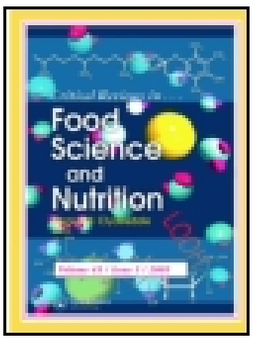

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Solid phase extraction as sample treatment for the determination of Ochratoxin A in foods: a review

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by two main types of fungi, *Aspergillus* and *Penicillium* species. OTA is a natural contaminant found in a large number of different matrices and it is considered as possible carcinogen for humans. So, low maximum permitted levels in foods have been established by competent authorities around the world, making essential the use of very sensitive analytical methods for OTA detection. Sample treatment is a crucial step of the analytical methodology to get clean and concentrated extracts, and therefore low limits of quantification.

Solid phase extraction (SPE) is a useful technique for rapid and selective sample preparation. This sample treatment enables the concentration and purification of analytes from the sample solution or extract by sorption on a solid sorbent. This review is focused on sample treatment procedures based on SPE prior to the determination of OTA in food matrices, published from 2010.

Keywords

Ochratoxin A, sample treatment, solid phase extraction, food

Abbreviations list

AFs: Aflatoxins

AFB_1: Aflatoxin B_1

AFB_2: Aflatoxin B_2

AFG_1: Aflatoxin G_1

AFG_2: Aflatoxin G_2

AFM_1: Aflatoxin M_1

AFM_2: Aflatoxin M_2

CE: Capillary electrophoresis

CCα: Decision limit

CCβ: Detection capability

DAD: Diode array detection

DLLME: Dispersive liquid-liquid microextraction

DON: Deoxynivalenol

DSPE: Dispersive solid phase extraction

EC: European Commission

ELISA: Enzyme-linked immunosorbent assay

FB_1: Fumonisin B_1

FB_2: Fumonisin B_2

FB_3: Fumonisin B_3

FLD: Fluorescence detection

FP: Fluorescence polarization

GC: Gas chromatography

GCB: Graphite carbon black

HLB: Hydrophilic-lipophilic balance

HPLC: High performance liquid chromatography

HT-2: HT-2 toxin

IAC: Immunoaffinity column

IARC: International Agency for Research on Cancer

ICH: International Conference on Harmonization

LC: Liquid chromatography

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

MAX: Mixed-mode/anion-exchange

MCX: Mixed-mode/cationic-exchange

MeCN: Acetonitrile

MeOH: Methanol

MeOTA: Methyl ochratoxin A

MIP: Molecularly imprinted polymer

MISPE: Molecularly imprinted solid phase extraction

MS/MS: Tandem mass spectrometry

NIP: Non-imprinted polymer

NIV: Nivalenol

OPA: O-phthalaldeyde

OS: Oligosorbents

OTA: Ochratoxin A

OTB: Ochratoxin B

OTC: Ochratoxin C

PAT: Patulin

PBS: Phosphate buffered saline

PCD: Photochemical derivatization

PLE: Pressurized liquid extraction

PSA: Primary-secondary amine

QTHQ: Ochratoxin hydroxyquinone

RSD: Relative standard deviation

SBME: Solid bar microextraction

SLE: Solid-liquid extraction

SPE: Solid phase extraction

SPME: Solid phase microextraction

TLC: Thin layer chromatography

TOF: Time of flight

TRF: Time-resolved fluorescence

T-2: T-2 toxin

UAE: Ultrasound assisted extraction

UHPLC: Ultra-high performance liquid chromatography

WAX: Weak anion-exchange

ZEA: Zearalenone

μ-SPE: Micro-solid phase extraction

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several *Aspergillus (A. ochraceus*, *A. niger*, *A. carbonarius*, *A. terreus)* and *Penicillium* species (*P. verrucosum*, *P. nordicum*). OTA is a pentaketide derived from the dihydrocoumarin family coupled to β-phenylalanine (Figure 1). The empirical formula of OTA is $C_{20}H_{18}O_6NCl$ with a molar mass of 403.8 g/mol and its chemical name is L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl)carbonyl] -(R)-isocoumarin. It is a white crystalline compound, highly soluble in polar organic solvents, slightly soluble in water and soluble in aqueous sodium hydrogen carbonate. The pKa values are in the ranges 4.2--4.4 and 7.0--7.3 (Anli & Alkis, 2010).

OTA has potent nephrotoxic, hepatotoxic, neurotoxic and teratogenic immunotoxic effects on several species of animals (Malir et al., 2014), and therefore the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen in group 2B (International Agency for Research on Cancer, 1993).

The occurrence of OTA in food and feed has been reported world-wide. Cereals and wine are considered one of the major food commodities susceptible to OTA contamination (Anli & Bayram 2009), however it also contaminates coffee, cocoa, beans, species or dried fruit (Wolff et al., 2000). OTA exhibits resistance to acidity and high temperatures, and because of its stability it is hardly impossible to remove it from contaminated foodstuffs (Khoury & Atoui, 2010).

In view of the foregoing, European Commission (EC) laid down maximum levels of OTA for cereal and cereal products, dried vine fruit, coffee, wine, grape juice, processed cereal based foods and baby foods for infants and young children, dietary foods for special medical purposes specifically for infants, spices and liquorice through Commission Regulation (EC) No 1881/2006

(European Commission, 2006), and subsequent amendments (Commission Regulation 105/2010 (European Commission, 2010) Commission Regulation 594/2012 (European Commission, 2012), Commission Regulation 2015/1137 (European Commission, 2015)). These maximum permitted levels are showed in Table 1. Also, provisions for methods of sampling and analysis for the official control of OTA are laid down in Commission Regulation (EC) No 401/2006 (European Commission, 2006), amended by Commission Regulation No 178/2010 (European Commission, 2010).

