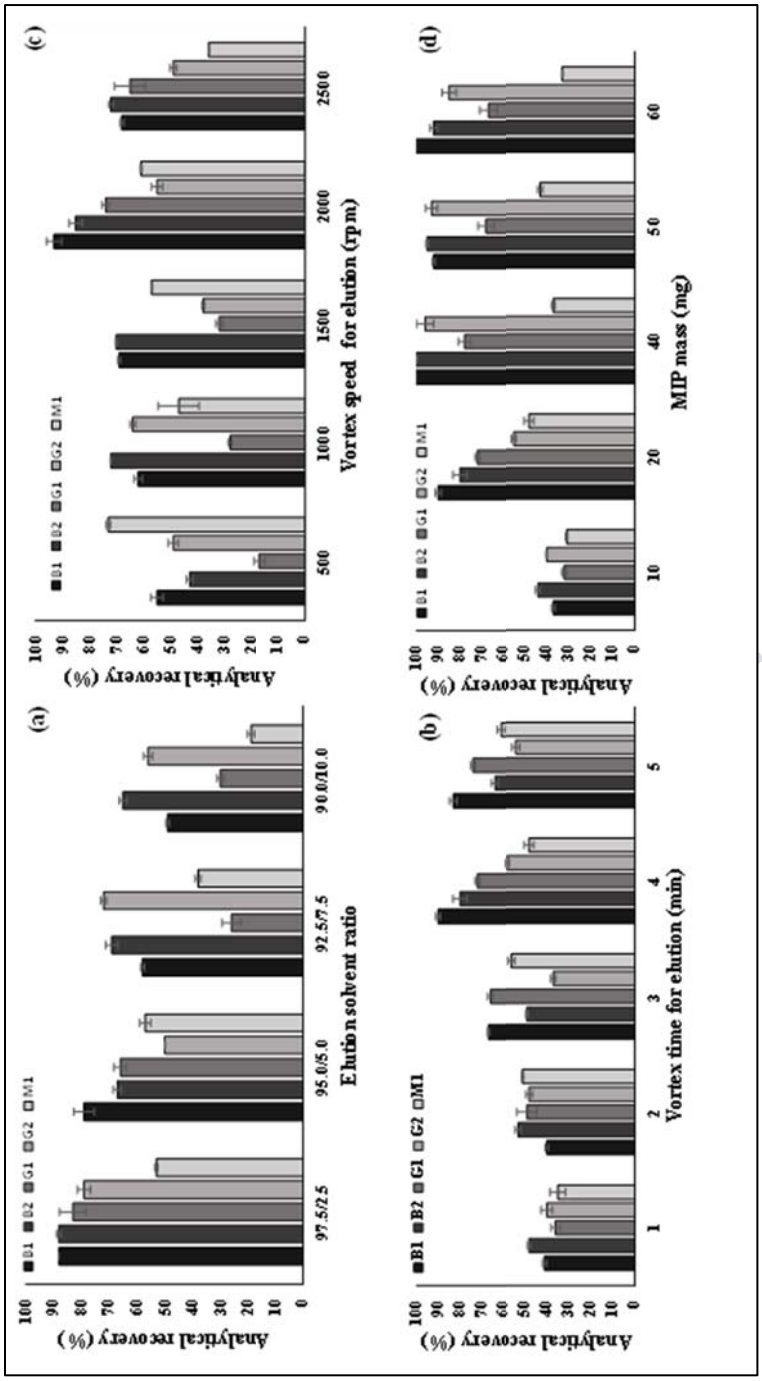

Figure 3: Effect of the elution solvent ratio (a), eluting vortex time (b), eluting vortex speed (c), and adsorbent mass (d) on the analytical recovery of AFs

3.3.1.4 Effect of the desorption time and the speed

The effects of the vortex time and speed for elution were tested by varying the vortex time from 1.0 to 5.0 min (97.5:2.5 acetonitrile/formic acid, 2000 rpm), and the vortex speed (97.5:2.5 acetonitrile/formic acid, 4.0 min) within the 500-2500 rpm range. Results (Figure 3(b)) show that AFs desorption is favoured when increasing the vortex time for elution, and 4.0 min is sufficient for a complete analyte desorption. Low desorption ratios at high vortex times (5.0 min) could be due to further analyte re-adsorption onto the adsorbent material. Regarding vortex speed for elution, the pre-selected speed of 2000 rpm was found to offer the highest analytical recoveries (Figure 3(c)) when using a vortex time of 4.0 min.

Finally, further experiments were performed to verify an efficient targets desorption, and the D-μ-MISPE was subjected again to the selected elution conditions (second elution). Negligible AFs concentrations in the eluates (second elution) were observed, which indicates the absence of carryover, and that the same adsorbent in D-μ-MISPE could be reused (a further study regarding re-usability of the MIP is discussed in later sections).

3.3.1.5 Effect of the MIP mass

Experiments described for selecting operating loading and elution conditions were performed using 20 mg of MIP as adsorbent in D-μ-MISPE. The number of accessible active sites on the adsorbent (MIP capacity) for interaction with target analytes depends on the amount of adsorbent dispersed in the sample solution. Therefore, the influence of the adsorbent mass was studied within the 10-60 mg range using the optimized conditions obtained for an MIP mass of 20 mg. Figure 3(d) shows that analytical recoveries are lower for experiments using 20 mg (and mainly 10 mg) of MIP, and the values are slightly increased when using 40 mg or higher of MIP. Therefore, 40 mg of adsorbent was selected for performing the D-μ-MISPE. Figure 3(d) shows that analytical recoveries are quantitative (close to 100%) for all AFs, except for AFG1 (approximately 80%) and AFM1 (~ 45%). The lower analytical recoveries for these AFs could be attributed to matrix effects and/or inefficient adsorption onto the MIP recognition cavities. These

issues will be clarified in further studies (method validation) in the following sections.

3.3.2 Cross-reactivity and imprinting effect

Parameters such as extraction efficiency (analytical recovery), distribution ratios, and selectivity coefficients (parameters defined in Table 2) were calculated to study the imprinting effect and the selectivity of the synthesized MIP used for D-μ-MISPE. The experiment (in triplicate) was performed using solutions (60:40 acetonitrile/0.1M KH_2PO_4 buffer, pH 7) containing 3.34 μg L^{-1} of each AF and DMC template, and 1.34 μg L^{-1} of AFM1 (concentrations that will be 100 and 20 μg L^{-1} by assuming a preconcentration factor of 15) under optimised D-μ-MISPE conditions using MIP and NIP as adsorbents. Table 2 shows extraction efficiencies close to 100% for DMC (template molecule) and for the five AFs when using MIP as an adsorbent; whereas, the extraction efficiency of the D-μ-MISPE with NIP as an adsorbent was lower than 45%. These findings prove that AFs (and DMC) adsorption occurs through the generated recognition cavities in MIP. Quantitative analytical recoveries for MIP are related to high distribution ratios and small selectivity factors, which contrasts with the values obtained for NIP (Table 2) and implies good imprinting effect of the prepared MIP.

Similarly, other compounds that could be present in fish extracts (see Table 2) were also investigated for assaying selectivity. Standards at 1.0 mg L^{-1} of all compounds were prepared in 60:40 acetonitrile/0.1M KH_2PO_4 buffer (pH 7.0) and were subjected to D-μ-MISPE using MIP and NIP as adsorbents. Concentrations of these compounds were assessed by flow injection analysis - MS/MS (without column, binary pump system and MS/MS acquisition parameters for each compound under study listed in Table S2, electronic supplementary information, ESI). The extraction efficiency for the investigated compounds was within the 5-40% range for both MIP and NIP adsorbents, which implies that retention of these compounds in MIP is not attributed to the selective imprinting cavities but is attributed to nonspecific adsorption (surface adsorption) as it occurs when using NIP as an adsorbent. Moreover, the low distribution ratios and high

selectivity coefficients for these compounds when using MIP and NIP prove high selectivity for AFs in the D-μ-MISPE process.

Table 2: Extraction efficiency, distribution ratio and selectivity coefficient for MIP D-μ-SPE and NIP D-μ-SPE

MIP			
	Extraction efficiency (%)[a]	Distribution ratio (D)[b]	Selectivity coefficient ($S_{(DMC/Q)}$)[c]
DMC	97	33	
AFB1	93	14	2.4
AFB2	90	11	3.0
AFG1	95	14	2.4
AFG2	94	16	2.1
AFM1	96	10	3.3
Vit D	8	0.082	402.4
Vit A	11	0.121	272.7
Carotene	24	0.332	99.4
Glycine	0	0	∞
Cysteine	30	0.434	76.0
Histidine	40	1.073	30.8
Lysine	2	0.025	1320.0
Methionine	40	0.86	38.4
Threonine	20	0.274	120.4
NIP			
DMC	31	0.447	
AFB1	34	0.528	63
AFB2	45	0.823	40
AFG1	45	0.877	38
AFG2	21	0.268	123
AFM1	43	0.78	42
Vit D	5	0.058	569

Table 2: Extraction efficiency, distribution ratio and selectivity coefficient for MIP D-μ-SPE and NIP D-μ-SPE (Continued)

NIP			
	Extraction efficiency (%)[a]	Distribution ratio (D)[b]	Selectivity coefficient ($S_{(DMC/Q)}$)[c]
Vit A	9	0.098	337
Carotene	29	0.636	52
Glycine	0	0	∞
Cysteine	38	0.634	52
Histidine	36	0.582	57
Lysine	2	0.022	1500
Methionine	40	0.856	39
Threonine	38	0.615	54

(a) % =(A_2/A_T) 100
(b) D =(A_2/A_1)
(c) $S_{(DMC/Q)}=D_{DMC}/D_Q$
A_1=Amount of analyte in the aqueous solution at equilibrium
A_2=Amount of analyte enriched by MIP/NIP at equilibrium
A_T =Total amount of analyte used in extraction
D_{DMC}= Distribution ratio of the Dimethoxycoumarine (template)
D_Q=Distribution ratio of other *Q* compounds (Vitamin A, Vitamin D, ß-Carotein, etc.)

3.3.3 Analytical performance

3.3.3.1 Calibration and matrix effect

Several calibration curves (n=6) were prepared using analyte concentrations of 2.5, 5.0, 10, 20, 50, 100, 200 μg L^{-1} for AFB1, AFB2, AFG1 and AFG2 (AFM1 concentration of 2.5, 5, 10, 20, 30, 40, 50 μg L^{-1}) and DMC (50 μg L^{-1}) as an internal standard. The mean slopes and standard deviations for each AF are listed in Table 3. Similarly, several standard addition curves (n=6) were also performed throughout the method validation process (fish extracts spiked with AFs standards within the 0.167-13.33 μg L^{-1} range, and within the 0.167-3.33 μg L^{-1} range for AFM1) subjected to the D-μ-MISPE process, which implies concentrations from 2.5 to 200 μg L^{-1} (from 2.5 to 50 μg L^{-1} for AFM1) by assuming a pre-concentration factor of 15. The mean slopes and

standard deviations of the standard addition calibrations are also listed in Table 3. The matrix effect expressed as the ratio (percentage) between calibration and standard addition slopes was around 20% for some AFs (16% for AFB1, 23% for AFB2, and 19% for AFG2), but it was very high for AFG1 (56%) and AFM1 (59%). AFG1 and AFM1 were found to give an analytical recovery of around 80% when performing D-μ-MISPE optimization (use of calibration for measurements), and the AFG1 and AFM1 un-quantitative recoveries could be attributed to the matrix effect. ANOVA test (95% confidence interval) showed that there was a significant difference between the mean slopes of the external calibration graph and standard addition graphs for all AFs (ANOVA tables in Table S3, ESI section). Therefore, the matrix effect is important and the standard addition method was selected for validation. Good linearity of the method was also obtained (regression coefficients were higher than 0.996 for all cases).

3.3.3.2 Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were based on the 3σ/10σ criteria (σ is the standard deviation of the measurement of eleven blank samples after applying D-μ-MISPE). Table 3 lists the LOD/LOQ values taking into account the mean slope for the standard addition, the pre-concentration factor of 15 (D-μ-MISPE), and fish sample mass and UAE process. Although there is no legislation for AFs maximum levels in fishery products, the calculated LOD/LOQ are lower than the maximum levels allowed by the EU regulation limit for AFs in animal feed (20 μg kg^{-1}).

The calculated LOD/LOQ can be improved by increasing the extract volume at the loading stage. Therefore, a study was performed by subjecting a fish extract (gilt-head bream flesh positive to AFB1) to the D-μ-MISPE procedure by using 1.5 mL of the fish extract (pre-concentration factor of 15 after extract evaporation and re-dissolution in 0.1 mL), 3.0 mL of the fish extract (pre-concentration factor of 30), and 5.0 mL of the fish extract (pre-concentration factor of 50). AFB1 concentrations of 0.89±0.09, 1.05±0.11, and 0.76±0.08 μg kg^{-1} were obtained for pre-concentration factors of 15, 30 and 50, respectively. The application of an ANOVA test (95% confidence interval) showed

that AFB1 concentrations after loading 1.5 (pre-concentration of 15) and 3.0 mL (pre-concentration of 30) and 5.0 mL (pre-concentration factor of 50) are not statistically different (Table S4, ESI section). From these results we can conclude that the D-μ-MISPE can be performed by loading up 5.0 mL of the fish extract without accuracy losses. These findings show the robustness of method, and the pre-concentration factor can be improved up to 50 (the improved LOD/LOQ by assuming a pre-concentration factor of 50 have also been listed in Table 3).

Table 3: Mean calibration and standard addition's slopes, and limit of detection (LOD) and limit of quantification (LOQ) of the method

	Mean slope (au^{-1} μg^{-1} L)			
	Calibration	Standard addition	LOD (μg kg^{-1})[a]	LOQ (μg kg^{-1})[a]
AFB1	1145±73	966±37	0.11 (0.033)	0.37 (0.11)
AFB2	263±20	203±15	0.20 (0.060)	0.67 (0.20)
AFG1	662±42	292±41	0.12 (0.036)	0.40 (0.12)
AFG2	138±14	112±4	0.20 (0.060)	0.68 (0.20)
AFM1	277±8	114±10	0.097 (0.029)	0.32 (0.064)

(a) Values in brackets correspond to a pre-concentration factor of 50

3.3.3.3 Precision and accuracy

The precision and accuracy of the method were evaluated based on the relative standard deviation (RSD %) and the analytical recovery in intraday and inter-day assays. Intraday precision and analytical recovery were obtained by performing three standard addition calibrations (D-μ-MISPE implying a pre-concentration factor of 15) in three different days, and replicating thrice each concentration level except one (the lowest, the highest and an intermediate concentration level) which were replicated seven times (0.167 μg L^{-1} for all AFs in the first standard addition, 1.33 μg L^{-1} for all AFs in the second standard addition, and 13.33 μg L^{-1} for AFB1, AFB2, AFG1 and AFG2, and 3.33 μg L^{-1} for AFM1 in the third standard addition). Similarly, inter-day assays (precision and analytical recovery) were obtained by preparing seven standard addition calibrations in seven different days but

replicating each concentration level thrice. Intraday RSDs (Table 4) were lower than 19%; whereas inter-day RSD values were lower than 14%. Analytical recovery for inter-day and intraday assays (Table 4) was within the 83-102% range for all cases.

To the best of the authors' knowledge, there is no any certified reference material (CRM) for AFs in fish. The proposed method was therefore applied to an ERM-BE-376 (compounds feeding stuff) to evaluate the accuracy. Several subsamples (n=6) were subjected to the UAE and to the optimized D-μ-MISPE conditions (pre-concentration factor of 15) before UHPLC-MS/MS. Good accuracy has been proved since the good agreement between the measured and the certified concentrations (Table S5, ESI).

Table 4: Intraday precision and inter-day precision (RSD%) and intraday and inter-day analytical recovery (AR%) of the method

		Intra-day		Inter-day	
		RSD (%)	AR (%)	RSD (%)	AR (%)
[AFB1] (μg L^{-1})	2.5	16	84±1	12	88±2
	5	-	-	13	93±1
	10	-	-	11	94±1
	20	10	102±2	8	96±2
	50	-	-	11	90±5
	100	-	-	8	87±7
	200	7	86±7	8	88±8
[AFB2] (μg L^{-1})	2.5	19	84±1	11	84±1
	5	-	-	7	89±1
	10	-	-	9	96±1
	20	5	87±1	8	92±2
	50	-	-	10	87±4
	100	-	-	10	89±8
	200	12	92±5	6	95±8

Table 4: Intraday precision and inter-day precision (RSD%) and intraday and inter-day analytical recovery (AR%) of the method (Continued)

		Intra-day		Inter-day	
		RSD (%)	AR (%)	RSD (%)	AR (%)
[AFG1] (µg L^{-1})	2.5	9	84±1	12	84±1
	5	-	-	10	83±1
	10	-	-	12	94±1
	20	6	89±1	8	85±2
	50	-	-	7	93±3
	100	-	-	4	88±4
	200	14	87±4	9	89±8
[AFG2] (µg L^{-1})	2.5	9	90±1	11	90±1
	5	-	-	11	88±2
	10	-	-	14	91±2
	20	8	88±2	6	94±2
	50	-	-	7	91±3
	100	-	-	5	91±5
	200	10	90±8	5	91±7
[AFM1] (µg L^{-1})	2.5	16	88±1	10	85±1
	5	-	-	8	89±1
	10	-	-	9	89±1
	20	5	92±3	6	90±1
	30	-	-	6	96±2
	40	-	-	5	96±2
	50	5	88±2	3	98±2

3.3.3.4 Reusability of the D-µ-SPE system

The number of loading/eluting cycles that can be performed with the same mass of MIP (40 mg) was studied using three different 40 mg sub-portions from the same synthesized MIP prepared in three Eppendorf tubes. Several aliquots from a fish extract spiked with 6.67 µg L^{-1} of AFB1 were subjected to the optimized D-µ-SPE procedure for

several days, and AFB1 concentrations were assessed. Analytical recoveries after each successive loading/elution cycles were found to be quantitative after ten to twelve successive loading/elution cycles (Figure S3, ESI), thus demonstrating the reusability of the D-μ-MISPE system.

3.3.4 Application

The applicability of the developed D-μ-MISPE method was tested by analysing the flesh of four cultured fish species and two fish livers (one from a gilt-head bream sample and another from a Japanese sea bass sample). Homogenized flesh and livers were subjected to the optimized UAE, D-μ-MISPE (pre-concentration factor of 15) and UHPLC-MS/MS in triplicate. AFB1 was quantified in the flesh of gilt-head bream (0.89±0.09 μg kg^{-1}), Japanese sea bass (0.67±0.07 μg kg^{-1}) and brown trout (0.39±0.01 μg kg^{-1}). AFG1 was also measured in the flesh of gilt-head bream (0.56±0.09 μg kg^{-1}). Other AFs were not detected in the flesh of the fish under study. AFB2 was quantified in the liver of gilt -head beam and Japanese sea bass (1.31±0.05 and 1.98 ±0.15 μg kg^{-1}, respectively). AFG1 was also found in the liver of Japanese sea bass (0.73±0.02 μg kg^{-1}). Although there are no EC regulation limits for AFs in fish, the AFs levels found in cultured fish are quite lower than the maximum limits established by EU for the presence of AFs in groundnut as an ingredient in food (8.0 μg kg^{-1} for AFB1 and 15 μg kg^{-1} for the sum of all AFs).