The low maximum levels established by legislation, ranging between 0.5 and 80 μg/kg, make necessary the use of very sensitive analytical methods for OTA quantification. OTA is a natively fluorescent molecule, thus conventionally sensitive methods for OTA determination are based on high performance liquid chromatography (HPLC) coupled to fluorescence detection (FLD) (Sáez et al., 2004; González-Osnaya et al., 2008; Tessini et al., 2010; Piccinelli et al., 2012; Remiro et al., 2010), including standardized method for the determination of OTA in different food commodities (European Committee for Standardization, 2009a; European Committee for Standardization, 2009b; European Committee for Standardization, 2010a, European Committee for Standardization, 2010b). Alternative detection methods, such as diode array detection (DAD) (Csutorás et al., 2013) or tandem mass spectrometry (MS/MS) (Campone et al., 2011) have also been proposed. Other less commonly applied techniques are thin layer chromatography (TLC) (Welke et al., 2010; Teixeira et al., 2011), capillary electrophoresis (CE) with UV/Vis detection (Almeda et al., 2008; González-Peñas et al., 2006), gas chromatography (GC) (Amelin et al., 2013), enzyme-linked immunosorbent assay (ELISA) (Anfossi et al., 2011; Zhang et al., 2011),

luminescence immunoassay (Hagan & Zuchner, 2011; Li et al., 2011) or tests based on colorimetric detection (Bazin et al., 2010).

Regardless the analytical technique used for the determination of OTA, sample treatment, including extraction and clean-up, is a crucial step in order to obtain enough cleaned and concentrated extracts to comply with the requirements of the current legislation. Several methods have been reported for the extraction of OTA from different food matrices, such as liquid-liquid extraction (LLE) (González-Osnaya et al., 2008), immune-ultrafiltration in cereal samples (Reiter et al., 2011), or pressurized liquid extraction (PLE) from cereals and derived products (Juan et al., 2005; González-Osnaya et al., 2006; Zinedine et al., 2010), animal derived food (Chen et al., 2012) or dried fruits (Campone et al., 2015). Recently, other emerging sample treatments have been proposed for the extraction of OTA, such as dispersive liquid-liquid microextraction (DLLME) in wine (Campone et al., 2011; Arroyo-Manzanares 2012) or cereals (Campone et al., 2012). Also, the so-called QuEChERS procedure (including a first liquid extraction/partitioning and a subsequent clean-up by dispersive solid phase extraction, DSPE) is being increasingly applied for the extraction of mycotoxins. Different QuEChERS-based extraction methods have been recently reported for the determination of OTA and other mycotoxins in cereal products (Desmarchelier et al., 2010; Arroyo-Manzanares et al., 2014), bread (Paíga et al., 2012), eggs (Garrido-Frenich et al., 2011), syrups (Arroyo-Manzanares et al., 2015), coffee (Nielsen et al., 2015), wine (Pizzutti et al., 2015), beer-based drinks (Tamura et al., 2011), human breast milk (Rubert et al., 2014), spices (Yogendrarajah et al., 2013) and other foods (Amelin et al., 2013). However, solid phase extraction (SPE), alone or combined with a previous extraction method, is by far the most popular technique used in routine mycotoxin

analysis for clean-up and pre-concentration of extracts (Turner et al., 2009; Cigic et al., 2009). Different sorbents, such as immunoaffinity columns (IACs), molecularly imprinted polymers (MIPs), oligosorbents (OS) or non-specific stationary phases, can be used depending on the specific application to purify OTA extracts.

Attempts have been made for comparing some of above mentioned sample treatments for determination of mycotoxins, including OTA (Sáez et al., 2004; Desmarchelier et al., 2010; Fabiani et al., 2010; Arroyo-Manzanares et al., 2011; Tozlovanu & Pfohl-Leszkowicz, 2010; Prelle et al., 2013).

The present review, gives an overview of existing analytical methodologies for sample preparation based on SPE before OTA determination in different food and beverages commodities, summarizing recent publications from 2010 to date on this interesting topic.

SOLID PHASE EXTRACTION

SPE is based on chromatographic principles and involved the use of a sorbent material ("stationary phase") usually packed in a column or supported in a disk, in which sample extract is loaded in order to trap the compounds of interest. Extract loading is usually followed by a rising step to remove contaminants, and a subsequent elution with a small volume of a different solvent to pre-concentrate the analytes of interest before instrumental analysis. This is the "capture the analyte" strategy, which is the most common SPE clean-up. Another possibility is trapping matrix contaminants, thus the extract containing the analyte of interest already cleaned is collected for analysis. This would be the "pass through" strategy, where all interfering substances are strongly retained by the stationary phase while target compounds are not retained by sorbent (Prelle et al., 2013). In this way, SPE can be used for two main purposes: i) sample

clean-up to remove matrix interferences; and ii) improving sensitivity by pre-concentration of the analytes.

SPE presents some advantages over LLE: it can be performed more rapidly, requires less solvent, and provides more concentrated extracts. Moreover, it is possible to choose among a range of sorbents that use different mechanisms for the extraction/retention of analytes, such as C8, C18, hydrophilic-lipophilic balance (HLB), mixed-mode/cationic-exchange (MCX), mixed-mode/anion-exchange (MAX) and weak anion-exchange (WAX). Recently, novel materials as sorbents in SPE have emerged as an alternative to clean-up complex matrices, trying to overcome the problems arising from complex food matrices. Some examples are MIPs, magnetic nanomaterials and carbon nanoparticles (Olariu et al., 2010; Wen et al., 2014).

Extraction and clean-up based on SPE can be applied directly to liquid samples (water, fruit juices, wine, beer, etc.) although in other cases a previous extraction by LLE with an appropriate solvent such as chloroform, acetonitrile (MeCN) or methanol (MeOH) is required. In the case of solid samples, solid-liquid extraction (SLE) is mandatory prior to the SPE step. The most used extracting solvent for vegetable samples is a mixture of either MeCN or MeOH with water, while in the case of animal origin matrices is either MeOH or chloroform. In addition to mechanical shakers more thorough SLEs have been carried out with the aid of blenders or Polytron (Ali et al., 2010; Rahmani et al., 2011; Turcotte et al., 2013), ultrasound (Jin et al., 2010; Roberts & Chang-Yen, 2014; Xue et al., 2014; Sun et al., 2015), or PLE (Juan et al., 2005; González-Osnaya et al.,2006; Zinedine et al., 2010; Chen et al., 2012; Campone et al., 2015). Other less common approaches for SLE previous to SPE, such as enzime-assisted extraction, have also been reported (Pietri et al., 2011).

Although the classical SPE is generally performed in cartridges filled with the sorbent material, in the last years some miniaturized SPE-related techniques have emerged (Costa, 2014), such as solid phase microextraction (SPME), micro-SPE (μ-SPE), solid bar microextraction (SBME) or DSPE.

The basic principle of SPME is the adsorption/desorption of analytes onto a fiber, which can be immersed either in a headspace or in a liquid matrix. SPME has been proposed for extraction of OTA from cheese (Zhang et al., 2009) and different nuts and cereal grains (Saito et al., 2012) prior to its determination by LC-MS/MS. Other approach is the so-called μ-SPE, which uses an envelope made from propylene membrane sheets with heat sealed edges packed with a proper sorbent. The extraction procedure involves the analyte adsorption by placing the μ-SPE device into the liquid sample, pH adjustment and stirring. The device is then removed and the analyte is desorbed by the addition of an adequate solvent. MIPs crushed monolith particles (Lee et al., 2012a) and Zeolite (Linde type L) (Lee et al., 2012b) have been used as specific sorbents in μ-SPE for the determination of OTA in coffee and grape fruit, and coffee and cereal samples, respectively, followed by HPLC-FLD. The advantages of these techniques are the minimized usage of solvent, simplicity, price and the high enrichment factors achieved. Other alternative, between SPME and μ-SPE, is the so-called SBME, where a few milligrams of a sorbent are wrapped in a hollow fiber micro-tube. Due to the porosity of the membrane, analytes are able to diffuse through and adsorb to the sorbent, developing the extraction in a way similar to μ-SPE. SBME was proposed for the determination of OTA in wheat and maize by HPLC-FLD (Al-Hadithi et al., 2015).

In DSPE a small amount of sorbent is added to the sample extract to remove matrix interferents. This comprises the second step of the well-known QuEChERS procedure, which has become very popular in the last years for determination of different compounds in food matrices (Rejczak & Tuzimski, 2015). QuEChERS method (including extraction and DSPE clean-up) has been proposed for determination of 14 mycotoxins (including OTA) in rice (Koesukwiwat et al., 2014) and for multi-mycotoxin determination in complex feed matrices (Dzuman et al., 2014) followed by UHPLC-MS/MS. On the other hand, novel DSPE approaches are intended to retain the analytes, instead of the interferent compounds. Through dispersion, the contact surface between sorbent and analytes is noticeably increased, resulting in higher extraction yields. Once isolated, the determination of the analytes can be carried out directly on the sorbent or in the eluate (Cruz-Vera et al., 2011). In this sense, a novel sorbent based on modified Fe_3O_4 magnetic nanoparticles has been proposed for DSPE of OTA from cereals (Mashhadizadeh et al., 2013). In this case, a strong external magnetic field is applied in order to pile up the particles and the suspension is discarded. In a similar approach, an aptamer was combined with magnetic nanospheres (nano-γ-Fe_2O_3) and used to develop a magnetic DSPE for determination of OTA in cereal, wheat flour and coffee extracts (Wu et al., 2011).

Despite those interesting applications of novel SPE-based methods, classic SPE developed in cartridges in still the most common sample treatment for determination of OTA. Thus, in the following sections, the different sorbents reported for SPE determination of OTA in food will be commented, highlighting the most representative publications, which are also summarized in Table 2.

Immunoaffinity columns

The most widely applied procedure for removing matrix component based on SPE has been the use of IACs. These columns are composed of OTA antibodies covalently immobilized on a solid sorbent, and they take advantage of the strong and specific antibodies-antigen (target analyte) interaction, providing a high selectivity and very clean extracts from complex matrices. On the other hand, IACs presents some drawbacks, as high cost, cross reactivity in some cases, and limited lifetime. A variety of these IACs for OTA are commercially available from different suppliers, such as: Ochraprep (R-Biopharm Rhône Ltd), OchraTest (Vicam), OchraStar (Romer Labs Diagnostic GmbH) or ToxinFast (Huaan Magnech Bio-Tech Co.), among others.

The use of IACs followed by HPLC-FLD has been proposed as reference method (European Committee for Standardization, 2009a; European Committee for Standardization, 2009b; European Committee for Standardization, 2010a, European Committee for Standardization, 2010b), and widely applied for the determination of OTA in a large number of food matrices. Generally it involves a previous extraction with a proper organic solvent, followed by dilution of the extract in phosphate buffered saline (PBS). A method based on IACs followed by HPLC-FLD has been validated for the simultaneous determination of OTA and its analogues, ochratoxin B (OTB), ochratoxin C (OTC) and methyl ochratoxin A (MeOTA) in red wine (Remiro et al., 2010). Sample preparation comprised pH adjustment, filtering by gravity and sample loading into the IAC, followed by column washing with PBS and elution with MeOH. Ochraprep columns were chosen in this study as they bound and retain the four analytes, providing good recoveries for all of them (from 73.4 to 93.5% over the concentration range $5 \cdot 10^{-4}$ -- 2 µg/L). The method was successfully applied to 20 red wine samples and provided very good sensitivity for OTA and its analogues, with limits of quantification (LOQs) established

as 0.5 ng/L. Ochraprep coupled to HPLC-FLD was also chosen for the determination of OTA in cocoa beans of high fat content (Roberts & Chang-Yen, 2014). In this case, ultrasound assisted extraction (UAE) and defatting of the extract was required previous to IAC purification. However, in other cases the lack of absolutely selectivity of IACs for individual mycotoxins could be a disadvantage. For instance, OTA content in coffee was underestimated because of OTB interfered when using IAC (Tozlovanu & Pfohl-Leszkowicz, 2010). In the same study it was suggested that OTA ring opens when alkaline medium is used during extraction, thus preventing an effective link in the IAC. Therefore, authors suggested that alkaline medium should be avoided during sample treatment to ensure better OTA recoveries. Following the manufacturer recommendations OchraStar IAC were also used for clean-up of coffee samples prior to HPLC-FLD. This analytical methodology was validated following the International Conference on Harmonization (ICH) recommendations, and the calculated recoveries ranged from 69% to 99% with a mean relative standard deviation (RSD) of 7.41% (Galarce-Bustos et al., 2014). IAC followed by HPLC-FLD has also been applied to develop and validate a method for OTA determination in meat products such as pork and dry-cured ham (Pietri et al., 2011). A comparison with SLE with chloroform previously reported for the analysis of meat products (Dall'Asta et al., 2010) with a novel enzyme-assisted extraction was carried out. The use of contaminant organic solvents is avoided in this method by applying Pancreatin hydrolyses proteins and thus OTA binding with them is prevented, while OTA in its ionised form is extracted by the aqueous solution. Other method was published for the determination of OTA in spices (Zhao et al., 2014), based on standardized methods, showing that, depending on the spice, a proper selection of the extraction solution, considering the PBS percentage, must be carried

out. Skarkova et al. validated a method based on IAC followed by HPLC-FLD using two different reference materials of naturally contaminated wheat (Skarkova et al., 2013). The method was applied to study OTA occurrence in 61 types of both, plant and animal origin food commodities such as cereal products, spices, coffee, cacao, beer, wine, raisins, pulses, mustard and edible offal, pork kidney, meat and liver. The method was sensitive and accurate, with LOQs and recoveries ranging from 0.01 to 0.3 µg/kg and 80-88%, respectively, depending on the matrix.