3.4 CONCLUSIONS

MIPs have been novelty used as adsorbents for vortex assisted D-μ-MISPE pre-concentration of AFs is extracts from fish (flesh and liver) samples. The pre-concentration method has been found to be highly selective for AFs, allowing for high pre-concentration factors (up to 50). Vortex stirring for assisting adsorbent dispersion guarantees target integrity and allows for a low-cost procedure for performing the pre-concentration stage. The proposed μ-SPE is therefore an appealing method for assessing very low concentrations of AFs in complex matrices such as those derived from fishery products. The adsorbent material (synthesized MIP) was found to be reusable, with each 40 mg

portion of the adsorbent allowing at least ten to twelve successive loading/eluting cycles. Although the proposed method has been found suitable from fish extract, the D-μ-MISPE pre-concentration method could be also useful for AFs enrichment from extracts from other foodstuffs. The high sensitivity of the method has allowed the assessment of very low AFs concentration in cultured fish (flesh and liver). AFB1 was quantified in the flesh of some fish specimens; whereas, AFB2 was present in the livers. The AFs levels found in these samples are quite low, but their presence demonstrates a direct relationship with the presence of AFs in the fish feeds used in the aquaculture sector.

Acknowledgment
This work was supported by the *Dirección Xeral de I+D – Xunta de Galicia Grupos de Referencia Competitiva* (project number ED431C2018/19), and Development of a Strategic Grouping in Materials - AEMAT (grant ED431E2018/08). The authors also wish to thank Dr. M. José Pazos-Guldrís (*Unidade de Microscopía)* at *Rede de Infraestruturas de Apoio á Investigación e ao Desenvolvemento Tecnolóxico* – Universidade de Santiago de Compostela for SEM technical support.

Conflicts of interest/Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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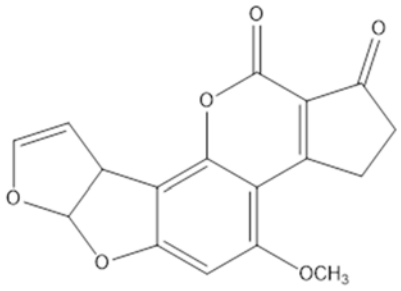
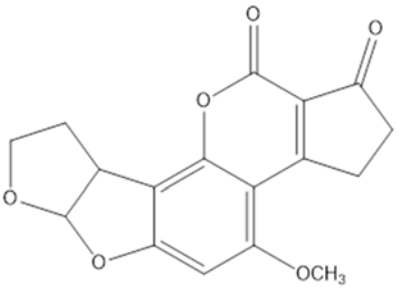
3.6 ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

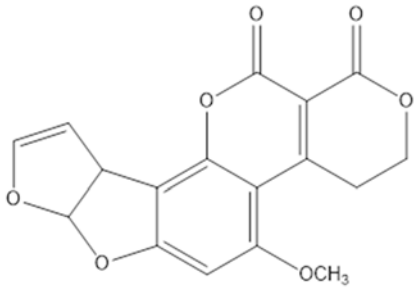
Table S1. Chemical structures of aflatoxins (B1, B2, G1, and G2) and dummy template



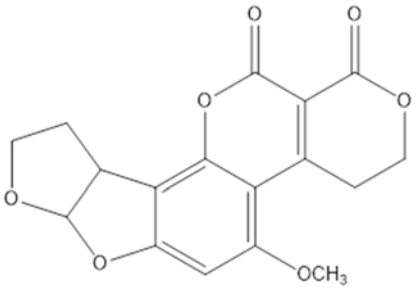
Aflatoxin B1



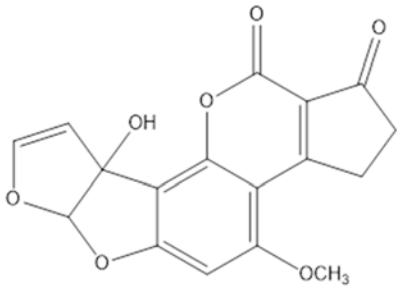
Aflatoxin B2



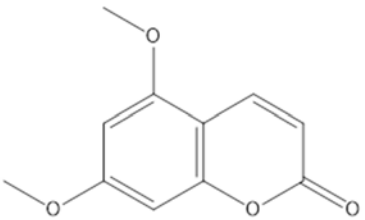
Aflatoxin G1



Aflatoxin G2



Aflatoxin M1



DMC

Table S2: Instrument parameters for FIA-MS/MS

MS/MS						
Compound[b]	Precursor ion (m/z)	Product ion (m/z)	DP(V)[a]	EP(V)[a]	CE(V)[a]	CXP(V)[a]
Vitamin D	385.400	367.300	40.110	10.150	15.000	5.000
Vitamin A	269.300	95.100	40.000	10.000	20.500	5.000
ß Carotene	537.300	295.100	86.000	9.000	45.100	20.000
Lysine	147.200	84.100	84.700	10.000	21.000	10.000
Methionine	150.100	104.000	6.320	8.500	18.000	3.000
Cysteine	241.100	152.000	20.000	10.650	19.000	12.000
Glycine	76.100	30.100	6.120	8.000	19.500	14.000
FIA						
Injection volume		20 µL				
Flow rate		60 µL min^{-1}				
Carrier		0.1 % formic acid in methanol				
Elution time		3.0 min				

(a) DP, Declustering potential; EP, Entrance potential; CE, Collision energy; CXP, Collision cell exit potential
(b) Electron spray operation conditions are: Ion spray voltage (IS), 5500 kV; Ion source temperature, 300 °C; nebulizer gas and curtain gas (N_2), 40 psi; collision gas (N_2), high

Table S3. ANOVA outputs when comparing slopes of calibration and standard addition

AFB1

Source	Sum of squares	Degree of freedom	Mean square	F ratio	p-value
Between groups	96123	1	96123	28.70	0.0003
Within groups	33490	10	3349		
Total (Corr.)	129613	11			

AFB2

Source	Sum of squares	Degree of freedom	Mean square	F ratio	p-value
Between groups	10800	1	10800	34.56	0.0002
Within groups	3125	10	312.5		
Total (Corr.)	13925	11			

AFG1

Source	Sum of squares	Degree of freedom	Mean square	F ratio	p-value
Between groups	410700	1	410700	238.43	0.0000
Within groups	17225	10	1722.5		
Total (Corr.)	427925	11			

AFG2

Source	Sum of squares	Degree of freedom	Mean square	F ratio	p-value
Between groups	2028	1	2028	19.13	0.0014
Within groups	1060	10	106		
Total (Corr.)	3088	11			

AFM1

Source	Sum of squares	Degree of freedom	Mean square	F ratio	p-value
Between groups	79707	1	79707	972.04	0.0000
Within groups	820	10	82.0		
Total (Corr.)	80527	11			

Table S4. ANOVA outputs when comparing D-μ-SPE procedures by using several loading sample volumes (pre-concentration factors of 15, 30 and 50)

AFB1					
Source	Sum of squares	Degree of freedom	Mean square	F ratio	p-value
Between groups	0.1266	2	0.0633	4.74	0.0583
Within groups	0.0802	6	0.0133667		
Total (Corr.)	0.2068	8			

Table S5: AFs concentrations in ERM-BE376 (compound feeding stuff) certified reference material

Compound	Certified value (μg kg^{-1})	Calculated value (μg kg^{-1})	Recovery %
AFB1	12.9±1.8	13.0±0.7	101
AFB2	0.68±0.10	<LOQ	_
AFG1	5.2±0.8	5.6±0.4	107

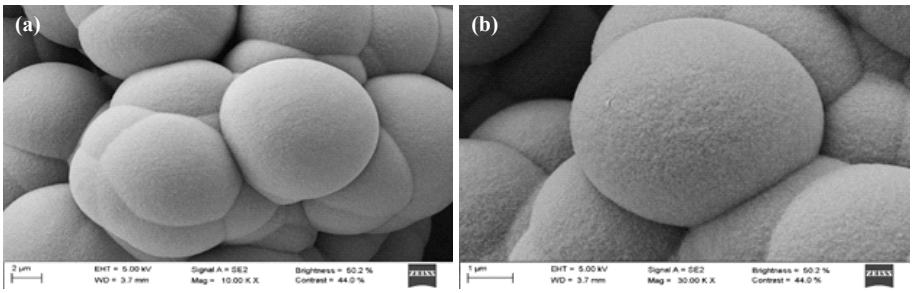


Figure S1: Scanning electron microscope (SEM) images for MIP (a) and NIP (b)

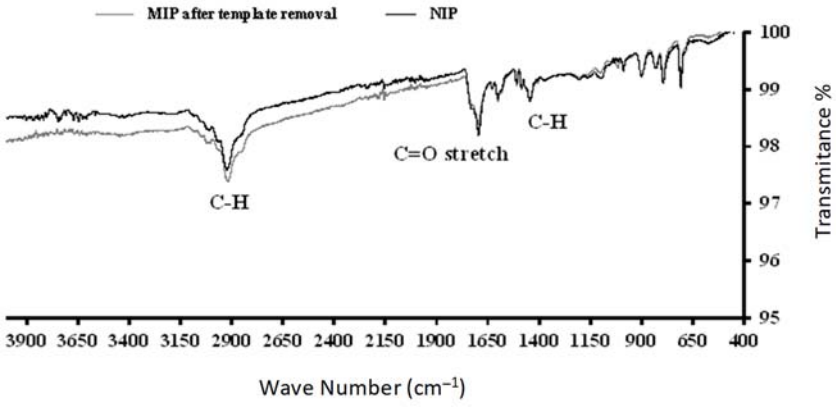


Figure S2: Fourier Transform Infrared Spectroscopy (FT-IR) Images of MIP after template removal and NIP

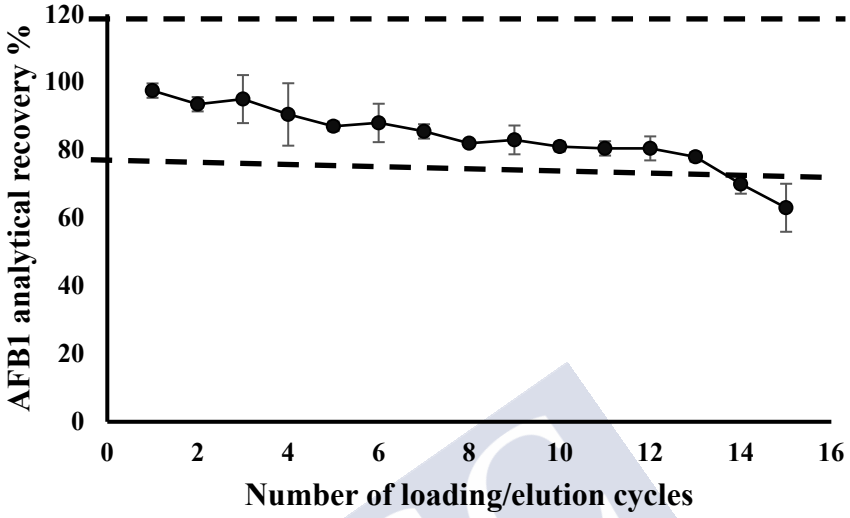
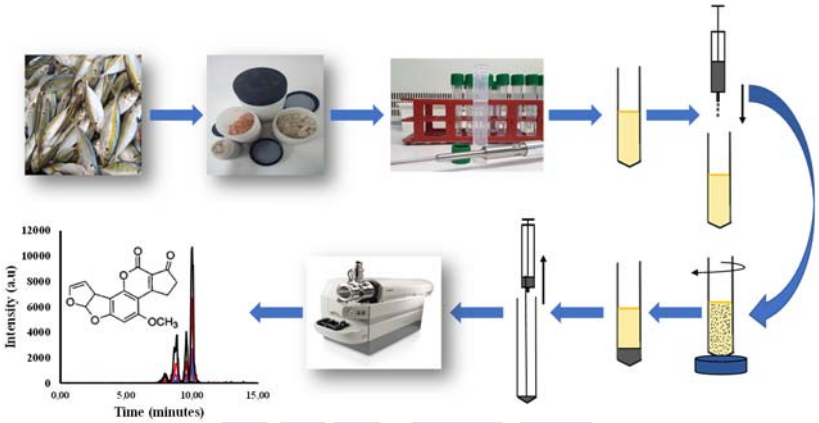


Figure S3. Effect of the number of loading/elution cycles on the analytical recovery of each D-μ-SPE (AFB1 concentration of 6.67 μg L^{-1})



CHAPTER 4

COMBINING ULTRASOUND ASSISTED EXTRACTION AND VORTEX ASSISTED LIQUID-LIQUID MICROEXTRACTION FOR THE SENSITIVE ASSESSMENT OF AFLATOXINS IN AQUACULTURE FISH SPECIES

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JOURNAL OF SEPARATION SCIENCE 43 (2020) 1331–1338

DOI: 10.1002/jssc.201901129

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Combining ultrasound assisted extraction and vortex assisted liquid-liquid microextraction for the sensitive assessment of aflatoxins in aquaculture fish species

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Abstract

Despite aflatoxins contamination in feedstuff is a well-known problem, and hence these residues are controlled in poultry products, there are scarce information regarding the presence of these toxic substances in aquaculture fish, facilities which use several feedstuff for fish breeding. A simple, rapid, and sensitive method has been therefore developed for aflatoxins (B1, B2, G1, and G2) assessment in aquaculture products by combining ultrasound probe assisted extraction and vortex assisted liquid liquid microextraction as a sample pre-treatment, and HPLC-MS/MS as a separation/detection system. Aflatoxins were extracted from fish flesh/liver with a 60:40 ACN:aqueous phosphate buffer (pH 7.0) mixture before pre-concentration and clean-up by vortex assisted liquid liquid microextraction under the following optimized conditions: 5.0 mL of fish extract at pH 7.0 and NaCl at 0.5 % (w/v), 400 µL of chloroform as extracting solvent, and vortex shaking at 2000 rpm for 1 min. The proposed method was shown to be precise and accurate, and the LOQs (from 0.20 to 1.10 µg kg^{-1}) were lower than the value established by the European Commission Regulation for aflatoxins in foodstuff. Results have shown that fish flesh is free of aflatoxins, but aflatoxins B2 and G1 were quantified in fish liver.

Keywords: Aflatoxins, Aquaculture fish, Liquid-liquid microextraction, Ultrasound assisted extraction, Liquid chromatography, Mass spectrometry

4.1 INTRODUCTION

Aflatoxins (AFs) contamination in foodstuff is a well-known public health hazard and regulations have been introduced by many countries to ensure food safety [1]. These compounds can be classified as difuranocoumarin derivatives, and they are naturally produced by *Aspergillus flavus* and *Aspergillus parasiticus*. There are four major AFs named as B1, B2, G1, and G2, being B1 the major AFs produced by toxin strains [2]. AFs are potential mutagenic and carcinogenic substances and they are included in the group 1 as human carcinogen substances by the International Agency of Research on Cancer (IARC) [3]. AFs may contaminate a wide variety of agriculture commodities, especially carbohydrate- and fat-rich foods such as maize and other cereals, spices, and nuts [4]. Fish feeds prepare from plant-derived nutrients (soybean meal, rapeseed meal, lupin seed meal, pea seed meal, sunflower oil cake, cottonseed meal, and sesame meal) [5] can be therefore susceptible to AFs contamination. Consumption of AFs contaminated feeds could therefore lead to possible AFs contamination of aquaculture fishery products, and the presence of B1 in the flesh and liver of some aquaculture fish species has been reported [6-8].

Because of the high AFs toxicity, very low maximum residue levels (MRL) have been stablished by several regulation authorities, such as EU guidelines that set a MRL of 15 μg kg^{-1} in foodstuff [9]. AFs assessment in solid foodstuff requires therefore advanced sample pre-treatment methods to avoid/diminish interferences (clean-up purpose) and to increase sensitivity (pre-concentration purpose). The chosen sample preparation technique depends on the nature of the sample [10], and it largely determines the quality of the results, especially analysis speed, precision, accuracy, sensitivity, resolution and cost. The use of large volumes of solvents is the main disadvantage of conventional sample pre-treatment methods when assessing ultra-trace targets, and the development of green activities by diminishing/replacing toxic

reagents, and miniaturizing and automating the sample pre-treatment process is of current interest. As a result, several microextraction techniques, grouped into sorbent- and solvent-based techiques, have been developed to replace classical extraction methods [10-12]. Among microextraction techniques, dispersive liquid-liquid microextraction (DLLME) [13-15] has emerged as a low-cost and fast procedure for pre-concentration/clean up proposes. The technique has been applied for extracting a wide range of organic and inorganic compounds in several sample matrices, especially liquid samples, such as environmental, food, clinic and forensic materials [16], and the technique has evolved towards assisted procedures to improve extractive efficiency. Several assistance mechanisms, such as those based on ultrasound [17] and microwave [18], have been proposed for enhancing the DLLME extraction efficiency. On other occasions, the extraction solvent dispersion is achieved by pulling in and pushing out the mixture of aqueous sample solution and extraction solvent repeatedly in a glass syringe (air-assisted dispersive liquid-liquid microextraction) [17,19,20]. DLLME extractive capabilities can be also increased by fast shaking using a vortex (vortex assisted liquid-liquid microextraction, VALLME) which leads to a fast extracting solving dispersion and a fast mass transfer from the aqueous phase to the organic extractant [21-27]. Advantages of VDLLME are mainly the low price of the shaking device (vortex are widely available) in comparison to ultrasound and microwaves apparatus. In addition, vortex stirring leads to more repeatable extractions than those obtained when using ultrasound assistance (ultrasound water-bath) because the ultrasound fluency is not the same in all the positions of the tank. Finally, the dispersing solvent is not needed for assisted-DLLME because extracting solvent dispersion is achieved by the external energy (ultrasound, microwave and mechanical stirring) which forces the dispersion process.

However, the application of DLLME, and its variant VALLME, for solid matrices require a previous stage for target isolation from the solid particles, and speed-up procedures based on using microwave assisted extraction (MAE), ultrasound assisted extraction (UAE) and pressurized liquid extraction (PLE) are commonly proposed [28,29],

and several DLLME proposals for AFs pre-concentration before AFs isolation from solid food matrices can be found in the literature [30-35]. Aquaculture fish products contain high amount of fat, and the reliable target extraction requires advanced extraction procedures for achieving clean extracts. VALLME is a fast and efficient process for targets pre-concentration from extracts containing large biomolecules such as extracts from fish flesh and liver. To the best of our knowledge, VALLME has been only applied for isolating AFs from rice wine [36] and brewed herbal tea [37], and applications for complex samples such as fish extracts has not been yet reported. Moreover, few studies regarding the presence of AFs in fishery products are available, and the current methodology offers a simple and sensitive way to perform the determination of these toxic substances in fishery products.