As mentioned before, the great advantage of IACs is the high specificity of antibodies to target analyte. The majority of commercially available IACs have antibodies specific to only one or a small group of closely related mycotoxins. However, several multi-functional IACs with specific antibodies for different mycotoxins have been commercialized, and used lately to develop multiresidue analytical methodologies for the simultaneous determination of OTA, aflatoxins (AFs) and other mycotoxins by HPLC-FLD. As fluorescence of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) is quenched in aqueous solvent, a derivatization preceding the detection is usually necessary in order to achieve enough sensitivity for these two analytes. Post-column photochemical derivatization (PCD) with mercury lamp (254 nm) has been the procedure of choice for the methods recently published. Turcotte *et al.* validated a method for the simultaneous determination of OTA and AFs in cocoa products using AflaOchra HPLC IAC (Vicam) for extract clean-up (Turcotte et al., 2013). The method was applied for the survey of 85 samples of cocoa products in the Canadian market. Results were confirmed by LC-MS/MS when concentrations of mycotoxins were above 2 ng/g. Also, a method based on HPLC-PCD-FLD and the use of AOZ HPLC IAC (Vicam) has been validated for the simultaneous determination of

OTA, AFs and zearalenone (ZEA) in cereals including rice, corn, wheat, oat and barley (Rahmani et al., 2010). The same IAC was used by Ibáñez-Vea *et al.* to validate a fast and sensitive method based on ultra-high performance liquid chromatography (UHPLC) for the determination of these mycotoxins in barley samples (Ibáñez-Vea et al., 2011). The short run time (13 min) was possible thanks to the use of a low volume column and high temperature (60 ºC), which allowed an increment of the mobile phase flow rate. Furthermore, derivatization was performed in a reactor coil (0.25 mL) smaller than usual in order to avoid excessive peak widening and maintaining good peaks resolution. In a different study twelve mycotoxins, including OTA, have been simultaneously determined in cereals by HPLC with DAD and FLD detectors on-line (Soleimany et al., 2011). Between the two detectors, two different derivatization systems were assembled. PCD was followed by a subsequent chemical post-column derivatization system, which consisted on a connection to merge the eluate with o-phthalaldeyde (OPA). OTA, four AFs (AFB_1, aflatoxin B_2 (AFB_2), AFG_1 and aflatoxin G_2 (AFG_2)), three fumonisins B_1 (FB_1), B_2 (FB_2), B_3 (FB_3) and ZEA were detected by FLD, while deoxynivalenol (DON), T-2 and HT-2 toxins were detected by DAD. The method implies a clean-up of sample extract by means of AOFZDT2 multi-functional IAC (Vicam). Recoveries from spiked corn was in the range 84-107% with RSD<12%. The method was finally applied to the analysis of 45 cereal samples including rice, wheat and maize flakes.

LC-MS/MS is currently spreading as a powerful technique for screening, identification/confirmation and quantification of mycotoxins. This analytical technique has been applied after IAC, to develop and validate an analytical method for the determination of OTA in different challenging food matrices, such as meat (Duarte et al., 2013). In addition stable isotope-

labelled OTA ($^{13}C_{20}$-OTA) is commercially available and therefore it can be used as internal standard to ensure high accurate quantification. Isotope dilution was also applied to determine OTA in Korean fermented soybean paste (Ahn et al., 2016) and in a broad range of food samples in the Canadian diet (Tam et al., 2011). Moreover, multi-functional IACs, in combination with LC-MS/MS have a great potential for effective and high-throughput multi-mycotoxin determinations. In this way, simultaneous quantification of several mycotoxins was successfully achieved in cereals and derived products (Liao et al., 2011; Lattanzio et al., 2014). Also, twelve mycotoxins have been recently determined in barley, maize breakfast cereals and peanuts by UHPLC-MS/MS (Vaclavikova et al., 2013). In order to get a fast method and to shorten the sample treatment procedure, a single extraction was carefully optimized, thus avoiding the double extraction with PBS recommended by the manufacturer. Acidic conditions were necessary for extraction of fumonisins, while these conditions reduced the efficiency of the IAC for some of the analytes, including OTA. Thus, solvent exchange was performed before the IAC loading. On the other hand, calibration with standard solutions treated as the samples were necessary in order to compensate for losses during treatment and to reduce bias of the method. A very fast UHPLC-MS/MS method was validated (Beltrán *et al*., 2011) for the determination of OTA and regulated AFs in baby food commodities and milk. In order to reach the concentration levels established by current European legislation for these matrices, a pre-concentration of extracts was necessary. In this study IACs proved to be much more efficient than Oasis HLB columns in removing matrix component. Using IACs allowed accurate quantification with external standard calibration, thus eliminating the need of any signal correction, i.e by using matrix-matched calibration.

Generally, one of the main drawbacks of IAC is that they are not reusable. For this reason, Meng *et al.* described the preparation of a reusable IAC for the determination of OTA in cereals (Meng et al., 2013). The monoclonal antibody was produced from a stable hybridoma cell line (4H10), which belongs to the immunoglobulin G1 (κ-light chain) isotype. This methodology has been validated in four different matrices (millet, maize, soybean, and swine finisher diet) with highly satisfactory results and applied to the analysis of market samples. The IAC columns were robust and could be regenerated and reused for another analysis observing no decrease of recovery even after repeated use (more than 20 times).

Recently, IACs have also been used combined with other extraction techniques, different than liquid extraction. Thus, Desmarchelier *et al.*, proposed the use of IAC after a QuEChERS-based extraction for the determination of OTA and AFs in cereals, cocoa, oil, spices, infant formula, coffee and nuts (Desmarchelier et al., 2014). The inclusion of IAC clean-up allowed achieving LOQs for OTA as low as 0.25 g/kg in cereals with recoveries ranging from 77 to 104% and precision with RSD between 2-19%. The method ruggedness was proved by a verification process conducted by another laboratory. Also, Xue *et al*. (Xue et al., 2014) determined simultaneously OTA and AFs in bee pollen by ultrasonic assisted extraction, followed by low-temperature fat precipitation and IACs, achieving LOQ of 0.5 µg/kg and recoveries between 79 and 83% (RSD <7.5%).

Molecularly imprinted polymers

MIPs are synthetic materials with artificial binding sites able to selectively recognize target molecules (Baggiani et al., 2008). These materials are synthesized by polymerization of chosen functional and cross-linking monomers in the presence of a template molecule (the target

compound, or even better an analogue, to avoid problems related to template leaking during SPE which may affect quantitation results). After the removal of the imprinted template from the polymer, specific binding cavities with shape and functional groups complementary to the template are created within the polymer matrix. Therefore, MIPs exhibit specific selectivity for the template molecule and its structurally relates molecules (Wulff, 1995; Sellergren 2007). MIPs have been proposed as sorbent in SPE (MISPE), SPME, SBSE, or magnetic MIP beads (Hu et al., 2013; Lasáková et al., 2009). The main advantages of the use of MISPE include chemical and thermal stability, compatibility with organic solvents, high target analyte capacity, reusability, long storage time without any loss of their properties and low price (Vidal et al., 2012; Ali et al., 2010). Considering the high complexity of food matrices, MIPs have been proposed for the determination of different compounds, including a reduced number of mycotoxins (Song et al., 2014), OTA among them. In this sense, the main strategies to synthesize MIPs for the selective extraction of OTA from different matrices, have been reviewed and discussed (Baggiani et al., 2008; Yu. & Lai, 2010).

A simple and rapid method, based on automatic on-line MISPE followed by FLD detection, has been recently proposed for the determination of OTA in wheat samples at ng/mL levels (Vidal et al., 2012). The MIP showed excellent specific interactions with OTA compared with the non-imprinted polymer (NIP). It was slurry packed with MeOH into a SPE column with a volume of about 1.7 mL, and used in a flow system coupled on-line with the detector. In this way the analyte is selectively retained in a glass column, while interfering molecules from wheat matrix are not. Then, OTA is removed from the column by increasing the eluting power of the mobile phase and monitored. After optimizing the loading, eluting and washing conditions (flow and

mobile phase nature), the reusability of the columns was estimated at five times at least. Recoveries obtained from two different wheat certified reference materials ranged from 92.1 to 104.0% with RSD lower than 9.6%.

Giovannoli *et al.* developed another extraction method based on MIP prepared through a mimic template approach, used for the determination of OTA in red wines (Giovannoli et al., 2014). This method proved to be a reliable alternative to the conventional IAC protocol obtaining recoveries ranging from 88 to 102% and allowing OTA pre-concentration well below the maximum permitted levels.

Commercially available MIPs specific for OTA extraction, Affinimip SPE Ochratoxin A (Polyintell), have been applied in sample treatment procedures with different matrices, followed by HPLC-FLD. In previous feasibility studies, the presence of selective cavities and specific interactions of this material was demonstrated by studying the retention of OTA on conventional crushed monoliths of both MIP and NIP (Ali et al., 2010). With this purpose, a SPE procedure was optimized and applied. Firstly, OTA standard in pure solvent (MeCN) was used for the study with a recovery of 107 ± 16% obtained on the MIP while only of 2% was obtained on the NIP (most of the analyte was eluted in the washing step). Then the sorbents were tested using spiked wheat extract (MeCN:water, 55:45, v:v) and results showed that although OTA retention was slightly modified due to matrix components, recoveries of 93% were obtained on the MIP and only of 2% on the NIP. After these promising results, the use of uniform MIP beads was considered and showed stronger retention of OTA than the crushed monolith, which allowed an even more efficient and cheaper product. Finally the authors compared the performance of this MIP and IAC for the cleaning-up of extracts from spiked wheat samples, concluding that

although the two sorbents showed similar selectivity, the MIP showed much higher capacity than IAC. The same MIP material has been used to optimize a method for the determination of OTA in beer, wine and grape juice by HPLC-FLD (Cao et al., 2013). The critical variables affecting the SPE (pH of loading solution, nature and volume of washing solution as well as nature and volume of eluting solution) were optimized using beer samples spiked with OTA. Recoveries in the range of 91.6-101.7% were obtained from spiked samples of each matrix (RSD<4.1%). The reusability of MISPE column was also assessed in the same study. With this purpose, 20 consecutive clean-up cycles of spiked bear extracts were carried out after regeneration with 10 mL of MeOH. A decreasing trend on recoveries was observed after the 8^{th} cycle; however the column can be re-used up to 14 times with recoveries higher than 80%. The authors conclude that MIPs are more convenient than IACs because similar recoveries are obtained with a cheaper methodology.

Oligosorbents

Aptamers are short single chains of oligonucleotides (DNA/RNA, 20-110 base pairs) that are able to fold into well-defined three-dimensional structures and specifically bind target molecules with very high affinities (Chapuis-Hugon et al., 2011; Rhouati et al., 2011). The high affinity of aptamers is a consequence of their ability to fold upon binding to their target molecule. Candidate aptamers are screened for their ability to bind a specific molecular target, and eventually selected from large populations of random sequenced oligomers (DNA/RNA libraries), by applying an iterative *in vitro* method called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Once the aptamer sequence is established, aptamer can be prepared by chemical synthesis. The use of these OSs in SPE for selective enrichment and clean-

up present several advantages, such as the regeneration within minutes, therefore increasing their reusability, longer shelf-life and superior stability under a broader range of conditions than traditional antibodies. They are chemically synthesized *in vitro*, resulting in little-to-no batch-to-batch variability and the possibility of customization to improve their stability and facilitate their immobilization. Most of the isolated sequences already identified are directed against large molecules such as peptides, proteins or nucleic acids, although a significant number of aptamers have also been selected for small molecules including OTA.