4.2 MATERIALS AND METHODS

4.2.1 Instrumentation

Aflatoxins were determined by using a 3200 Q TRAP LC/MS/MS system (ABSciex, Concord, Canada) equipped with an electrospray ionization source, a Flexar FX-15 UHPLC binary pump with integrated vacuum degasser (Perkin Elmer, Waltham, MA, USA), and a Flexar UHPLC autosampler (Perkin Elmer). Chromatographic separation was done with a Zorbax C-18 reverse phase column (100 mm length, 4.6 mm i.d, 3.5 μm particle diameter) from Agilent Technologies (Santa Clara, CA, USA) connected to a C-18 guard column (4 mm length, 3.0 mm i.d) from Phenomenex (Torrance, CA, USA) under controlled temperature (40 °C) using a GECKO 2000 column heater (Amchro GmbH, Hattersheim, Germany). UHPLC-MS/MS data processing was performed with MultiQuant 2.1 software (ABSciex). AFs isolation from fish was performed with a VibraCell VCx 130 ultrasound probe (Sonics, Newtown, CT, USA). VALLME was performed using a Reax Top D-91126 (0 – 2500 rpm rotation speed range) from Heidolph (Schwabach, Germany). A Nahita model 2600 centrifuge (Beriain, Spain) was used for phase (organic/aqueous) separation after VALLME. Extract evaporation after VALLME was performed by an EC1 metal block thermostat and nitrogen sample concentrator from VLM (Leopoldshohe-Greste, Germany). Basic laboratory equipments

such as pH-meter (Basic20, Crison, Barcelona, Spain), analytical balance (Classic ML, Mettler Toledo, Columbus, OH, USA), glass vacuum filtration assembly (Labbox, Barcelona, Spain), and domestic Taurus blade tissues grinder (Barcelona, Spain) were also used throughout the current work.

4.2.2 Reagents

Ultrapure water, 18 MΩ cm of resistivity, was obtained from a Milli–Q purification device (Millipore Co., Bedford, MA, USA). AFs stock standard solutions (1000 mg L^{-1}) were prepared from solid AFs (B1, B2, G1, and G2) from Sigma-Aldrich (Steinheim, Germany) dissolved in LC-MS grade methanol (Merck, Darmstadt, Germany). 5,7-dimethoxycoumarin (DMC) from Sigma-Aldrich was also prepared in methanol (stock standard solution of 1000 mg L^{-1}) and it was used as an internal standard. All standard solutions were stored at -20 °C in the dark. Sodium hydroxide, monopotassium phosphate, sodium chloride, and ACN (gradient grade for liquid chromatography) were from Merck (Darmstadt, Germany); whereas, chloroform was from Panreac (Barcelona, Spain). Certified reference material ERM-BE376 (compound feeding stuff) was purchased from European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (Geel, Belgium). Other used consumables were Durapore 0.20 µm membrane filters (Millipore), 0.45 µm nonsterile mixed cellulose ester syringe filters (Sterlitech, Kent, WA, USA), and disposable syringe (sterile, 2 mL) from Dispomed (Gelnhausen, Germany).

4.2.3 Fish samples

Cultured fish species, gilt-head bream (*Sparus aurata*), Japanese sea bass (*Lateolabrax japonicus*), brown trout (*Salmo trutta*), and turbot (*Scophthalmus maximus*) were collected from local fish markets from Santiago de Compostela (Spain). Fish samples were washed with clean ultrapure water and the gut, head, bones, fins, and scales were removed. Fish flesh and liver were then separated and homogenised by using a domestic blender, and finally stored at -20 °C in pre-cleaned sealed

plastic bottles. Method optimization and validation were performed using the flesh from several gilt-head bream fish samples.

4.2.4 Ultrasound assisted extraction procedure (aflatoxins extraction from fish)

Homogenized samples (1.000 g) were weighted into 30 mL centrifuge tubes before adding 5.0 mL of 60:40 ACN/0.1 M KH_2PO_4 buffer pH 6 (pH adjusted by using 0.1 M NaOH). The final extracting solution leads to a pH of 7.0. The mixture was ultrasonicated at 40% amplitude (40% of the sonicator power/frequency (130 W/20 kHz)) for 7.0 min. The mixture was then vacuum filtered (0.22 µm) and the filtered extract was used for further VALLME.

4.2.5 Vortex assisted liquid-liquid micro-extraction procedure

The whole fish extracts (5.0 mL) after UAE (pH extract of 7.0) were spiked at 6.0 µg L^{-1} for each AF and DMC for optimization and validation studies (application of the method was performed by spiking the fish extracts only with DMC – internal standard). NaCl (25 mg) was then added (NaCl concentration of 0.5 %(w/v) in the 5 mL extract) before adding 400 µL of chloroform (AFs extracting solvent). The mixture was finally vortexed at 2000 rpm for 1 min, and the cloudy solution containing the dispersed fine droplets of the extraction solvent was centrifuged (2500 rpm, 10 min) for phase (organic/aqueous) separation. After aqueous removal, the chloroform extract was fully dried under N_2 flow, and the residue was re-dissolved in 300 µL of methanol (pre-concentration factor of 16.7). Methanolic extracts were finally 0.45 µm filtered before HPLC-MS/MS analysis.

4.2.6 HPLC-MS/MS measurement

Chromatographic separation was performed by gradient elution (Table S1, Supporting Information) using 0.1% formic acid in ultrapure water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) at a flow rate of 250 µL min^{-1}. Multi Reaction Monitoring (MRM) was used for data acquisition (parameters in Table S1, Supporting Information).

AFs standards in methanol (2.5, 5, 10, 20, 50,100 and 200 μg L^{-1}) were used for VALLME optimization. Validation and sample analysis were however performed by using the standard addition technique (fish extracts spiked with 0.15, 0.6, 1.2, 20, 3.0, 6.0 and 12 μg L^{-1}, taking into account a pre-concentration factor of 16.6). DMC (6.0 μg L^{-1} before pre-concentration,) was used as an internal standard. MRM chromatograms for a 100 μg L^{-1} standard and a Japanese seabass liver sample are shown in Figure S1 (Supporting Information).

4.3 RESULTS AND DISCUSSION

4.3.1 Optimization of vortex assisted liquid-liquid microextraction parameters

Polar solvents such as chloroform and dichloromethane (polarity index, P, of 4.1 and 3.1, respectively) are preferred for AFs extraction since AFs exhibit moderate polarity (predicted octanol-water partitioning coefficients, LogP, from 0.37 for AFB2 to 0.73 for AG1, data generated using the ACD/Labs Percepta Platform - PhysChem Module [38]). Therefore, a preliminary experiment was performed for comparing the extractive capabilities of two polar solvents (chloroform and dichloromethane), two solvents with moderate polarity (toluene and carbon tetrachloride, P' of 2.4 and 1.6, respectively), and a non-polar solvent (hexane, P' of 0.1). A volume of 300 μL of each solvent was used, which gave a disperser/extractant ratio of 10 (experiments were performed with 5.0 mL of 6:4 of acetonitrile/0.1 M KH_2PO_4 fish extract). Fish extracts (pH 7.0) were used directly (without pH adjustment) after AFs spiking (6.0 μg L^{-1} for each AF and DMC), and VALLME was performed by vortexing at 2000 rpm for 5.0 min. AFs analytical recoveries for experiments in triplicate showed the best extraction efficiencies for chloroform and dichloromethane as extractants. Carbon tetrachloride and toluene gave moderate analytical recoveries; whereas, hexane has been found unsuitable. Experimental data agree therefore with expected results based of LogP of AFs and the polarity of the extracting solvents. Chloroform was finally selected because the high extractive capacity, and also because the use of this solvent leads to a highly stable cloudy solution, and it has been shown to offer better repeatability than dichloromethane. Our findings are in

good agreement that those reported by other authors regarding AFs extraction by DLLME procedures, which have found dichloromethane [36], and mainly chloroform [31-37] as the most suitable extracting solvents.

4.3.1.1 Effect of the pH

Sample extract pH plays a unique role to transfer the target molecules into the extractant phase in DLLME/VALLME procedures. Fish extract pH [an ACN/0.1 M KH_2PO_4 (6:4) solution] was found to be 7.0 after UAE. Lower pHs were achieved by adding small volumes of formic acid to the fish extract; whereas, higher pHs were achieved by adding small volumes of a 0.1 M sodium hydroxide solution. The fish extract pH was studied within the 4.0-10 pH range (experiments in triplicate) using 300 μL of chloroform and assisting the extraction by vortexing at 1500 rpm for 5 min. Figure 1(a) shows that poor recoveries at high and low extract pH values, and best extraction yields are observed when extract pH is adjusted at 7.0. AFs chemistry implies hydrogen acceptor properties (six hydrogen bond acceptors for AFB1 and AFB2, and seven hydrogen bond acceptors for AFG1 and AFG2 [38]) for AFs, and low protonation degree at neutral pHs). Mass transfer from the ACN/aqueous phase to the polar organic solvent (chloroform) would be favoured at acid pHs (partial protonation) but lower extraction efficiency was observed for pHs lower than 7.0. Extraction impairment at acid pHs must to be attributed to the lack of stability of AFs at strong acidic or basic conditions [39, 40], and a pH of 7.0 was therefore selected as the best extract pH for VALLME (this pH is also the fish extract pH after UAE).

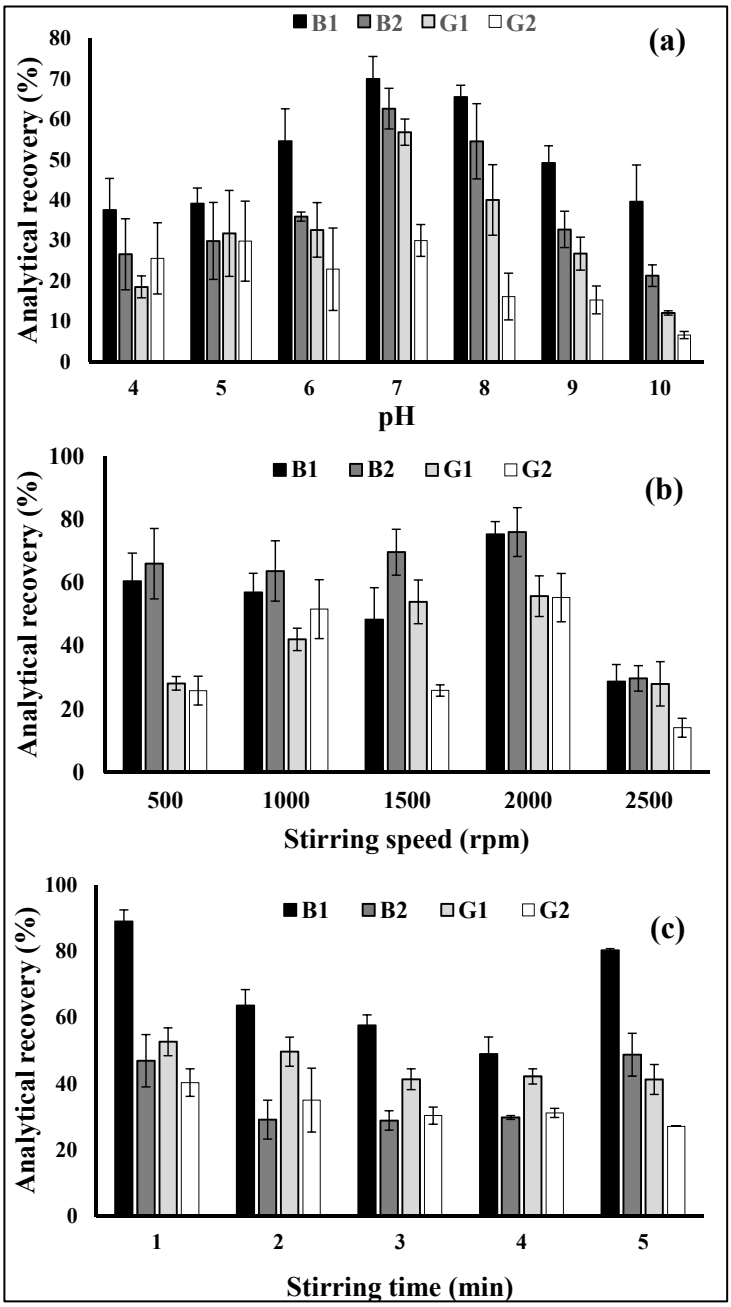

Figure 1 : Effect of the pH of the fish extract (a), the stirring (vortex) speed (b), and the stirring (vortex) time (c) on the AFs recover

4.3.1.2 Stirring time and speed

Several vortexing speeds and times were tested to obtain the best interaction for AFs extraction. Experiments in triplicate involved a fish extract pH of 7.0 (300 μL of chloroform) and a stirring speed of 1500 rpm when studying vortexing times within the 1.0-5.0 min range (results in Figure 1(b)), or a stirring time of 5.0 min when studying the stirring time within the 500-2500 rpm range (Figure 1(c)), respectively. Results regarding the stirring time shows a gradual extraction efficiency impairment at vortexing times up to 1.0 min, although a slight improvement on recovery can be observed when using 5.0 min as a stirring time (Figure 1(b)). After selecting 1.0 min as the best stirring time (fast VALLME process), the extractive procedure was performed at several vortexing speeds. Extraction yields increased gradually as the stirring speed increased from 500 to 2000 rpm (Figure 1(c)). However, experiments at 2500 rpm led to lower extraction efficiencies. Extraction efficiency worsening when using high stirring speeds and long extraction times is common to many microextraction techniques, and it has been attributed to the back diffusion phenomena [41]. Therefore, a vortexing speed of 2000 rpm was finally selected for further experiments.

4.3.1.3 Salting out effect

Previous experiments were performed by using directly the fish extract obtained after UAE. However, the addition of salts (commonly sodium chloride) has been reported to enhance target transfer from the aqueous phase to the extractant solvent in liquid-liquid, and also in liquid-liquid based microextraction procedures. Therefore, several experiments were performed by adding sodium chloride at final concentrations in the fish extract within the 0.5-2.0% (w/v) range. Figure 2(a) shows that a small amount of sodium chloride increases the extraction yields of all AFs, except G2 Recoveries for B1 and B2 were improved from nearly 80% in absence of sodium chloride to 90-100% when adding 0.5% (w/v) sodium chloride, and G1 recovery was also increased from 60% to nearly 80%. B1 recovery was found to remain constant at high sodium chloride amounts, however, B2 and G1 recoveries were impaired when adding sodium chloride concentrations higher than 1.0% (w/v) and 0.5% (w/v), respectively. High sodium

chloride amounts did not led to significant improvement/worsening on G2 extraction efficiency. Since the highest sodium chloride amounts could not improve the extraction, sodium chloride 0.5% (w/v) was selected.

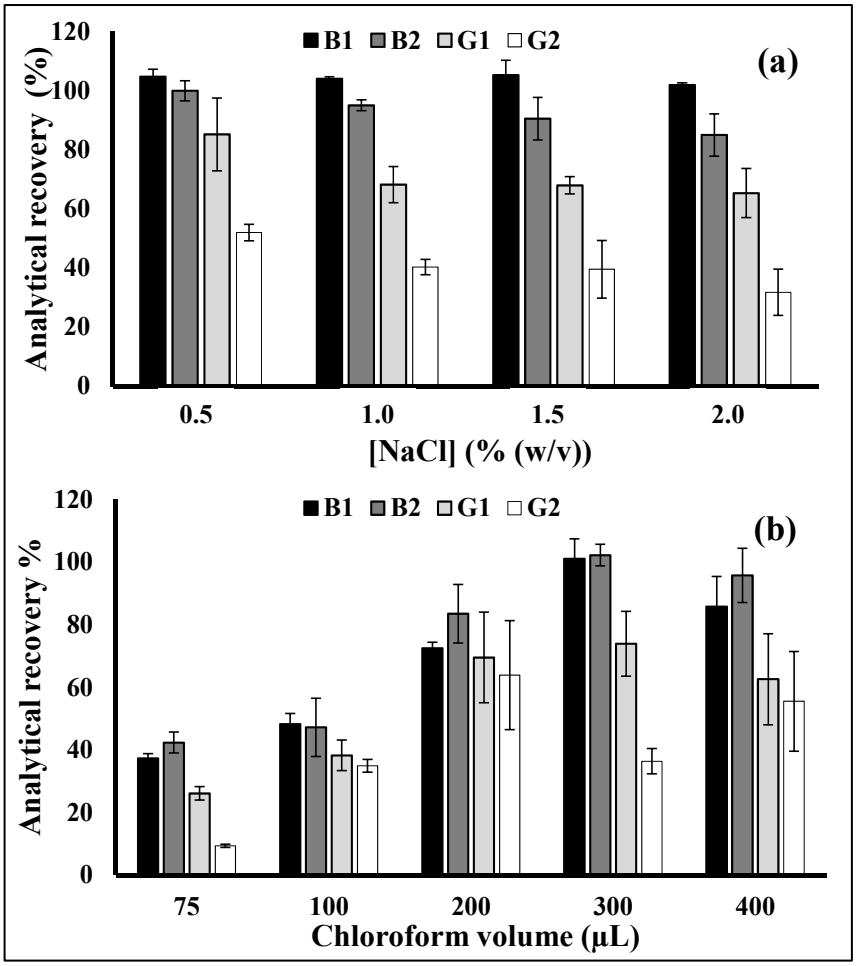

Figure 2: Effect of the NaCl concentration (a), and the extractant volume (disperser/extractant ratio) (b) on the AFs recovery

4.3.1.4 Effect of the extraction solvent volume

Since fish extract consists of a 6:4 ACN/aqueous buffer mixture, 5.0 mL aliquots imply the presence of 3.0 mL of ACN (disperser solvent). Therefore, previous experiments using 300 µL of chloroform leads to a disperser/extractant ratio of 10. The effect of the extractant volume (the effect of the disperser/extractant ratio) was studied by using 75, 100, 200, 300, and 400 µL of chloroform which means disperser/extractant ratios of 40, 30, 15, 10, and 7.5, respectively. Figure 2(b) show that the extraction efficiency is improved when using disperser/extractant ratios lower than 15 (chloroform volumes higher than 200 µL). Best performances were obtained when using 300 and 400 µL of chloroform, and despiste of slight lower recoveries for B1 and B2 when using 400 µL than those obtained for 300 µL, compromise conditions (slight higher G2 recovery) have led to select 400 µL (disperser/extractant ratio of 7.5) as extractant solvent volume.

4.3.1.5 Calibration and matrix effect

Matrix effect has been estimated by comparing the slopes of calibration graphs (AFs standards prepared in methanol) and the slopes of standard addition graphs. Several calibration graphs were prepared covering concentration of 2.5, 5, 10, 20, 50, 100, 200 µg L^{-1} for each AF and using DMC (100 µg L^{-1}) as an internal standard. Standard addition graphs were prepared by spiking several fish sub-samples (1.00 g) mixed with 10 mL of 6:4 ACN:buffer solution (final pH 7.00) with increasing AFs concentrations (0.15, 0.3, 0.6, 1.2, 3.0, 6.0, and 12 µg L^{-1}) which lead to AFs concentrations within the 2.5 – 200 µg L^{-1} range (pre-concentration factor of 16.7) and with a fixed DMC concentration (6.0 µg L^{-1}, which implies 100 µg L^{-1} after pre-concentration). Mean slopes and standard deviations for seven calibration and standard addition graphs are listed in Table 1. Regarding B1, B2 and G1, similar slopes for calibration and standard addition graphs can be observed, which implies negligible matrix effect (relative error of 3, 18 and 17% when using the calibration instead of the standard addition technique for B1, B2, and G1, respectively). These findings agree with those obtained after VALLME optimization because quantitative B1 and B2 analytical recoveries under the optimized

VALLME conditions were obtained (AFs concentrations, and hence analytical recoveries, were obtained using calibrations). However, differences between calibration and standard addition graphs' slopes for G2 are quite important (Table 1), and the relative error when using calibration instead of using the standard addition technique is close to 43%. These similarities/differences were verified by applying an ANOVA test (p values higher than 0.05 when comparing slopes for B1, B2 and G1, and a p-value of 0.0319 for G2 comparisons, 95% confident level). Therefore, the matrix effect is important when assessing the G2 and the standard addition graph used for further AFs assessments. Furthermore, the regression coefficients were higher than 0.995 for all AFs, which shows good linearity.

4.3.1.6 LOD and LOQ

The LOD and LOQ were determined based on the 3/10 standard deviation and calibration's slope criteria as follows:

$$LOD = 3\frac{Sd}{m} \qquad LOQ = 10\frac{Sd}{m}$$

where *Sd* is the standard deviation of eleven measurements of a blank, and *m* is the mean slope of the standard addition graph.

Instrumental LOD and LOQ values were then expressed as μg kg^{-1} taking into account the pre-concentration factor of 16.7 after VALLME, and they are listed in Table 1. Good sensitivity is therefore obtained because LODs are lower than the European Commission (EC) regulation limit for AFs in foodstuff (MRL of 15 μg kg^{-1}) [9].

Table 1. Mean slopes of calibration and standard addition, and LOD and LOQ values

	Slope (mean±Sd)			
	Calibration[a]	Standard addition[a]	LOD (μg kg^{-1})[b]	LOQ (μg kg^{-1})[b]
B1	471.8±9.3	456.8±82,0	0.09	0.29
B2	130.6±6.7	106.5±14.8	0.32	1.08
G1	367.8±13.9	307.1±27.1	0.07	0.23
G2	70.1±2.0	40.0±0.9	0.36	1.10

[a] n=7, [b] pre-concentration factor 16.7

4.3.1.7 Precision and accuracy

Intra- and inter-day precision and recovery were determined by spiking several aliquots of the fish sample with AFs concentrations at several concentration levels (0.15, 0.3, 0.6, 1.2, 3.0, 6.0, and 12 μg L^{-1}) which led to AFs concentrations of 2.5, 5.0, 10, 20, 50, 100, 200 μg L^{-1} after VALLME pre-concentration. Intra-day assay was assessed by replicating (n=7) three AFs concentration levels (2.5, 20 and 200 μg L^{-1}) in three different days, whereas the other concentration levels were replicated thrice. Similarly, inter-day assays were calculated by preparing seven standard addition in seven different days but replicating each concentration level thrice. Results (Table 2) show good intra- and inter-day precision (RSD values lower than 20%), and also good intra- and inter-day recovery (within the 80-100 % range).

Since there is not a certified reference material (CRM) for AFs in fish commercially available, the developed method was applied to a ERM-BE 376 (AFs in feeding stuff) CRM to check accuracy. The sensitivity of the method was high enough for assessing B1 and G1 (measured values of 11.8±0.3 and 5.8±0.06 μg kg^{-1} for B1 and G1, respectively), which are in good agreement with the certified concentrations (12.9±1.8 and 5.2±0.8 μg kg^{-1} for B1 and G1, respectively). However, sensitivity of the method was not good enough to assess B2, which certified concentration was lower than the LOD of the method for this AF.

Table 2. Intra-day precision and inter-day precision (RSD%) and intraday and interday analytical recovery (AR%±SD) of the method (n=7)

	Intra-day assay		Inter-day assay	
	RSD (%)	AR (%)	RSD (%)	AR (%)
B1				
2.5	18	95±1	13	89±1
5	--[a]	--[a]	9	90±1
10	--[a]	--[a]	8	83±1
20	10	103±2	9	96±2
50	--[a]	--[a]	10	90±4
100	--[a]	--[a]	9	96±7
200	6	98±5	6	95±8
B2				
2.5	18	78±1	11	88±1
5	--[a]	--[a]	8	95±0
10	--[a]	--[a]	8	91±1
20	5	99±2	7	93±2
50	--[a]	--[a]	10	94±4
100	--[a]	--[a]	6	90±5
200	6	93±9	7	94±10
G1				
2.5	17	84±1	7	93±1
5	--[a]	--[a]	9	94±3
10	--[a]	--[a]	5	93±1
20	8	107±2	8	94±1
50	--[a]	--[a]	6	93±3
100	--[a]	--[a]	8	98±7
200	8	94±5	7	96±10
G2				
2.5	19	92±1	13	94±0
5	--[a]	--[a]	6	90±1
10	--[a]	--[a]	10	83±1
20	13	97±3	5	93±1
50	--[a]	--[a]	9	87±4
100	--[a]	--[a]	7	92±6
200	3	86±5	3	91±6

[a] Not assessed

4.3.2 Application

Applicability of the proposed UAE-VALLME method was tested by analysing gilt-head bream (flesh and liver), Japanese seabass (flesh and liver), brown trout (flesh) and turbot (flesh) aquaculture fish samples. Each sample was subjected to the optimized procedure in triplicate, and AFs concentrations were found to be lower than the LOD of the method for all fish flesh samples, except for flesh from gilt-head bream which showed a B1 concentration within the LOD-LOQ range (LOD=0.09 μg kg^{-1} < 0.17±0.03 μg kg^{-1} < 0.29 μg kg^{-1}). However, B2 was quantified in the two fish liver samples (1.14±0.08 μg kg^{-1} in gilt-head bream, and 1.50±0.21 μg kg^{-1} in Japanese seabass), and G1 was also found in Japanese seabass's liver (0.33±0.02 μg kg^{-1}). Despiste there are no regulation regarding MRL of AFs in fish, found AFs concentrations are lower than the MRL established by the EU regarding AFs in groundnut as an ingredient in foodstuff (8 μg kg^{-1} for AFB1 and 15 μg kg^{-1} for the sum of all AFs) [9].

4.4 CONCLUDING REMARKS

Targets isolation, extract clean-up, and targets enrichment can be obtained by combining several sample pre-treatments such as UAE and VALLME, and the capabilities of each single sample pre-treatments are therefore improved in the combined procedure. The validated HPLC-MS/MS method after combined UAE-VALLME has demonstrated to be quite precise, accurate and sensitive for AFs assessment in fish flesh and fish liver. Therefore, the quantification of some AFs such as B2 and G1 in aquaculture fish livers, as well as the detection of B1 in the flesh from gilt-head bream, has been possible because of the low LOD/LOQ values of the current proposal. The developed method is therefore a potential strategy for AFs monitoring in aquaculture products.

Acknowledgment

This work was supported by the Dirección Xeral de I+D – Xunta de Galicia Grupos de Referencia Competitiva (project number 6RC2014/2016 and ED431C2018/19), and Development of a Strategic Grouping in Materials - AEMAT (grant ED431E2018/08).

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4.6 ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Table S1. Instrument parameters for HPLC-MS/MS

MS-MS						
Compound[a]	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)[b]	EP (V)[b]	CE (V)[b]	CXP (V)[b]
DMC	206.8	121.1	46.6	7.6	34.1	3.0
B1	313.0	241.0	70.4	4.8	52.3	7.2
B2	315.0	259.2	84.9	2.8	39.3	2.2
G1	329.0	200.1	59.9	4.2	55.1	4.5
G2	331.0	213.2	68.8	4.1	35.0	3.0

HPLC			
Column[c]	Zorbax Eclipse C_{18} reverse phase column (100 mm length × 4.6 mm i.d, 3.5 µm particle diameter)		
Injection volume	20 µL		
Flow rate	250 µL min^{-1}		
Mobile phase composition	0.1% formic acid in ultrapure water (A) and 0.1% formic acid in methanol (B)		
	Total time (min)	A%	B%
	0.1	50	50
	3.0	50	50
	8.0	0	100
	12.0	0	100
	13.0	50	50
	15.0	50	50

[a] Electron spray operation conditions are: ion spray voltage, 5500 kV; ion source temperature, 300 °C; nebulizer gas and curtain gas (N_2), 40 psi; collision gas (N_2), high
[b] DP-Declustering potential, EP-Entrance potential, CE-Collision energy, CXP-Collision cell exit potential
[c] Column oven temperature 40°C

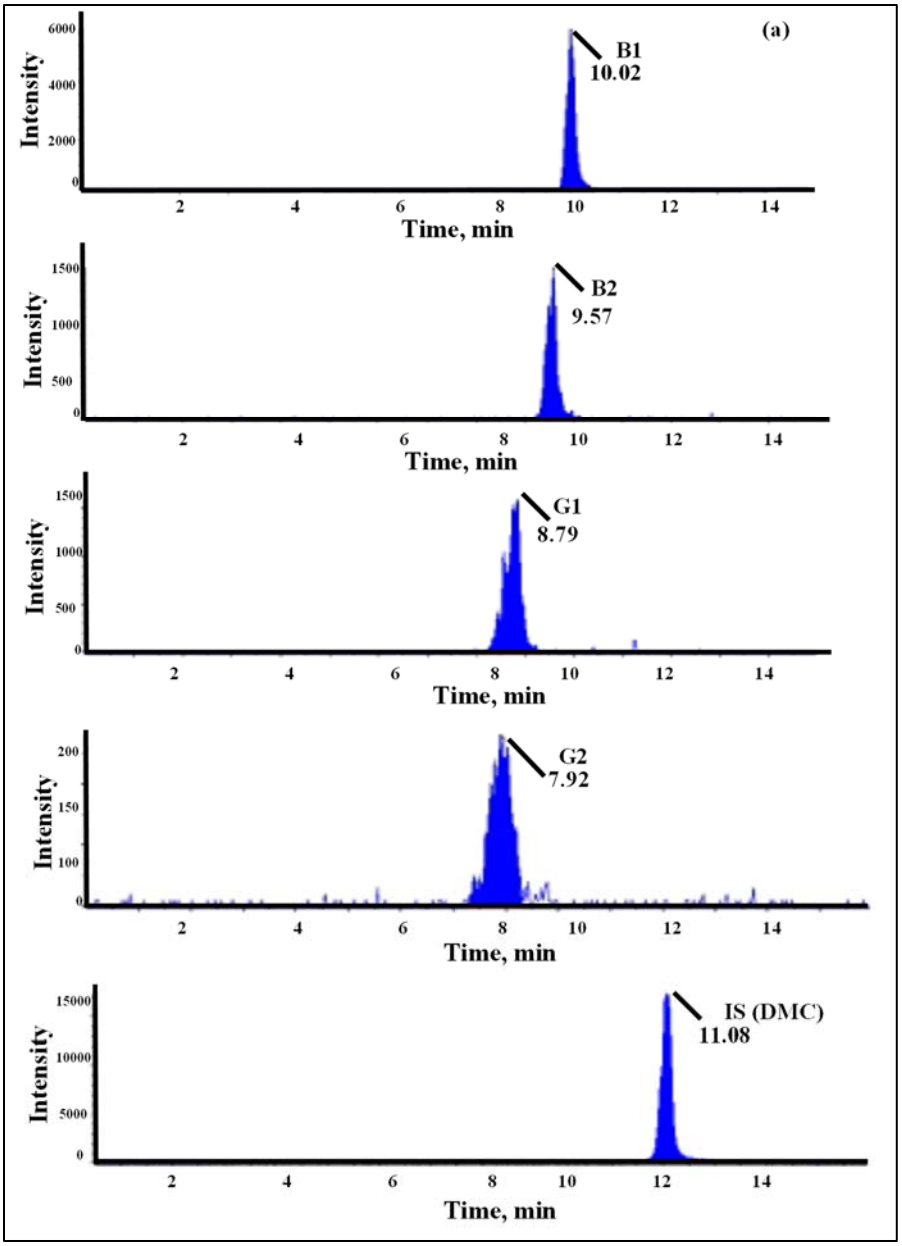

Figure 1a. MRM chromatograms of a 100 µg L^{-1} B1, B2, G1 G2 and DMC standard solution.

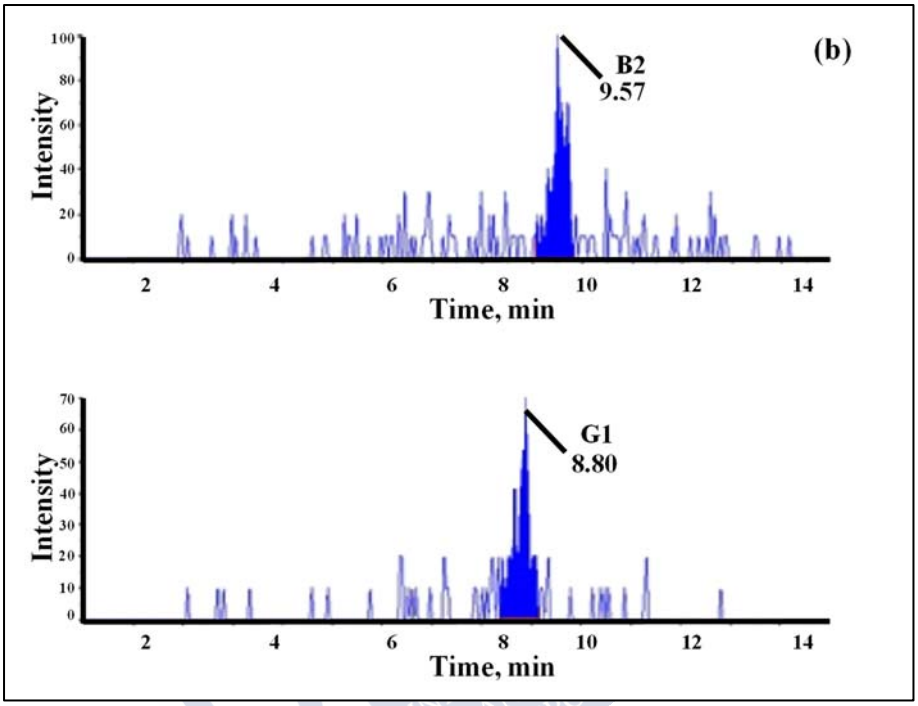

Figure 1b. MRM chromatograms of B2 and G1 in a fish liver sample

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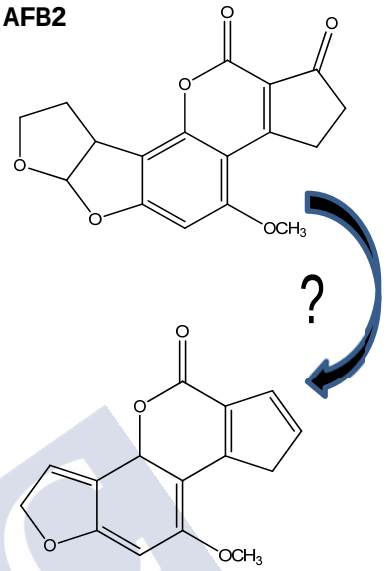

Dialysable fraction

CHAPTER 5

BIOAVAILABILITY OF AFLATOXINS IN CULTURED FISH AND ANIMAL LIVER USING AN *IN VITRO* DIALYSABILITY APPROACH

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Bioavailability of aflatoxins in cultured fish and animal liver using an *in vitro* dialysability approach

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Abstract

The present study aims to investigate the bioavailability of aflatoxins from fish, and chicken and rabbit liver using an *in vitro* dialysability approach. Ultra-high-performance liquid chromatography-tandem mass spectrometry was used to assess aflatoxins (AFB1, AFB2, AFG1, and AFG2) content in samples and in dialysate and residue fractions after the *in vitro* procedure. A vortex assisted dispersive liquid-liquid microextraction technique was used for pre-concentrating aflatoxins before chromatographic separation/quantification. Raw samples showed bioavailability ratios of 41-45% for AFB1, 28-38% for AFB2, and 42% for AFG2. The culinary process (steaming cooking and frying) was found to change aflatoxins bioavailability, and higher bioavailability ratios than those obtained for raw samples were assessed. The highest AFB1 bioavailability value was found in fried gilt head beam (average of 59%); whereas, the lowest ratio was detected in raw rabbit and chicken liver samples (average of 41%). In the case of AFB2, a mass balance study between the AFB2 concentrations in samples and the sum of AFB2 concentrations in dialysate and residue fractions was not consistent, which implies that AFB2 is transformed into other compounds during the *in vitro* process. AFB2 transformation/degradation has been investigated/ confirmed by high-resolution mass spectrometry.

Keywords: Bioavailability, Aflatoxins, Transformation products, fish, High-resolution mass spectrometry

5.1 INTRODUCTION

Moulds of *Aspergillus* genus are among the most important causing for food and feed spoilage since they produce mycotoxins as toxic secondary metabolites [1]. Aflatoxins (AFs) are a group of mycotoxins produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* [2]. The major AFs are AFB1, AFB2, AFG1, and AFG2, which occurs in food like nuts, cereals, and cereal-based products such as animal feed. Milk can be contaminated with AFM1 which is a metabolite from AFB1 as a result of animals exposed to contaminated feed [3]. Aflatoxins were categorized under class 1A human carcinogen by the International Agency for Research on Cancer (IARC) [4]. The toxic effect of the AFs in both animals and humans depend on several factors such as duration of exposure, intake level, metabolism and defence mechanisms of the body, and individual susceptibility. The disease caused by AFs is known as aflatoxicosis, and acute aflatoxicosis results in death; whereas, chronic intoxication causes cancer, especially in the liver, immune suppression and slow pathological conditions [5]. Due to the multiple adverse health effects of the AFs on human and animals, many countries have regulations on AFs levels in food and feed. The European Regulation (Commission Regulation (EC) No. 1881/2006) set a maximum limit of 8 μg kg^{-1} for AFB1 and 15 μg kg^{-1} for the sum of all AFs [6].

Ingredients used to prepare feedstuff for aquaculture facilities have been found to contain frequently AFs [7-9]. Currently, the increase on ingredients from plant origin instead of animal origin when preparing aquafeeds fish farms has intensified the potential onset for aflatoxicosis among the cultured animals [10]. Animal tissues can retain AF residues, AF metabolites included, giving rise to potential public health [10]. Despite the several studies on the presence of AFs in foodstuff, there are few data regarding AFs in fish. Nevertheless, fish have been found to be as highly susceptible species for AFs after controlled AFB1 exposition experiments with several freshwater fish species [11-16].