The first aptamer identified to strongly and specifically bind any mycotoxin was firstly introduced by Cruz-Aguado *et al.* (Cruz-Aguado & Penner, 2008). In this study, the feasibility of using aptamer as sorbent for SPE was demonstrated. The high affinity of the aptamer toward OTA was evidenced by the estimated dissociation constant in the nano-molar range. Furthermore, it did not bind compounds with structures similar to OTA, such as N-acetylphenylalanine or warfarin, and bound with a 100-fold less affinity OTB, thus providing high OTA specificity. This aptamer sequence can be currently commercially found (OTA-Sense aptamer-affinity columns) (De Girolamo et al., 2012), and has been used to obtain different OSs to be applied in the extraction of OTA from different matrices such as wheat, wheat flour, wine or beer. For instance, De Girolamo *et al*. obtained an OSs, which was employed for the optimization of a procedure to extract OTA from wheat samples by SPE (De Girolamo et al., 2011). In this case, the aptamers were not 5´phosphorylated, and therefore the immobilization was probably through bonds to the internal phosphate groups. Several variables such as the OS volume, column size and breakthrough volume (maximum eluting solvent) were assessed. The optimized OS-based SPE method followed by HPLC-FLD showed lower sensitivity than the

IAC-based method, although it was suitable for the cleaning and pre-concentration of wheat extracts, at OTA concentration levels that naturally occur in this matrix. OTA recoveries ranged between 74-88% with RSD lower than 6%. The same sample treatment was followed to develop a successful method for the direct determination of OTA in wheat extracts. In this case time-resolved fluorescence (TRF) response was used for detection, which was produced by the complex made up of terbium, a fluorescent lanthanide with a long lifetime, OTA and the aptamer (De Girolamo et al., 2012). TRF measurements were performed in a 96-well microplate reader and the procedure allows the simultaneous analysis of several samples in less than 30 min.

The same sequence aptamer was used to obtain other two OSs for the selective extraction of OTA from red wine previous to its determination by HPLC-FLD (Chapuis-Hugon et al., 2011). With this purpose, different immobilization strategies of aptamers on functionalized supports, based on either non-covalent or covalent binding, were studied. The non-covalent immobilization was based on the interaction of streptavidin-activated agarose with a 5′-biotinylated OTA aptamer, while the covalent immobilization took place by binding cyanogen bromide (CNBr)-activated sepharose with a 5′-amino-modified OTA aptamer. After optimization of the extraction procedure in aqueous media, similar capacity and recoveries close to 100% were found for both materials, while non-specific interactions were found for any of the control supports (functionalized support without immobilized aptamer). However, the immobilization by covalent bonding was preferred for OTA extraction from red wine samples, because retention and selectivity were less affected by ethanol content, and therefore a more robust material was obtained following this strategy. Using these OSs, fewer interference compounds were co-extracted compared to the extraction by C18, and recoveries similar to those obtained with IAC

were obtained (close to 100%). An OS obtained following the same strategy was used as sorbent for the extraction of OTA from beer (Rhouati et al., 2011). The working range of the method was comparable to those found with conventional methods (0.5-10 ng/mL), and good recoveries (91-98%, RSD<3%) were obtained from spiked beer samples. In this case the cartridge could be used 3 times without performance decrease. Yang *et al*. also applied this sequence prepared in-house through a covalent immobilization strategy for the determination of OTA in ginger powder by HPLC-FLD (Yang et al., 2014). The OTA-aptamer was immobilized on NHS-activated Sepharose 4 Fast Flow (a pre-activated agarose matrix) through the covalent reaction of 5'-amino group in the aptamer and NHS on the sepharose. LOQ was 1.5 µg/kg, with recoveries above 81% (RSD<5%). Finally, the reusability of OSs and IACs was compared to determine OTA in spiked ginger powder samples and it was concluded that the aptamer-affinity column could be reused eight times without notable effect on aptamer-binding efficiency, while IAC could only be reused four times.

Further experiments have been carried out to improve the performance of this OS by optimizing its synthesis (Ali & Pichon, 2014). The characterization of different OSs showed that grafting the aptamer from either of its two ends (5´ or 3´ side) had no effect on the OS capacity, whereas increasing the length of the spacer arm separating the sequence form the sepharose (from C6 to C12) increased the OS capacity by 50%. This study also revealed that increasing the amount of aptamers in the grafting solution, increases the OS capacity up to a limit, because steric hindrance effects limit the amount of aptamer efficiently grafted to the solid phase. The resulting OS showed no cross-reactivity to either OTB or ochratoxin hydroxyquinone (QTHQ), and was

applied for the extraction of OTA from a contaminated reference sample with recoveries close to 90% and estimated reusability for real samples of 15 times.

Recently, Giovannoli *et al.* synthetized OTA-binding hexapeptide on different commercial solid supports based on silica or organic polymers (Amberlite IRC-50, Lewatit CNP105, Toyopearl CM-650 M, porous silica gel beads and micrometric glass beads) (Giovannoli et al., 2015). They investigated the thermodynamic and kinetic binding properties of stationary phases, obtaining the highest values of the equilibrium binding constant (K_{eq}) and binding site concentration (B_{max}) with Lewatit CNP105. So, Lewatit CNP105 was compared with Amberlite IRC-50 as solid support in SPE extraction of OTA from wine samples and the extracts were analyzed by HPLC-FLD. Limit of detection (LOD) and LOQ of 0.45 and 1.5 μg/L, respectively, and good recoveries between 71% and 108% for Amberlite IRC-50 and 91% and 101% for Lewatit CNP105, were obtained. Although both supports showed comparable extraction accuracy, a statistically significant difference was found in terms of extraction precision, confirming that the solid phase based on Lewatit CNP105 performs better than the solid phase based on Amberlite IRC-50.