In order to know the possibility of AFs transfer from food to the human body, the knowledge of AFs amount in the foodstuff is not enough, and studies that could highlight the fraction of AFs absorbed by the human body are needed. Human bioavailability approaches, mainly encompassing processes such as digestion, absorption, transport, utilization, and elimination, are useful strategies to understand the fraction of nutrients and pollutants that can theoretically be released in the gastrointestinal tract and become available for intestinal absorption [17, 18]. Human bioavailability approaches are split in *in vivo* and *in vitro* assays, the former require humans or animals are based on using labelled targets (e.g., isotopes when investigating trace elements bioavailability) [18]. Although *in vivo* assays offer the best estimation of the bioavailability of a target, the inherent ethical issues of these experiments have led to the preferable use of *in vitro* bioavailability methods. Two different *in vitro* approaches, bio-accessibility and dialysability, have been developed for performing bioavailability studies [17,18]. Bio-accessibility is the simplest *in vitro* method and it indicates the maximum fraction of a substance that can theoretically be released from the foodstuff in the gastrointestinal tract (bio-accessible fraction) and becomes then available for intestinal absorption (enter into the bloodstream). The second *in vitro* assay refers to the fraction of a substance that reaches the systemic circulation from gastrointestinal tract (bioavailability fraction) and it is available to promote its activities in the exposed organism. Cell lines are mainly used for simulating intestinal absorption, but methods based on dialysis membranes (dialysability) for simulating the intestinal absorption are preferred because they are more straightforward, simple of operation and cheaper than methods based on cell lines.

Regarding AFs human bioavailability in foodstuff, few data are available when comparing to other mycotoxins [19], and the published bioavailability studies have been based on bio-accessibility assays for assessing AFB1 bioavailability in peanut and buckwheat [20] and in pistachio nuts, buckwheat and infant formula- sunflower oil mixtures [21]. The same bio-accessibility approach has been used for AFB1, AFB2, AFG1, and AFG2 in edible nuts, dried fruits, paprika, and cereals [22], and AFM1 in milk [23]. All reported studies implied a

previous artificial contamination process with variable amounts of AFs prior to the bio-accessibility assay [20-23], although naturally contaminated peanut and buckwheat [21] and milk [23] were also analysed. High bio-accessibility ratios (higher than 80%) were observed [20-23], although the presence of probiotic bacteria during the *in vitro* process was found to reduce the AFs bio-accessibility [21-23]. Data regarding bioavailability of AFs in fish and chicken/rabbit livers are not yet available, and also, bioavailability studies based on *in vitro* dialysability (simulated intestinal absorption by using a dialysis membrane) have not been yet performed for AFs. The current study focuses on the development and application of an *in vitro* dialysability assay to evaluate the AFs (AFB1, AFB2, AFG1, and AFG2) bioavailability in cultured fish and chicken and rabbit liver under naturally contamination conditions (basal AFs present in the food samples). The effect of the culinary process (steaming cooking and frying) on the AFs dialysability has been also investigated. Finally, some attempts have been carried out to discover transformation products from AFB2 during *in vitro* procedure.

5.2 MATERIALS AND METHODS

5.2.1 Instrumentation

Aflatoxins content was measured with a 3200 Q TRAP LC/MS/MS system (ABSciex, Concord, Canada) equipped with Flexar FX-15 (UHPLC) binary pump, vacuum degasser and Flexar UHPLC autosampler (Perkin Elmer, Waltham, MA, USA). UHPLC/MS/MS data processing was performed with MultiQuant 2.1 software (ABSciex). Derivates (transformation products) from AFB2 after the *in vitro* process has been characterized with an Agilent 1260 Quant Pump VL coupled with a UV–Visible detector 1260 VWD VL (Agilent Technologies, Santa Clara, CA, USA) and manual injection with a Rheodyne 7725i (Cotati, CA, USA) injector (loop volume of 20 µL) and using a 250 µL Hamilton Gastight 1725 syringe (Bonaduz, Switzerland). An LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled to an Accela pump (Thermo) and to an Accela autosampler (Thermo) was also used for confirming the presence of transformation products from AFB2. In all

cases, a Zorbax C_{18} reverse phase column (100 mm length, 4.6 mm diameter and 3.5 µm particle diameter) from Agilent connected to a C_{18} guard column (4 mm length, 3.0 mm diameter) from Phenomenex (Torrance, CA, USA) was used to achieve chromatographic separation. Chromatographic separations were performed under controlled temperature (40 °C) by using a GECKO 2000 column heater from Amchro GmbH (Hattersheim, Germany). A Boxcult temperature-controlled incubation chamber (Stuart Scientific, Surrey, UK) equipped with a Rotabit orbital-rocking platform shaker (J. P Selecta, Barcelona, Spain) was used for the *in vitro* enzymolysis procedure. A VibraCell VCx 130 ultrasound probe from Sonics (Newtown, CT, USA) was used for AFs isolation from samples (flesh and liver). A Laborcentrifugen 2K15 centrifuge from Sigma (Osterode, Germany) was used for extracts and dialysates isolation. An EC1 metal block thermostat nitrogen sample concentrator from VLM (Leopoldshohe-Greste, Germany) was used for extracts evaporation. Other laboratory devices such as a Taurus blade grinder (Barcelona, Spain), a Basic20 pH meter with a glass calomel-electrode (Crison, Barcelona, Spain), a Classic ML analytical balance from Mettler Toledo (Columbus, OH, USA), and a Milli-Q water purification device (Millipore, Bedford, MA, USA) were also used throughout the work. The chemometrics package used for data analysis was STATGRAPHICS Centurion XVIII (Manugistics Inc., Rockville M.D., USA); whereas ChemDraw 15.0 (Cambridge Soft Corporation, Waltham, MA, USA) was used for molecular structures design.

5.2.2 Reagents

Aflatoxins (AFB1, AFB2, AFG1, and AFG2) and 5,7-dimethoxycoumarine (DMC) solid standards were from Sigma-Aldrich (Steinheim, Germany), and AFs and DMC stock solutions (1000 mg L^{-1}) were prepared by using LC-MS grade methanol from Merck (Darmstadt, Germany). All standard solutions were stored in amber bottles at -20 °C in the dark. Digestive enzymes (p-7000 porcine pepsin, and p-1750 porcine pancreatin), bile salt (approximately 50% sodium chlorate and 50% sodium deoxycholate), and piperazine-NN-bis (2-ethane-sulfonic acid) di-sodium salt (PIPES) were from Sigma-Aldrich.

Hydrochloric acid 37%(m/m) and chloroform were from Panreac (Barcelona, Spain); whereas, sodium hydrogen carbonate, sodium hydroxide, monopotassium phosphate, sodium chloride, and acetonitrile (gradient grade for LC) were from Merck. Dialyzability was performed by using Spectra/Por® standard grade regenerated cellulose dialysis membranes [10 kDa molecular weight cut off (MWCO), 45 mm flat width, 29 mm dry diameter, 20 µm thickness, and 6.4 mL cm^{-1} volume to length ratio] from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). Other consumables were disposable 2.0 mL sterile syringes from Dispomed (Gelnhause, Germany), and Clear PVDF syringe filters (0.22 µm, 4 mm) from Perkin Elmer.

5.2.3 Samples

Cultured fish samples in the current study consist of three flesh and two liver samples from gilt-head beam (*Sparus aurata*); two flesh, one liver, and one egg samples from Japanese sea bass (*Lateolabrax japonicus*); two flesh samples from brown trout (*Salmo trutta*); and one flesh sample from turbot (*Scophthalmus maximus*). One rabbit liver and one chicken liver were also included in the study for comparison purposes. Fish samples were washed with clean tap water and fish muscle, liver and eggs were separated. Fish samples and rabbit and chicken livers were homogenised separately using the domestic blender, and the homogenized samples were stored in plastic bottles at -20 °C until further use.

5.2.4 Culinary methods

Fish flesh, rabbit liver and chicken liver samples were divided into three portion and two portions of the samples were treated with two culinary treatments such as steaming and frying (without oil or any spices). Based on the literature, the steaming cooking was done for 25 min and frying was performed for 6.0 min [24, 25]. After performing the two culinary treatments, samples were led to cool at room temperature, and they were then homogenised using a mortar and pestle before storing in the sealed plastic bottles at -20 °C.

5.2.5 Moisture analysis

The moisture content of the raw and cooked samples was assessed by following the 950.46 (2000) AOAC official method [26]. Experiments were performed thrice, and moisture was calculated in accordance with equation 1:

$$Moisture\ \% = \frac{W_1 - W_2}{W_1} X100 \quad equation\ 1$$

where, W_1 and W_2 are the weights before and after drying, respectively.

5.2.6 AFs extraction, pre-concentration, and UHPLC-MS-MS determination

AFs content in muscle and liver samples were analysed following the method published by Jayasinghe et al. [27]. Briefly, 1.0 g of homogenised samples (raw and cooked) were placed together with 5 mL of 60:40 acetonitrile/0.1 M aqueous KH_2PO_4 (pH 7.0) into a 30 mL centrifuge tube and the mixture was subjected to ultrasonication at an amplitude of 40% (power of 130 W and frequency of 20 kHz) for 7.0 min. After vacuum filtration, the extract was subjected to a pre-concentration method based on a vortex assisted liquid-liquid microextraction (VALLME) procedure. VALLME consisted of mixing 25 mg of NaCl with 5.0 mL of extract and adding 400 µL of chloroform as an extractant before vortexing at 2000 rpm for 1 min. The cloudy solution containing the dispersed fine droplets of extractant was centrifuged at 2500 rpm for 10 min for the organic phase separation. After removing the aqueous phase, the chloroform extract was fully dried under N_2 flow, and the residue was further dissolved in 100 µL of methanol (pre-concentration factor of 50). The extract was finally filtered (0.22 µm filters) before UHPLC-MS-MS analysis (Table 1).

Table 1: UHPLC-MS/MS operating condition

MS-MS						
Compound[a]	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)[b]	EP (V)[b]	CE (V)[b]	CXP (V)[b]
DMC	206.80	121.10	46.59	7.63	34.15	3.00
AFB1	313.00	241.00	70.39	4.80	52.34	7.23
AFB2	315.00	259.20	84.90	2.82	39.32	2.24
AFG1	329.00	200.12	59.94	4.18	55.14	4.50
AFG2	331.00	213.20	68.78	4.11	35.00	3.00

HPLC			
Column[c]	Zorbax Eclipse C_{18} reverse phase column (100 mm length × 4.6 mm i.d, 3.5 µm particle diameter)		
Injection volume	20 µL		
Flow rate	250 µL min^{-1}		
Mobile phase composition	0.1% formic acid in ultrapure water (A) and 0.1% formic acid in methanol (B)		
	Total time (min)	A%	B%
	0.1	50	50
	3.0	50	50
	8.0	0	100
	12.0	0	100
	13.0	50	50
	15.0	50	50

[a] Electrospray operation conditions are: Ion spray voltage (IS), 5500 kV; Ion source temperature, 300 °C; nebulizer gas and curtain gas (N_2), 40 psi; collision gas (N_2), high

[b] DP, Declustering potential; EP, Entrance potential; CE, Collision energy; CXP, Collision cell exit potential

5.2.7 *In vitro* dialyzability procedure

The *in vitro* dialyzability procedure was performed according with Moreda-Piñeiro et al. [28] by placing 2.0 g of homogenised raw/cooked sample and 20 mL of ultrapure water into 100 mL Erlenmeyer flasks. Experiments with AFs standards implied 20 mL of single AFs (AFB1, AFB2, AFG1 and AFG2) aqueous standard solutions at 4.0 μg L^{-1} for preliminary studies, and 20 mL of AFB2 at 200 μg L^{-1} for studies regarding the elucidation of transformation products by HPLC-UV and HPLC with high resolution mass spectrometry (HRMS). All experiments were performed in triplicate and at least one blank was prepared for each experiment set. The pH of the dispersed sample/standard solution was adjusted at 2.0 by adding a small amount of 6.0 M HCl, followed by adding 150 μL of gastric solution (16% (m/v) pepsin in 0.1 M HCl). The flasks were then placed under orbital horizontal shaking at 150 rpm and 37 °C for 2.0 h. After finished the gastric digestion, the flasks were immediately placed in an ice bath to stop the enzymatic reaction. The procedure was continued by adding 5.0 mL of intestinal solution consisted of a 4.0% (m/v) pancreatin and 2.5% (m/v) bile salt solution prepared in 0.1 M sodium hydrogencarbonate. At this point, a dialysis membrane (10 kDa MWCO) containing 20 mL of 0.15 N PIPES solution at pH 7.5 was placed inside the flask and it was sealed with a plastic clamp. Intestinal digestion took place under orbital horizontal shaking (150 rpm and 37 °C) for 2 h, and the enzymolysis was stopped by cooling down (ice-bath). The solution inside the dialysis membrane (dialysate or dialysable fraction) and remaining slurries/solutions in the flask (residue/non-dialysable fraction), were weighted separately and transferred to polyethylene vials before kept at -20 °C.

5.2.8 Aflatoxins determination in dialysable and non-dialysable fractions

AFs content in the dialysate and residue were assessed following the VALLME pre-concentration method described in section 2.6 with some modifications. Experiments involving AFs aqueous standards were performed using 500 μL of dialysates together with 4500 μL of ultrapure water, 1500 μL of acetonitrile as a dispersive solvent, and 400

µL of chloroform as an extract (preliminary experiments led to better pre-concentration by using 400 µL of chloroform). Experiments for dialysates from foodstuff involved 5.0 mL of the dialysate and the same volumes of acetonitrile (1500 µL) and chloroform (400 µL). Because of the pH of the dialysate (PIPES solution, pH 7.5) was close to the optimum pH required for VALLME (pH of 7.0), dialysates pre-concentration was performed without pH adjustment (pH of the PIPES buffer was quite stable and pH change after adding acids was difficult). However, pH of the residues was adjusted to 7.0 after adding small volumes of 0.1M HCl (pHs of residues from samples were within the 9.0-9.5 range, and the pH of the residues from AFs aqueous standards was close to 8.0). Similarly, experiments for residues from AFs aqueous standards (500 µL) were performed by adding 4500 µL of ultrapure water, 1500 µL of acetonitrile, and 400 µL of chloroform; whereas, VALLME pre-concentration for residues from foodstuff (5.0 mL) required 1500 µL of acetonitrile and 400 µL of chloroform.

For all cases, the mixtures were vortexed at 2000 rpm for 1.0 min, and the cloudy solution was centrifuged at 2500 rpm for 10 min for phases separation. The upper (aqueous) solution was removed, and the extract (chloroform phase) was evaporated to dryness under N_2 flow and re-dissolved in 100 µL of methanol. After filtration, the clear extract was analysed by UHPLC-MS-MS (Table 1).

The bioavailability (dialyzability) ratios were calculated according to equation 2:

$$Bioavailability\ \% = \frac{[AF]_{dialysable}}{[AF]_{total}} \times 100 \quad equation\ 2$$

where $[AF]_{total}$ is the AF concentration in the sample, and $[AF]_{dialysable}$ is the dialysable AF concentration (AF content found in the dialysate) referred to the sample weight.

5.2.9 Mass balance study

In order to assess the accuracy of the bioavailability study, a mass balance approach was applied. After performing the *in vitro* procedure for AFs aqueous standards and food samples in triplicate (section 5.2.7), AFs concentrations were determined in the dialysates and in the residue fractions (section 5.2.8). The concentration of each AF in

the dialysable and non dialysable fractions was summed, and the values were statistically compared (Student's *t* test and ANOVA test, 95% confidence interval) with the AF concentration in the standards/ foodstuff before the *in vitro* bioavailability approach.

5.3 RESULTS AND DISCUSSION

5.3.1 AFs in flesh and liver from cultured fish and liver from chicken and rabbit

AFs, mainly AFB1 and AFB2, were quantified in four samples (the flesh from two gilt-head beams, a flesh from one Japanese sea bass, and a liver from one gilt-head beam) from a total of twelve fish samples under study, and also in the livers from chicken and rabbit (Table 2). Table 2 also lists the AFs concentrations in the samples containing detectable AFs concentrations after cooking (steaming and frying). Regarding raw samples, AFB1 was quantified in three fish flesh samples (within the 0.71–1.24 μg kg^{-1} range), and one fish liver sample (1.70±0.02 μg kg^{-1}). AFB1 in rabbit liver was 1.41±0.15 μg kg^{-1}, whereas a higher AFB1 level was found in chicken liver (3.30±0.11 μg kg^{-1}). AFB2 was quantified in one gilt-head beam flesh (0.76±0.11 μg kg^{-1}) and liver (1.04±0.26 μg kg^{-1}), and AFG2 in the same gilt-head beam liver (1.04±0.26 μg kg^{-1}) and rabbit liver (0.77±0.18 μg kg^{-1}) samples. AFG1 was not detected in any sample under study. Cooked (steaming and frying) fish flesh, and chicken and rabbit liver samples were analysed for the AFs and the AFs content was found to be highly varied among steaming cooked and fried samples (the highest AFs content was found in fried samples, Table 2). Differences are expected because during the culinary process the moisture content of the sample was reduced drastically. The moisture percentage in the raw samples, steaming cooked and fried samples was between 78-83%, 69-76% and 53-59%, respectively (Table 3). Hence the AFs in the fried samples were higher compared with steaming cooked and raw samples.

Table 2: Aflatoxins content in raw, steaming cooked and fried samples (n=3)

	[AFs] in raw samples (μg kg^{-1})				[AFs] in steaming cooked samples (μg kg^{-1})				[AFs] in fried samples (μg kg^{-1})			
	B1	B2	G1	G2	B1	B2	G1	G2	B1	B2	G1	G2
Gilt-head beam -1	1.24±0.18	[a] –	[a] –	[a] –	1.63±0.09	[b] –	[b] –	[b] –	2.20±0.21	[b] –	[b] –	[b] –
Gilt-head beam -2	0.88±0.03	0.76±0.11	[a] –	[a] –	1.20±0.06	1.44±0.14	[b] –	[b] –	1.38±0.03	1.51±0.03	[b] –	[b] –
Gilt-head beam -3	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Japanese sea bass-1	0.71±0.07	[a] –	[a] –	[a] –	1.13±0.07	[b] –	[b] –	[b] –	1.45±0.02	[b] –	[b] –	[b] –
Japanese sea bass-2	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Turbot	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Brown Trout-1	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Brown Trout-2	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Gilt-head beam-liver-1	1.70±0.02	1.04±0.26	[a] –	1.04±0.26	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Gilt-head beam-liver-2	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Japanese sea bass-liver	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Japanese sea bass-eggs	[a] –	[a] –	[a] –	[a] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –	[b] –
Rabbit-liver	1.41±0.15	[a] –	[a] –	0.77±0.18	1.96±0.17	[b] –	[b] –	1.18±0.18	2.57±0.10	[b] –	[b] –	2.01±0.05
Chicken-liver	3.30±0.11	[a] –	[a] –	[a] –	3.86±0.33	[b] –	[b] –	[b] –	4.50±0.04	[b] –	[b] –	[b] –

[a] Lower than LOD of the method
[b] Not determined

Table 3: Moisture of raw, steam and fried samples (n=3)

	Moisture (%)		
	Raw	Steaming cooking	Frying
Gilt-head beam -1	80±3	76±1	59±1
Gilt-head beam -2	79±2	73±1	57±2
Japanese sea bass-1	78±1	73±1	51±2
Rabbit liver	82±1	72±1	45±1
Chicken liver	83±2	69±2	43±2

5.3.2 AFs bioavailability ratios. Preliminary experiments with AFs standards

Preliminary experiments were led to assess the bioavailability of AFs in single AFs (AFB1, AFB2, AFG1, and AFG2) aqueous standards in order to observe target (AFs) transformations during the *in vitro* dialyzability process. Single AFs aqueous standards of 4.0 μg L^{-1} were performed in triplicate, and the AF concentration was assessed in the dialysable and non-dialysable (residue) fractions. A mass balance study based on statistical comparisons (Student's *t* test, 95% confidence interval) between the initial AF concentration (4.0 μg L^{-1}) and the sum of AF concentrations in the dialyzable and non-dialysable fractions (Table 4) showed that there are not AFs losses and/or AFs transformation during the *in vitro* process for AFB1, AFG1, and AFG2. The bioavailability ratio for these AFs was found to be quite similar and close to 40% (41±4, 37±9, and 42±7% for AFB1, AFG1, and AFG2, respectively). However, the sum of the AFB2 concentration in the dialysate and in the residue (dialysable and non-dialysable fractions) were found to be statistically different than the initial AFB2 concentration, which could imply AFB2 losses and/or AFB2 transformation into other compounds during the dialysability assay. The bioavailability ratio assessed for AFB2 (23±4%) was therefore lower than those obtained for the other AFs.

Several experiments were performed to verify if the VALLME pre-concentration procedure works properly for AFB2 by using volumes of AFB2 standards smaller than 500 µL (200, 300 and 400 µL) and chloroform (extractant) volumes higher than 400 µL (500, 600 and 800 µL). However, similar AFB2 concentrations in the dialysates and residues were obtained (bioavailability ratios close to 20%) and the mass balance assays showed that the sum of the AFB2 concentrations in the dialysable and non-dialysable fractions were statistically significantly different than the initial AFB2 concentration. Therefore, mass balance failure for AFB2 must be attributed to AFB2 changes/transformation during the *in vitro* process, and a further study will be carried out to find possible transformation products from AFB2.

Table 4: AFs concentrations in the dialyzable (d) and non-dialysable fractions (r), and AFs bioavailability for AFs aqueous standards (n=3)

	[AF] (µg L^{-1})[a]	$[AF]_{d+r}$ (µg L^{-1})[b]	$[AF]_d$ (µg L^{-1})[c]	$[AF]_r$ (µg L^{-1})[d]	Bioavailability (%)
AFB1	4.0	3.9±0.2	1.7±0.1	2.2±0.2	41±4
AFB2	4.0	2.5±0.2	0.9±0.1	1.6±0.1	23±4
AFG1	4.0	4.0±0.3	1.5±0.3	2.5±0.1	37±9
AFG2	4.0	3.8±0.1	1.8±0.1	2.0±0.1	42±7

[a] Initial AFs concentration
[b] AFs concentration as a sum of AF concentrations in the dialysable fraction (dialysate) and non-dialysable fraction (residue)
[c] AFs concentration in the dialysable fraction (dialysate)
[d] AFs concentration in the non-dialysable fraction (residue)

The assessed bioavailability ratios based on *in vitro* dialysability for AFs standards are half lower than the bio-accessibility ratios previously reported for AFs in artificially contaminated food [20-23] which is attributed the additional stage consisting of AFs crossing through the dialysis membrane for simulating intestinal absorption. In addition, AFB2 transformation/degradation into other compounds was not reported in previous bio-accessibility studies [22].

5.3.3 AFs bioavailability in cultured fish and chicken/rabbit liver samples

AFs concentrations in the dialysates from foodstuff (raw and cooked samples) as well as AFs dialysability ratios, are listed in Table 5. Slight higher dialysable AFs concentrations from cooked samples, mainly fried samples, than those obtained from raw food were found. These findings agree with those obtained when assessing the AFs content in raw and cooked samples (section 5.3.1), and they are explained taking into account the moisture content of samples which is reduced from 78- 83% in raw samples to 69-76% in steaming cooked samples and to 43- 59% in fried samples.

Dialysability ratios in raw samples were found to be quite similar among fish flesh and chicken and rabbit liver, and were within the 41-45 % range for AFB1, and from 28 to 38% for AFB2 (AFG2 was only found in one sample and the dialysability ratio was 42%). Considering the results of cooked samples, the steam and fried samples were showed higher bioavailability compare with raw samples. As previously commented for AFs aqueous standards, lower dialysability was obtained for AFB2, and it could be attributed to AFB2 transformations into other compounds.

The culinary process was found to affect differently the AFs dialysability (Table 5). The steaming cooking does not affect the AFs bioavailability since dialysability ratios (42-54% for AFB1, 29% for AFB2 and 43% for AFG2) were quite similar to those found in raw samples (findings verified after applying an ANOVA test at a confidence interval of 95% that were statistically). However, frying increases the AFs bioavailability, and AFB1 dialysability was found to be within the 54-59% range, and values of 37 and 56% were obtained for AFB2 and AFG2, respectively. Dialysability ratios for fried samples were found to be statistically significantly higher ($p<0.05$) than those found in raw and steaming cooked samples, and in AFs aqueous standards.

Bioavailability ratios in the analysed samples (fish flesh/liver and chicken and rabbit liver) based on a dialysability approach have been found to be lower than those reported when applying bio-accessible assays for buckwheat, edible nuts, dried fruits, wheat and maize, among

others [20-23]. Despite the food composition, mainly major nutrients such as protein and fat, affect the bioavailability of AFs [22], the lower bioavailability ratios reported in the current research must be mainly attributed to the simulated absorption stage through the dialysis membrane which is not considered in bio-accessible approaches.

5.3.4 Mass balance study

A mass balance study was performed to assess the accuracy of the method by determining the AFs contents in the dialysable (dialysates) and non-dialysable (residue) fractions. AFs concentrations as a sum of the AFs levels in the dialysable and non dialysable fractions have been statistically compared to the AFs content in the samples by using an ANOVA test at the 95% confidence interval.

The results are listed in Table 6, and p-values higher than 0.05 were calculated for AFB1 and AFG2, which means that there is no statistically significant differences between AFB1 and AFG2 concentrations in the samples and sum of the AFs concentrations in the dialysates and residues. However, a p-value lower than 0.05 was obtained for AFB2, implying statistically significant differences between the AFB2 concentrations in the samples and the sum of AFB2 concentrations in the dialysable and non-dialysable fractions. These results agree with those obtained when using AFB2 aqueous standards and should be attributed to AFB2 transformation/degradation into other compounds during the *in vitro* assay. As a consequence, the amount of AFB2 which could be bioavailable (dialysable) is lower than the amount of the other AFs. However, the health risk from AFB2 is not less important than risk derived from the other AFs since the AFB2 products could be also highly dialysable.

Table 5: AFs concentration, dialysable AFs concentration, and AFs bioavailability in raw, steam and fried samples (n=3)

	B1			B2			G2		
	Total (µg kg^{-1})	Dial.[a] (µg kg^{-1})[a]	Bioa.[b] (%)	Total (µg kg^{-1})	Dial.[a] (µg kg^{-1})[a]	Bioa.[b] (%)	Total (µg kg^{-1})	Dial.[a] (µg kg^{-1})[a]	Bioa.[b] (%)
Raw sample									
Gilt-head beam -1	1.24±0.18	0.54±0.09	44±7	–[c]	–[d]	–[d]	–[c]	–[d]	–[d]
Gilt-head beam -2	0.88±0.03	0.40±0.03	45±6	0.76±0.11	0.21±0.02	28±3	–[c]	–[d]	–[d]
Japanese sea bass-1	0.71±0.07	0.31±0.04	44±5	–[c]	–[d]	–[d]	–[c]	–[d]	–[d]
Gilt-head beam-liver-1	1.70±0.02	0.71±0.06	42±7	1.04±0.26	0.39±0.02	38±9	0.76±0.21	0.32±0.01	42±5
Rabbit-liver	1.41±0.15	0.59±0.04	41±8	–[c]	–[d]	–[d]	0.77±0.18	0.31±0.06	42±8
Chicken-liver	3.50±0.11	1.42±0.07	41±2	–[c]	–[d]	–[d]	–[c]	–[d]	–[d]
Steaming cooking sample									
Gilt-head beam -1	1.63±0.09	0.88±0.07	54±5	–[c]	–[d]	–[d]	–[c]	–[d]	–[d]
Gilt-head beam -2	1.20±0.06	0.63±0.02	53±5	1.44±0.14	0.42±0.03	29±6	–[c]	–[d]	–[d]
Japanese sea bass-1	1.13±0.07	0.48±0.06	42±8	–[c]	–[d]	–[d]	–[c]	–[d]	–[d]
Rabbit-liver	1.96±0.17	0.94±0.11	48±8	–[c]	–[d]	–[d]	1.18±0.18	0.51±0.09	43±8
Chicken-liver	3.86±0.33	1.65±0.15	43±5	–[c]	–[d]	–[d]	–[c]	–[d]	–[d]

Table 5: AFs concentration, dialysable AFs concentration, and AFs bioavailability in raw, steam and fried samples (n=3) (Continuned)

	B1			B2			G2		
	Total (µg kg^{-1})	Dial.[a] (µg kg^{-1})[a]	Bioa.[b] (%)	Total (µg kg^{-1})	Dial.[a] (µg kg^{-1})[a]	Bioa.[b] (%)	Total (µg kg^{-1})	Dial.[a] (µg kg^{-1})[a]	Bioa.[b] (%)
Fried sample									
Gilt-head beam -1	2.20±0.21	1.29±0.08	59±7	_[c]	_[d]	_[d]	_[c]	_[d]	_[d]
Gilt-head beam -2	1.38±0.03	0.75±0.02	54±5	1.51±0.03	0.56±0.05	37±7	_[c]	_[d]	_[d]
Japanese sea bass-1	1.45±0.02	0.77±0.04	53±4	_[c]	_[d]	_[d]	_[c]	_[d]	_[d]
Rabbit-liver	2.57±0.10	1.18±0.31	56±4	_[c]	_[d]	_[d]	2.01±0.05	1.31±0.03	56±7
Chicken-liver	4.50±0.04	2.41±0.26	54±4	_[c]	_[d]	_[d]	_[c]	_[d]	_[d]

[a] Dial. AFs concentration in the dialysable fraction (dialysate)
[b] Bioa. Bioavailability
[c] Concentration lower than the LOD of the method
[d] Not assessed

Table 6: Mass balance of the *in vitro* dialysability procedure for raw, steaming cooked and fried samples (n=3)

	Raw samples			Steaming cooked samples			Fried samples		
Sample type	[AF] (µg kg^{-1})	[AF]$_{d+r}$ (µg kg^{-1})[a]	p-value[b]	[AF] (µg kg^{-1})	[AF]$_{d+r}$ (µg kg^{-1})[a]	p-value[b]	[AF] (µg kg^{-1})	[AF]$_{d+r}$ (µg kg^{-1})[a]	p-value[b]
AFB1									
Gilt-head beam -1	1.24±0.18	1.14±0.04	0.4007	1.63±0.09	1.61±0.07	0.4136	2.20±0.21	2.02±0.11	0.2588
Gilt-head beam -2	0.88±0.03	0.84±0.04	0.2381	1.20±0.06	1.13±0.04	0.1680	1.38±0.03	1.36±0.06	0.1078
Japanese sea bass-1	0.71±0.07	0.71±0.09	1.0000	1.13±0.07	0.93±0.13	0.0788	1.45±0.02	1.38±0.12	0.6913
Gilt-head beam-liver-1	1.70±0.02	1.61±0.12	0.2693	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]
Rabbit-liver	1.41±0.15	1.38±0.13	0.8064	1.96±0.17	1.95±0.13	0.9546	2.57±0.10	2.49±0.31	0.9204
Chicken-liver	3.50±0.11	3.47±0.15	0.7938	3.86±0.33	3.41±0.19	0.1088	4.50±0.04	4.27±0.31	0.2715
AFB2									
Gilt-head beam -2	0.76±0.11	0.46±0.02	0.00970	1.44±0.14	0.89±0.09	0.0046	1.51±0.03	1.03±0.13	0.0034
Gilt-head beam-liver-1	1.04±0.26	0.63±0.05	0.00455	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]
AFG2									
Gilt-head beam-liver-1	0.76±0.21	0.71±0.07	0.7156	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]
Rabbit-liver	0.77±0.18	0.78±0.04	0.9297	1.18±0.18	0.99±0.12	0.153	2.01±0.05	1.91±0.19	0.4278

[a] AFs concentration as a sum of AF concentrations in the dialysable fraction (dialysate, d) and non-dialysable fraction (residue, r)
[b] p-value after ANOVA (confidence interval of 95%, p-values lower than 0.05 indicate statistically significant differences)
[c] Not assessed

5.3.5 Characterization of AFB2 transformation products

Attempts for characterizing AFB2 transformation products during the *in vitro* dialyzability process were carried out by HPLC-UV (monitoring at 365 nm), UHPLC-MS, and HPLC-HRMS (operating conditions are listed in Tables S1, S2 and S3, electronic supplementary information – ESI). Experiments consisted of subjecting 20 mL of AFB2 standard solutions at 200 μg L^{-1} to the dialysability procedure, and pre-concentrating 10 times the AFB2 present in the dialysate and in the residue by applying the described VALLME procedure.

Results from HPLC-UV experiments are shown in Figure 1, where AFB2 eluates at nearly 12 min (Figure 1(c-e)), and several chromatographic signals at retention times higher than 12 min are observed when analysing the dialysate and the residue fraction (Figure 1(d) and Figure 1(e), respectively). Chromatographic signals at retention times different than the AFB2 retention time (12 min) are not attributed to UV artifacts and/or PIPES (dialysable fraction) or enzyme residues (non-dialysable fraction) because the *in vitro* dialysability/residue blanks are free of chromatographic signals (Figure 1(a, b)).

Possible transformation products from AFB2 were designed using a ChemDraw software taking into account possible changes (mainly oxidation and hydroxylation) of the molecule as consequence of the enzymolysis process (Figure 2). Most of the proposed structures for the AFB2 transformation products were based on the literature regarding AFB1 metabolisms [29] establishing similar metabolisms (transformation) schemes for AFB2. Experiments by UHPLC-MS were then performed on the basis of the molecular weights of the proposed structures (the mass spectrometer was set in negative and positive modes for monitoring the subsequent m/z ratios). Sensitive detection was found when working in positive mode and some intense chromatographic signals at retention times different than that observed for AFB2 (retention time of 10.08 min under UHPLC-MS conditions) were observed when setting the mass acquisition at certain m/z ratios. Extracted chromatograms at certain m/z ratios were found to have high intensities when working in positive mode acquisition.

As shown in Figure 3, intense signal at a m/z ratio of 257.05 was obtained when analysing the dialysate; whereas, high signals at m/z ratios of 247.05, 289.08, and 298.08 were observed for residue analysis. These m/z ratios match with some structures proposed on the basis of AFB2 metabolisms (structures coded as *5* for 257.05 in Figure 2, and structures *4*, *3* and *2* in Figure 2 for 247.05, 289.08, and 298.08, respectively).

A further HPLC-HRMS study (operating conditions in Table S3) was performed to verify/find the proposed molecular weights (exact masses) in the dialysate and residue fractions. Extracted chromatograms for the exact mass of AFB2 in the dialysable and non-dialysable fractions (exact m/z ratio of 315.0863, which implies an exact mass of 314.0783) showed a retention time of 11.7 min. As can be seen in Total Ion Chromatogram (TIC) when analysing the dialysate and residue fractions (Figure 4, 5, respectively), other chromatographic signals at higher retention times than 11.7 min are observed. Intensities higher than 1.010^3 units in some extracted chromatograms at selected m/z ratios were found and for the m/z ratio of 257.0737 (retention time of 12.22 min in the dialysate and 12.23 in the residue, Figure 4, and %, respectively) the molecular formula $C_{15}H_{13}O_4$ of the proposed structure *5* (Figure 2 and Figure 3(c)) matches properly (mass error lower than 2 ppm). A second extracted chromatogram at a m/z ratio of 273.0757 (molecular formula of $C_{15}H_{12}O_5$, proposed structure *6* in Figure 2) was found in the dialysate but the intensity of the signal was lower than 1.010^3 units and confident conclusion could not be attained. Similarly, low intense extracted chromatograms when analysing the residue fraction were obtained at the m/z ratios of 328.0657 ($C_{17}H_{12}O_7$), 298.0835 ($C_{17}H_{14}O_5$), 288.0706 ($C_{15}H_{12}O_6$), and 247.0600 ($C_{13}H_{10}O_5$), which are related to proposed degradation products coded as *1*, *2*, *3*, and *4* in Figure 2, respectively. However, the low intensity of the signals has not allowed conclusive identifications/structure proposals.