Other specific materials

Multifunctional cartridges, containing a combination of materials with different retention mechanism (e.g. charcoal, ion-exchange resins, etc.,) such as MycoSep and MultiSep (Romer Lab) have been widely used for mycotoxin clean-up. The major advantages of these columns are the reduced amount of solvent and avoidance of time-consuming rinsing steps required in single-phase SPE (Cigic et al., 2009). MycoSep and MultiSep columns allow a one-step clean-up within 30 s. MycoSep column is pushed into a test tube containing the sample extract, forcing the extract to filter upwards through the packing material of the column. The interferences adhere to

the chemical packing in the column and the purified extract, containing the analytes of interest, passes through the column. Whereas that, the sample extract is applied on top of the MultiSep column and the interferences adhere to the chemical packing in the column and the purified extract, containing the analytes of interest, can be collected in a suitable vessel (http://www.romerlabs.com/en/products/mycotoxins/mycosep-multisep/) (for more details, see Figure 2)

Several studies have compared these columns with IACs and non-specific sorbents. Prelle *et al.* compared MycoSep 229 Ochra with IAC, MIP and a non-specific sorbent (HLB) in the purification of OTA from wine, beer, roasted coffee and chili (Prelle et al., 2013). MycoSep showed the best results in wine (recoveries rate: 82-103%) and chili (recoveries rate: 91-103%), however authors selected IAC column as sample treatment because of the lower recoveries rate of Mycosep for the rest of matrices (beer: 62-68%, roasted coffee: 50-55%). This study also described the first use of MycoSpin cartridge clean-up, other "pass through" specific sorbent, on OTA monitoring. MycoSpin columns showed good recoveries only for wine samples (74-80%), observing lower recovery at lower concentration levels.

Fabiani *et al.* compared three sample treatments (IAC, MycoSep 229 Ochra and LLE) and two analytical techniques (HPLC-FLD and ELISA) to determine OTA in wines (Fabiani et al., 2010). The best recoveries were obtained with IACs (88--115%) both at high and low concentrations of OTA, close to those of MycoSep (84--126%), while LLE gave lower recoveries (64--96%). Moreover, samples cleaned-up with MycoSep were analysed by HPLC-FLD and ELISA, and ELISA reported lower concentrations of OTA than HPLC-FLD. Finally, MycoSep 229 Ochra column has also been applied to determine OTA in spices as red paprika and black pepper using

HPLC-FLD analysis. The method was validated obtaining a LOQ of 3 µg/kg, while recoveries were not lower than 85% in all concentration ranges tested (Bononi et al., 2010).

These columns have also been applied in multi-mycotoxin determination. Thus, Rahmani *et al*. compared the efficiency of MycoSep, AOZ multi-functional IAC and Oasis HLB for the simultaneous purification of AFs, OTA and ZEA from cereal samples and subsequent detection using HPLC-FLD (Rahmani et al., 2011). They demonstrated that MycoSep 226 AflaZON + column, showed the worst recovery value and clean-up performance for OTA, although it must be highlighted that this sorbent is specific for AFs and ZEA, so a low recovery rate for OTA was predictable. Also, MultiSep 229 Ochra was applied as clean-up after QuEChERS-based extraction for the determination of OTA and other mycotoxins in corn grits by LC-MS/MS (Tamura et al., 2014). This method was proposed in order to minimize carryover and recoveries of 78.4% with LOQ of 0.4 µg/kg were obtained.

Other commercially available specific cartridges are ISOLUTE Myco SPE columns (Biotage), which contain a novel polymer-based sorbent designed to isolate a wide variety of different mycotoxins; thus, in this case the mycotoxin is selectively bind to the sorbent, whilst washing off matrix components that could interfere with the analysis (just the opposite to the MycoSep and MultiSep) (http://www.biotage.com/product-page/isolute-myco). ISOLUTE Myco SPE columns were tested for the determination of OTA in red wine (Mariño-Repizo et al., 2015). The SPE was optimised by experimental designs and the method was validated according to European Commission 2002/657/EC achieving a decision limit (CCα) of 0.07 µg/L and recoveries of 95.7-107.2% for OTA.

Non-specific stationary phases

Despite the current and increasing interest in more selective binding material (such as IAC, MIP, OS or the previously commented MycoSep or Isolute Myco SPE), non-specific stationary phases (reverse phase, normal phase, ion exchange), such as C-18, florisil, phenyl, aminopropyl or ion exchange, are still usually employed to perform SPE, specially to develop multiresidue methods for the simultaneous confirmation and quantification of different mycotoxins, generally by LC-MS/MS. This clean-up procedure eliminates many interferences and significantly reduces matrix effects (ionization suppression or enhancement) allowing the quantification of mycotoxins in complex food matrices, although usually these multiresidue methods involve matrix-matched calibration in order to reduce systematic errors. HLB (a styrene-divinylbenZEAe polymer) has been the sorbent of choice in the last years for food analysis. In a recent study Oasis HLB (Waters) was preferred over MycoSep 226 AflaZON to develop a method for the simultaneous determination of OTA, aflatoxin M_1 (AFM_1), ZEA and α-zearalenol in raw milk, liquid milk and milk powder by LC-MS/MS, as the MycoSep did not allowed to quantify accurately OTA and α-zearalenol (Huang et al., 2014). Recoveries for OTA in the range 93.9-101.2% with RSD lower than 10% were obtained. In other study, Oasis HLB was also the purification method of choice, after its comparison with MycoSep 226 AflaZON and Bond Elut Mycotoxin (Agilent, a multifunctional SPE column recommended for trichothecene and zearalenon determination) to develop and validate a method for the determination of six AFs and OTA in different matrices such as muscle, liver, kidney and fat of swine, bovine and sheep, muscle and liver of chicken, muscle and skin of fish, hen eggs and dairy milk by LC-MS/MS (Chen et al., 2012). The method involves a previous extraction applying PLE using MeCN/hexane as extraction solvent. In addition to automated sample handling and time-saving, other advantages such as reduced

solvent consumption, better sensitivity and higher recoveries were achieved using PLE instead of shaking extraction. Recoveries ranged from 68.3 to 105.7%, with inter-day RSD lower than 17%. The decision limit (CCα) and detection capability (CCβ) for OTA were ≤ 0.59 and 1.21µg/kg, respectively. Other authors selected this SPE sorbent because it is water-wettable and maintains high retention and capability even when it is dried-up, as for instance in the simultaneous determination of bisphenol A, AFB_1, patulin (PAT) and OTA in butter peanut, cereal-based baby food and apple and grape juices (Song & Moezzi, 2013). Recently Oasis HLB cartridges were applied for multi-mycotoxin determination in bottled water by UHPLC-MS/MS, without any other sample treatment (Mata et al., 2015), and in a wide range of fresh fish and dried seafoods by LC-MS/MS after UAE (Sun et al., 2015). In this case, recovery efficiencies of Oasis HLB, Mycosep 226 AflaZON and Cleanert C18 cartridges (Agela) for clean-up were compared. Although generally one single SPE step is required for achieving clean extracts, a subsequent clean-up with multifunctional Multisep^{TM} 229 Ochra column after Oasis HLB was needed for an efficient removal of pigments and polar matrix compound from red wine (Tamura et al., 2012). The method allowed the determination of 14 mycotoxins by UHPLC-MS/MS in red and white wines, providing LOQs well below the EU regulatory levels, with OTA LOD and LOQ of 0.06 and 0.2 µg/L, respectively.