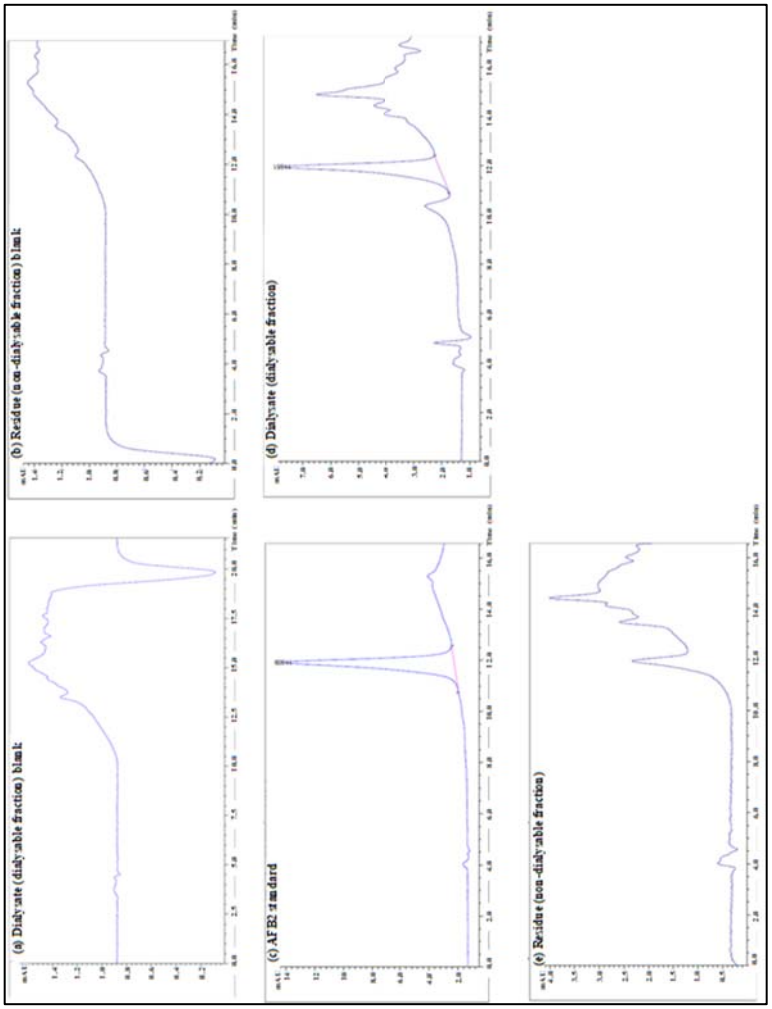

Figure 1: HPLC-UV chromatograms (a) dialysate blank, (b) residue blank, (c) AFB2 standard (10 mg L^{-1}), (d) dialysate from AFB2 standard (10 mg L^{-1}), (e) residue from AFB2 standard (10 mg L^{-1})

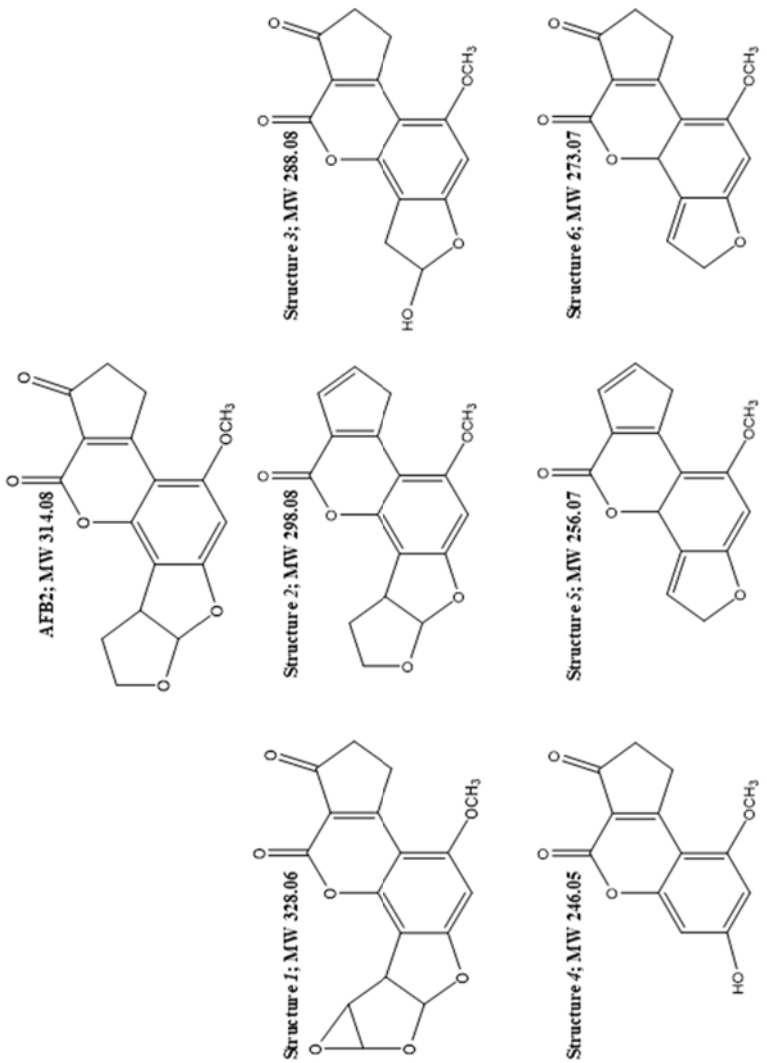

Figure 2: Structures of possible transformation products from AFB2

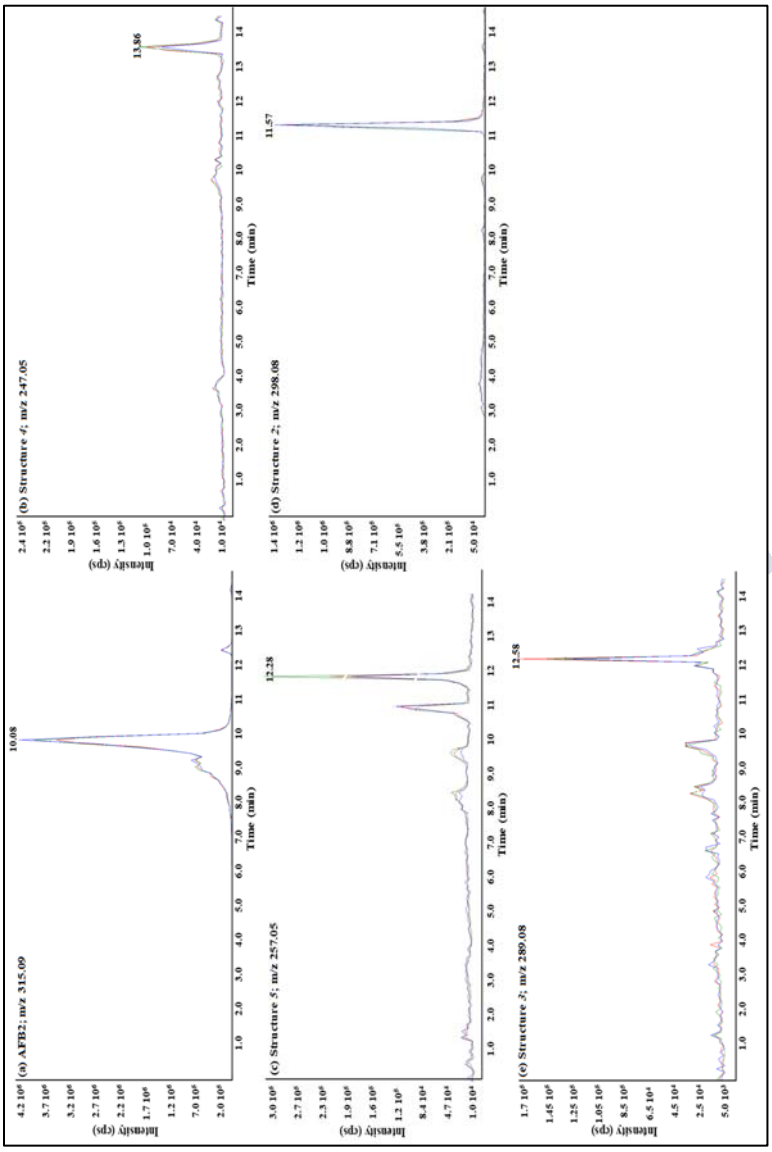

Figure 3: Extracted HPLC-MS chromatograms for dialysable AFB2 (a), non-dialysable degradation products (b, d, and e), and dialysable degradation products (c) from AFB2 standard (200 µg L^{-1})

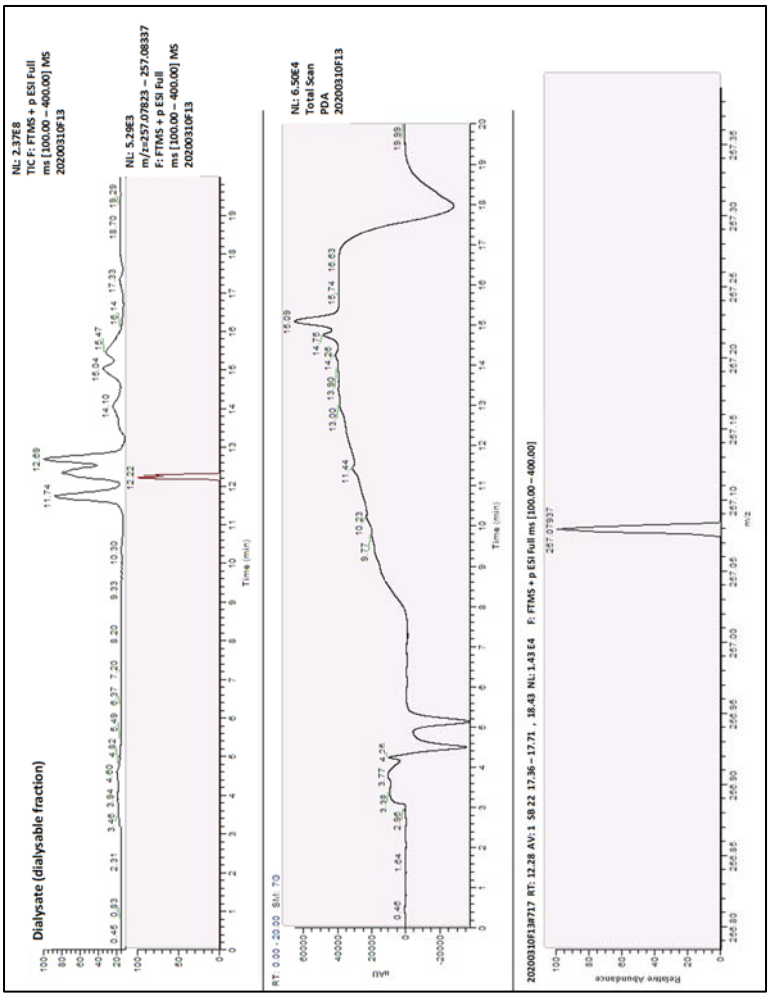

Figure 4: Total ion and extracted HPLC-HRMS chromatograms, UV chromatogram, and ESI full scan mass spectra for a transformation product from AFB2 standard (200 µg L^{-1}) in the dialysable fraction.

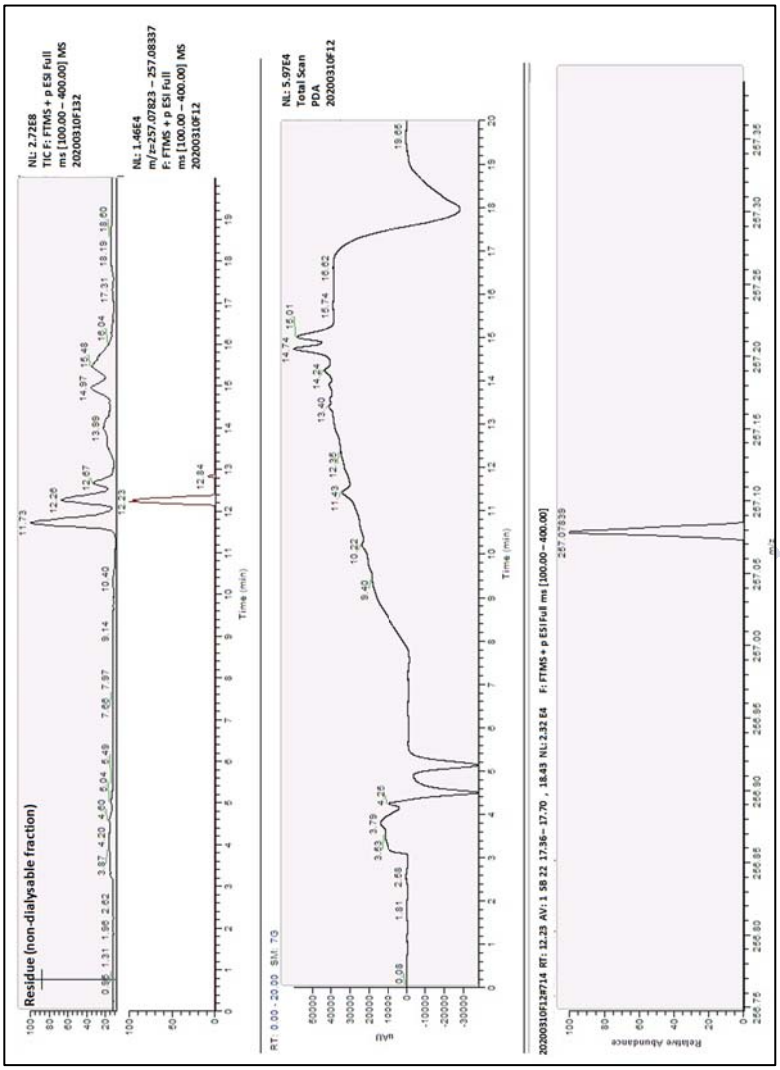

Figure 5: Total ion and extracted HPLC-HRMS chromatograms, UV chromatogram, and ESI full scan mass spectra for a transformation product from AFB2 standard (200 µg L^{-1}) in the non-dialysable fraction

Therefore, AFB2 is degraded during *in vitro* dialysability process and several degradation products are present in both dialysable and non-dialysable fractions. These finding explains the lower dialysability of AFB2 than those assessed for other AFs, and also explains a poor mass balance. A degradation product of exact mass of 256.0657 and tentative molecular formula of $C_{15}H_{13}O_4$ and molecular structure given in Figure 2 (structure coded as 5) was found. This species is partially dialysable since it is present in both dialysable and non-dialysable fractions.

5.4 CONCLUSION

The dialysability approach have been novelty applied for studying AFs bioavailability in cultured fish and chicken and rabbit liver. Dialysability approaches for assessing bioavailability of AFs have led to moderate AF bioavailability ratios which were half lower than reported estimations based on bio-accessibility assays. The use of a dialysis membrane during the simulated intestinal digestion stage for mimicking intestinal absorption led to lower, but more realistic, bioavailability ratios. AFs content was significantly different ($p<0.05$) in cooked samples than in raw samples because of water losses during the cooking process. In addition, AFs bioavailability has been found to be affected by the culinary process, and AFs bioavailability was slightly higher after frying, whereas steaming cooking led to similar AF bioavailability than raw samples. A mass balance study performed for AFs aqueous standards and food samples showed good accuracy of the dialysability approach for all AFs, except for AFB2. Lack of accuracy for AFB2 was not attributed to an inefficient pre-concentration/ isolation from the dialysate and residue fractions by VALLME since the results obtained were the same when changing parameters such as the extractant volume (chloroform) and the AFB2 concentration (dialysate/residue solution volume). Lack of accuracy for AFB2 was found to be attributed to AFB2 transformation during the *in vitro* dialysability process. Despite the several possible transformation products (mainly in the non-dialysable fraction) observed by HPLC-UV and UHPLC-MS, only one possible degradation compound formed by dihydrofuran residue loss from the AFB2 molecule was proved by HPLC-HRMS. This degradation product was found to be partially

dialysable; therefore, although AFB2 bioavailability (28-38%) was found to be lower than other AFs (41-59%), the health risk of AFB2 is similar to the other AFs since the main degradation product from AFB2 was also bioavailable. Toxicity evaluation of the transformation products from AFB2, as well as a better characterization of these compounds, require therefore further research.

Acknowledgment
This work was supported by the *Xunta de Galicia* (*Grupo de Referencia Competitiva*, grant number ED431C2018/19; and *Program for Development of a Strategic Grouping in Materials – AEMAT*, grant number ED431E2018/08). The authors are grateful to Dr. Gerardo Fernández-Martínez (Chromatographic Techniques Unit – Scientific Research Support Services at the University of A Coruña) for HPLC–HRMS technical support.

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IV. CONCLUSIONS

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IV.1 STUDY OF AFS IN FISH FEED

The first steps of the thesis have been focused on developing sample pre-treatments and analytical methods for aflatoxins (AFs) assessment in fish feed. Two analytical methods have been optimized: the first approach was based on molecularly imprinted polymers (MIPs) – Mn-doped ZnS QDs – room temperature phosphorescence (RTP) nanosensor for a low-cost screening of AFs; and the second approach was based on molecularly imprinted polymers micro-solid-phase extraction, (MIMSPE) and ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) as sample pre-treatment/pre-concentration method and analytical technique, respectively.

IV.1.1 Room temperature phosphorescent determination of aflatoxins in fish feed based on molecularly imprinted polymer - Mn-doped ZnS quantum dots.

Due to the luminescence properties of QDs, simple and inexpensive screening methods based on fluorescence and phosphorescence measurements can be developed. The main advantage of the synthetized MIP-PEG-Mn-doped ZnS QDs composite is the enhanced selectivity since RTP quenching is only observed when AFs interacts with the recognition cavities in the MIP layer over the PEG-Mn-doped ZnS QDs nanoparticles. Moreover, other outstanding advantages referrer to nanomaterial stability, and low-cost preparation.

RTP measurements offers higher sensitive determination than those offered by fluorescence-based techniques, and scattering phenomena is avoided. Based on these advantages, the proposed technique was successfully applied to AFs determination in fish feed at very low concentrations, and the proposed method has also high potential for other foodstuff and biological samples after an adequate AFs extraction method. Finally, we can conclude that the proposed RTP method can be used as a highly sensitive, selective and low-cost (worldwide applicability) method for the determination of AFs in fish feed.

IV.1.2 Ultrasound-assisted combined molecularly imprinted polymer for the selective micro-solid phase extraction and determination of aflatoxins in fish feed using liquid chromatography-tandem mass spectrometry

A combined procedure based on using ultrasounds for target isolation followed by porous membrane-protected micro solid-phase extraction using a molecularly imprinted polymer (MIP) as an adsorbent has been developed as a highly selective pre-concentration and clean-up procedure for AFs before UHPLC-MS/MS.

The MIP beads were synthetized by using the precipitation polymerization method and the MIP-μ-SPE device consisted of a cone-shape bag made of polypropylene (PP) containing the synthetized MIP beads inside. The proposed method was found to offer good clean-up efficiency since the PP membrane acts as a barrier and avoids the interaction of large biomolecules in the sample extract with the MIP's particles. In addition to the high selectivity and pre-concentration capacities, each MIP-μ-SPE device can be re-used at least 20 times which is also an important practical advantage over commercially available single-use SPE cartridges. The proposed material can be useful for AFs enrichment from foodstuff after target extraction. The current study was shown to be an accurate and precise sensitive, highly selective, and cost-effective method for the determination of AFs in the fish feed.

IV.2 STUDY OF AFS IN CULTURED FISH

The second part of the Thesis has been devoted to sample pre-treatment/pre-concentration methods for AFs assessment in cultured fish. Two different methods, one of them based on MIPs (vortex assisted molecularly imprinted polymer dispersive micro-solid phase extraction, D-μ-MISPE), and a second consisting of a solvent based technique (vortex assisted dispersive liquid-liquid microextraction, VALLME) have been developed.

IV.2.1 Vortex assisted- dispersive molecularly imprinted polymer micro-solid phase extraction for aflatoxins isolation from cultured fish before UHPLC-MS-MS assessment

MIPs have been novelty used as adsorbents for vortex assisted D-μ-SPE (D-μ-MISPE) pre-concentration of AFs from fish (flesh and liver) extracts. The absorbent (20 mg of the synthesized MIP) was found to be reusable at least ten to twelve time (ten to twelve successive loading/eluting cycles) and the pre-concentration method was highly robust since the pre-concentration factor can be easily increased by loading larger volumes of extracts without losing the analytical performances (precision and analytical recovery) Vortex stirring for assisting absorbent dispersion guarantees target integrity and allows the advantage of the low-cost device. The proposed D-μ-MISPE is an appealing method for assessing very low concentrations of AFs in complex matrices derived from fishery products.

Moreover, AFs levels found in the analysed samples were quite low, but their presence demonstrates a direct relationship with the presence of AFs in the fish feeds used in aquaculture facilities.

IV.2.2 Combining ultrasound-assisted extraction and vortex-assisted liquid-liquid microextraction for the sensitive assessment of aflatoxins in aquaculture fish species

The main advantage of this method leads to a fast extracting solvent dispersion and a fast mass transfer from the aqueous phase (fish extract) to the organic extractant. Besides, vortex stirring leads to more repeatable extractions when compared to DLLME methods assisted by ultrasound. Through this method, ultrasound-assisted extraction was proposed for AFs isolation from fish muscle and liver, and vortex assisted was proposed for target extraction from sample extract to organic extractant.

The combination of ultrasound assisted extraction (UAE) for AFs isolation from fish matrices and VALLME has demonstrated to be a simple, quick, and quite repeatable method for AFS extraction/pre-concentration, and the use of UHPLC-MS/MS has led to highly precise, accurate, and sensitive method for AFs assessment in the fish flesh/liver. The developed method is therefore a potential strategy for

AFs monitoring in aquaculture products, and it was found to be adequate and robust for AFs pre-concentration from dialysates (PIPES extracts) obtained after *in vitro* dialysability procedures.

IV.3 STUDY OF THE BIOAVAILABILITY OF AFS IN CULTURED FISH AND ANIMAL LIVER USING AN IN-VITRO DIALYSABILITY APPROACH

After performing *in vitro* dialysability approach for human bioavailability of AFs from fish, the VALLME technique was found to offer the best performances to pre-concentrate trace amounts of AFs in dialysable (dialysate) and non-dialysable (residue) fractions. Bioavailability ratios of AFs were found to be moderate (from 28 to 45%) and the culinary (frying and steaming) process was found to affect differently the AFs dialysability (steaming cooking does not affect the AFs bioavailability, but frying increases the AFs bioavailability).

Good mass balance of the over-all *in vitro* dialysability process was obtained for AFB1, AFG1, and AFG2 (the AFs contents as a sum of the AFs concentrations in the dialyzable and non-dialyzable fractions were not significantly different than the AFs contents in the raw/cocked food). However, an unsuccessful mass balance was obtained for AFB2, which means that AFB2 transformation/degradation occurs during the *in vitro* dialysability process. Despite the several possible transformation products found in the dialysates and residues, only one possible degradation compound formed by dihydrofuran residue loss from the AFB2 was proved by HPLC-HRMS.

There is no idea about the toxicity of the new transformation products and further studies will be highly required for toxicity evaluation of the transformation products from AFB2 as well as a better characterization of these compounds.

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V. ANNEX I

RESUMEN Y DISCUSIÓN

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Los mohos del género *Aspergillus* son la causa más importante del deterioro de alimentos y piensos ya que pueden producir en condiciones ambientales adecuadas toxinas como metabolitos secundarios. Las aflatoxinas (AFs) son un tipo de estas micotoxinas biosintetizadas por vía policétida, y químicamente se pueden considerar derivados de la furanocumarina. Las AFs fueron encontradas por primera vez en 1960 tras la enfermedad y posterior muerte generalizada de pavos debido a piensos contaminados procedentes de Brasil. Principalmente, las AFs son generadas por dos especies principales de *Aspergillus* como son el *A. flavus* y el *A. parasiticus*. Estos mohos producen más de catorce AFs distintas, pero cuatro de ellas (AFB1, AFB2, AFG1 y AFG2) son las más preocupantes debido a su alta toxicidad. Otras AFs como las AFM1 y AFM2 son los metabolitos de las AFs AFB1 y AFB2, y se encuentran principalmente en productos como la leche. Las AFs han demostrado ser potentes agentes carcinógenos (especialmente en el hígado), mutagénicos, neurotóxicos e inmunosupresores. Por lo tanto, la Agencia Internacional para la Investigación del Cáncer (*International Agency for Research on Cancer*, IARC) clasifica estas sustancias como carcinógenos del grupo 1A. La aparición y producción de AFs depende de las condiciones geográficas y climáticas, tales como la humedad, la temperatura, el pH del suelo, las fuentes de nutrición en el suelo, la radiación solar, el tipo de cultivo, la edad del cultivo, el transporte y condiciones de almacenamiento del producto.

La contaminación por AFs representa un problema importante no solo en los alimentos para el Hombre sino también en los alimentos destinados a animales. En relación a estos últimos, podemos distinguir entre alimentos de origen animal (por ejemplo, harina de pescado) y alimentos de origen vegetal. Los alimentos de origen vegetal son los más comunes en nutrición animal y se basan en el maíz, la cebada, las semillas de algodón, el trigo y la avena. Los factores que promueven la formación de AFs en el alimento dependen del tipo de alimento y la calidad de las condiciones de almacenamiento del mismo. Además, la contaminación puede ocurrir durante la etapa previa o posterior a la cosecha de los ingredientes (secado inadecuado del grano), a la etapa de procesamiento de los productos, a las malas condiciones de almacenamiento, al ataque de insectos, entre otras.

Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (*Food and Agriculture Organization*, FAO), la producción acuícola mundial ha registrado una alta producción anual desde 2000, y el volumen de productos de la acuicultura supera al de la pesca en muchos países. De esta forma, la acuicultura representa actualmente un segmento de rápido crecimiento de la agricultura y la industria de fabricación de piensos, particularmente en China continental y en otros países asiáticos con el 90% de la producción mundial. Debido al origen vegetal de muchos piensos para acuicultura, las AFs pueden estar presentes en los mismos y suponer un riesgo de salud pública. El empleo de piensos contaminados con AFs en las actividades acuícolas produce efectos adversos para la salud de los peces, así como una deficiente tasa de crecimiento y lesiones graves y microscópicas en los tejidos. Esto conduce a pérdidas económicas importantes debido a la baja producción, morbilidad, mortalidad, y baja calidad de pescado y de sus derivados. Además, la exposición crónica a través de una alimentación prolongada con bajas concentraciones de AFs causa tumores en el hígado y en el riñón de los peces.

El Hombre puede estar expuesto a concentraciones variables de AFs debido al consumo de productos acuícolas contaminados. La Comisión Europea ha establecido el límite máximo de AFB1 en 20 μg kg^{-1} para todos los alimentos y piensos. Por lo tanto, el desarrollo de métodos analíticos rápidos, altamente selectivos y sensibles para determinar AFs en alimentos, y en concreto en productos de la acuicultura, es de especial importancia. Por otra parte, es también necesario conocer la fracción biodisponible de estos contaminantes. El objetivo del presente trabajo ha sido explorar las posibilidades de usar diferentes tecnologías de extracción y preconcentración como herramientas avanzadas para la determinación de AFs en piensos para pescado y en pescado de acuicultura, así como en conocer la fracción biodisponible de AFs tras el consumo de productos acuícolas.

En general, los métodos de cribado (*screening*) tienen como característica principal el de requerir un mínimo tratamiento de la muestra, el de aportar información analítica cualitativa o de grupo de especies (por ejemplo, AFs totales), el ser rápidos, y el de ofrecer una información para la toma de decisiones inmediata pudiendo requerir

una posterior confirmación con otra metodología. Además, este tipo de métodos permite determinaciones empleando instrumentos de bajo coste y moderado mantenimiento. Ejemplo de métodos de cribado son los inmunoensayos y los sensores químicos como los compositos basados en polímeros de impronta molecular (*molecularly imprinted polymer*, MIP) y nanopartículas luminiscentes como los quantum dots (QDs). En este sentido, se ha explorado de manera novedosa el potencial de un composito MIP@Mn-ZnS QDs luminiscente para la determinación conjunta de AFs (AFB1+AFB2+AFG1+AFG2) en piensos por espectrometría de fosforescencia a temperatura ambiente (*room temperature phosphorescence*, RTP). Existen algunos estudios basados en el análisis de fluorescencia de CdTe-QDs para AFB1 como métodos simples, rápidos y de bajo coste. Desafortunadamente, esos enfoques de detección directa son altamente sensibles, pero carecen de suficiente selectividad. A través de este estudio, se mejoró la selectividad al cubrir los QDs con una capa de MIP, el cual ofrece las cavidades de reconocimiento selectivas a las AFs. Por otra parte, al llevar a cabo las determinaciones por RTP en lugar de por fluorescencia, se consigue una mayor sensibilidad en las determinaciones. Los estudios de precisión y exactitud a través de un material de referencia certificado (ERM-BE-376) ofrecieron resultados de alta calidad. Los resultados obtenidos con esta metodología indican que el nanosensor RTP desarrollado se puede utilizar para el cribado de AFs en piensos, siendo una herramienta versátil, de excelente sensibilidad y selectividad, fácil preparación y bajo coste.

La técnica de extracción en fase sólida (*solid phase extraction*, SPE) se usa comúnmente para fines de limpieza de extractos y preconcentración de cantidades traza de analito, y las tendencias recientes se centran en miniaturizar el proceso de extracción (*micro-solid phase extraction*, μ-SPE). En una de las modalidades de este grupo de técnicas, el absorbente se encierra en el interior de una membrana de polipropileno (PP) la cual permite la difusión de analitos libremente a su través para absorberse en una pequeña cantidad de absorbente en el interior de la membrana). Empleando esta técnica, se ha propuesto el empleo de MIP específico a AFs como absorbente en μ-SPE para mejorar la selectividad y permitir altos factores de

preconcentración. El procedimiento desarrollado MIP-μ-SPE (*molecularly imprinted micro-solid phase extraction*, MIMSPE) se combinó con una técnica auxiliar de ultrasonido para aumentar la extracción previa de las AFs en muestras de piensos. Las partículas poliméricas se sintetizaron mediante un método de polimerización por precipitación que garantiza una distribución homogénea del tamaño de partículas y la integridad de las cavidades de impronta generadas. El procedimiento de extracción asistido por ultrasonidos y preconcentración MIMSPE ha resultado ser una combinación ventajosa para minimizar el efecto de matriz, problema inherente a otros procedimientos de preconcentración basados en SPE. La elevada eficiencia de limpieza se atribuye principalmente a la alta selectividad del absorbente y también al efecto de la membrana de PP que actúa como una barrera a moléculas de alto peso molecular. La reutilización de los dispositivos MIMSPE es otra de las ventajas del procedimiento, encontrándose que los dispositivos desarrollados pueden reutilizarse al menos veinte veces sin pérdidas de las capacidades de retención del material (los cartuchos SPE comerciales son dispositivos de un solo uso). Por otra parte, la metodología desarrollada en combinación con la cromatografía líquida de alta resolución con espectrometría de masas en tándem (*ultrahigh performance liquid chromatography – tandem mass spectrometry*, UHPLC-MS/MS) ofrece buenos parámetros analíticos de precisión y exactitud, así como excelente sensibilidad para cuantificar AFs piensos para la acuicultura.

De manera similar, el polímero sintetizado se usó como absorbente en una nueva SPE miniaturizada como es la microextracción dispersiva en fase sólida (*dispersive micro-solid phase extraction*, D-μ-SPE) combinada con la agitación en vórtex para asistir la dispersión y acelerar los procedimientos de carga/elución. Esta metodología de pretratamiento empleando el MIP sintetizado como absorbente (*molecularly imprinted polymer - dispersive micro solid phase extraction*, D-μ-MISPE) se aplicó a la preconcentración de AFs en productos de la acuicultura. La agitación mecánica por vórtex es una técnica de agitación suave que facilita la transferencia de masa desde el extracto a la fase absorbente. En comparación con los ultrasonidos, técnica también empleada para facilitar la dispersión en los

procedimientos D-μ-SPE, la agitación por vórtex es más homogénea que la agitación ultrasónica, principalmente en los baños de ultrasonidos donde la irradiación con ultrasonido no es igual en todas las posiciones del tanque ni a las distintas alturas o profundidad de la inmersión de los viales. Por otra parte, la alta energía de las sondas de ultrasonidos puede degradar los analitos y el absorbente durante el proceso. Finalmente, la técnica asistida por vórtex es simple, y el dispositivo es de alta disponibilidad y el bajo precio sen comparación con las técnicas asistida por ultrasonido. Al igual que en el procedimiento MIMSPE, la metodología D-μ-MISPE también permite la reutilización de la pequeña cantidad de absorbente empleada en el proceso de preconcentración y la mayor miniaturización del sistema permite conseguir mayores factores de enriquecimiento (hasta 50 sin pérdida de las capacidades de absorción). Este factor es muy importante ya que los niveles de AFs en la carne de pescado es extraordinariamente baja. Los parámetros de validación tras UHPLC-MS/MS, tales como los bajos límites de detección y cuantificación, alta precisión y exactitud, alta selectividad y reutilización hacen que el método propuesto sea una metodología de alto rendimiento para la preconcentración de AFs en extractos de pescados.

El uso de grandes volúmenes de disolventes es la principal desventaja de los métodos convencionales de pretratamiento de muestras cuando se evalúan analitos a nivel ultratraza. Las medidas encaminadas a la protección del medioambiente hacen que en el laboratorio analítico se evite o se minimice el volumen de reactivos tóxicos y disolventes, miniaturizando y automatizando el proceso de pretratamiento de la muestra que implique disolventes. Como resultado, se han desarrollado varias técnicas de microextracción para reemplazar el método de extracción líquido-líquido (*liquid-liquid extraction*, LLE). Entre las técnicas de microextracción, la microextracción dispersiva líquido-líquido (*dispersive liquid-liquid microextraction*, DLLME) ha surgido como un procedimiento rápido y de bajo costo para la preconcentración de trazas y la limpieza de extractos. La técnica DLLME se ha aplicado para extraer una amplia gama de compuestos orgánicos e inorgánicos en varias matrices Los investigadores han propuesto varios mecanismos para asistir (acelerar) el proceso

extractivo, tales como los ultrasonidos y las microondas, y se ha propuesto igualmente el empleo de la agitación vórtex (*vortex assisted liquid-liquid microextraction*, VALLME) para facilitar la dispersión. La metodología VALLME se adecuó para la preconcentración de AFs en extractos de pescado (carne e hígado), y el método desarrollado demostró ser una forma simple y sensible de llevar a cabo el enriquecimiento de las AFs previa a su etapa de determinación por UHPLC-MS/MS. Las principales ventajas del método fueron su bajo coste, simplicidad de operación, bajo uso de disolventes orgánicos y bajos residuos. En condiciones óptimas, el método desarrollado presentó características de validación satisfactorias (linealidad, sensibilidad, recuperación analítica y precisión) y se aplicó con éxito al análisis de carne e hígado de pescado.

Se ha investigado la biodisponibilidad humana de las AFs en muestras de pescados, hígado de pescado, e hígado de conejo y pollo utilizando un procedimiento de dializabilidad *in vitro*. Existe muy poca información acerca de la biodisponibilidad humana de estos contaminantes y la escasa bibliografía se refiere a procedimientos *in vitro* de bioaccesibilidad, los cuales no tienen en cuenta la etapa de absorción intestinal en el procedimiento simulado. Los porcentajes de biodisponibilidad (dializabilidad) se establecieron tras la determinación del contenido de AFs en el dializado (fracción biodisponible) y en el contenido total de las mismas en el alimento. La técnica de preconcentración que ofreció las mejores prestaciones fue la metodología VALLME. El procedimiento D-μ-MISPE resultó ser también adecuado para la preconcentración de AFs en el dializado (valores cuantitativos de las AFs), pero sin embargo el procedimiento de preconcentración MIMSPE no ofreció resultados cuantitativos, incluso incrementando la cantidad de MIP en el interior de la membrana. La explicación de la escasa aplicabilidad de los dispositivos MIMSPE puede deberse a que las AFs estén asociadas a los restos de enzimas empleadas la digestión *in vitro*, y éstas no puedan atravesar los poros de la membrana de PP.

La biodisponibilidad se evaluó en la muestra en crudo, y también tras aplicar dos procedimientos culinarios diferentes (cocinado al vapor y a la plancha). El estudio del balance de masa se realizó para evaluar

la precisión del método y se evaluó comparando estadísticamente la suma de la concentración de AF en la fracción dializable y en el residuo (fracción no dializable) con la concentración de AFs en las muestras. Se ha encontrado un excelente balance de masas para AFB1, AFG1 y AFG2 (concentraciones estadísticamente comparables a un nivel de significación del 95%). Sin embargo, el balance de masas no fue adecuado para AFB2, lo que implica posibles transformaciones de AFB2 durante el procedimiento *in vitro*. Se llevaron así estudios de caracterización de los posibles productos de transformación empleando HPLC-UV, UHPLC-MS y HPLC-HRMS, pudiendo proponer al menos un producto de degradación cuya masa exacta fue confirmada por HRMS.

En general, las muestras de pescado cocidas mostraron una biodisponibilidad similar a las muestras crudas; sin embargo, las diferencias fueron significativas con las muestras sometidas a fritura. La biodisponibilidad de AFs en peces e hígado de animales (pollo y conejo) basada en un procedimiento de dializabilidad *in vitro* es menor que la encontrada en la bibliografía para cereales empleando el método de la bioaccesibilidad. La diferencia se debe fundamentalmente a la etapa simulada de absorción intestinal, la cual supone una barrera y no es simulada en los procedimientos de bioaccesibilidad. En general, debido a la baja biodisponibilidad y a los bajos niveles de AFs en productos de la acuicultura se puede considerar éstos como seguros para la salud de los seres humanos.

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VI. ANNEX II

LIST OF PUBLICATIONS

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List of publications

1. **Jayasinghe G.D.T.M**, R. Domínguez González, Pilar Bermejo Barrera, Antonio Moreda Piñeiro, Combining ultrasounds assisted extraction and vortex assisted liquid-liquid microextraction for the sensitive assessment of aflatoxins in aquaculture fish species, Journal of Separation Science 43 (2020) 1331–1338.
2. **Jayasinghe G.D.T.M**, R. Domínguez González, Pilar Bermejo Barrera, Antonio Moreda Piñeiro, Room temperature phosphorescent determination of aflatoxins in fish feed based on molecularly imprinted polymer-Mn-doped ZnS quantum dots, Analytica Chimica Acta 1103 (2020) 183-191.
3. **Jayasinghe G.D.T.M**, R. Domínguez González, Pilar Bermejo Barrera, Antonio Moreda Piñeiro, Ultrasound assisted combined molecularly imprinted polymer for the selective micro-solid phase extraction and determination of aflatoxins in fish feed using liquid chromatography-tandem mass spectrometry, Journal of Chromatography A, 1609 (2020) 460431.

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