HLB has also been proposed for multiresidue determination of several contaminants, including mycotoxins. In this sense, a generic sample treatment approach was proposed for the simultaneous determination of 60 multi-class pesticides and 9 mycotoxins, including OTA, in wines (Pérez-Ortega et al., 2012). A comparison of matrix effect after SPE with Oasis HLB and Bond Elut Plexa was carried out, selecting the former. After clean-up, the analytes were

identified and quantified by LC- time of flight (TOF). Under optimal conditions 70% of the target analytes showed relatively low matrix effects. Average recovery of 117% was obtained for OTA with RSD of 4%.

Although most of the SPE applications using non-specific sorbents for OTA determination are based on HLB, other sorbents have also been reported. Thus, in a comparison study, Ocnaru *et al.* concluded that C18 cartridges were more convenient that IAC, previous to the determination of OTA in wine samples by HPLC-FLD, in terms of price and precision (Ocnaru et al., 2014). Interestingly, C18 cartridges have also been used in automatic on-line SPE coupled to UHPLC-MS/MS for simultaneous determination of AFs and OTA in dried fruits after PLE (Campone et al., 2015). With this whole analytical procedure the presence of target analytes can be assessed in less than 40 min obtaining LOQ between 0.05 and 0.09 µg/L and recoveries above 94% for OTA. C18 was also the sorbent chosen for SPE in a method developed for determining 15 mycotoxins (including OTA) by UHPLC-MS/MS in beer-based products (Tamura et al., 2011). In this case, a previous QuEChERS-based extraction was proposed and different SPE cartridges were tested: C18, primary-secondary amine (PSA), and graphite carbon black (GCB). Poor recovery rates were observed for OTA using PSA and GCB cartridges. OTA was absorbed by PSA because of the ionic affinity between the primary and/or secondary amines in the PSA support and the carboxyl groups in OTA; and by GCB due to π--π interactions between the sp^2 hybrid orbitals in GCB's six-membered rings and the planar aromatic rings in this mycotoxin. Consequently the samples were cleaned-up through a C18 cartridge, with an average recovery of 110.2%.

Mixtures of sorbents have also been reported. For instance, Jin *et al.* used laboratory-made mixed SPE cartridges, containing silica gel, florisil and kieselguhr (diatomaceous earth), for determining ten mycotoxins (including OTA and OTB) in cereal grains by UHPLC-MS/MS (Jin et al., 2010). In this case the isotopically labelled $^{13}C_{15}$-DON was used as internal standard, compensating target losses and eliminating matrix effect.

Anion exchange sorbents have also been used for the determination of OTA in different matrices. Zhong *et al.* developed a method for the evaluation of the risk assessment of OTA contamination from wine by HPLC-FLD, obtaining LOQ of 0.03 μg/L and recoveries above 97% (RSD 4%) (Zhong et al., 2014). Pascale *et al.* proposed other method where wheat samples were extracted with MeCN/water (60:40, v/v) and purified by a rapid SPE procedure using an aminopropyl column prior to the fluorescence polarization (FP) immunoassay (Pascale et al., 2014). This sorbent proved to be quite versatile as it can be used in either normal phase or reversed phase mode. It retains the analytes either by a polar adsorption (from non-polar solution) or by weak anion exchange (from aqueous solution). This method allowed reaching a very high sensitivity in real samples (LOD of 0.8 μg/kg) and despite the purification step, the whole analytical procedure was performed in less than 20 min. Also, Longobardi *et al.* reported a method for quantification of OTA in wines where a IAC was coupled to an aminopropyl SPE column, prior to direct fluorometric measurement after spectral deconvolution (Longobardi et al., 2013). Average recoveries ranged between 94.5-105.4% while LOD was 0.2 ng/mL, with a total time of analysis of 30 min. The method was compared with an AOAC Official Method based on IAC clean-up and HPLC-FLD, and a good correlation was observed. Other method published by Nielsen *et al.* was based on a mixed-mode reversed phase-anion exchange SPE after a

QuEChERS-based extraction for the determination of OTA and fumonisins (B_2, B_4 and B_6) in coffee (Nielsen et al., 2015). OTA was detected at levels down to 1 μg/kg by UHPLC-MS/MS with recoveries above 76%.

Moreover, size-exclusion SPE has also been proposed by Wang *et al.*, who developed tailor-made SPE column with C4 and NH_2 mixed macropore silica gel packed into a guard cartridge (Wang & Li, 2015). This sorbent was introduced as an on-line clean-up column coupled to UHPLC-MS/MS for screening of OTA and other three mycotoxins (ZEA, AFB_1 and AFM_1) in liquid milk and milk powder. Permanent post-column infusion of mycotoxin standards was used to quantify matrix effects throughout the chromatographic run. Recovery of OTA was in the range 109--120% (RSD 2--9%).

CONCLUSIONS

Increasingly demanding legal requirements concerning food contaminants (such as OTA) force to the development of more and more sensitive analytical methods. In this sense, sample treatment is still the bottleneck of method development in food analysis. Although recent methodologies (as QuEChERS) are nowadays commonly used for extraction and clean-up of multi-mycotoxins in different matrices, SPE is still the method of choice in most routine laboratories, especially when only OTA is the analyte of interest. Recent advances of this robust sample treatment, including the development of more selective stationary phases and miniaturization, still make SPE a valuable technique of choice in this field of food control.

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Table 1. Maximum permitted levels by EC for OTA in foodstuffs (European Commission, 2006; European Commission, 2010; European Commission, 2012; European Commission, 2015)

	Foodstuffs	Maximum level (μg/kg)
1.	Unprocessed cereals	5.0
2.	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs listed in 9,10 and 13	3.0
3.	Dried vine fruit (currants, raisins and sultanas)	10.0
4.	Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5.0
5.	Soluble coffee (instant coffee)	10.0
6.	Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15% vol) and fruit wine	2.0
7.	Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2.0
8.	Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2.0

9.	Processed cereal-based foods and baby foods for infants and young children	0.50
10.	Dietary foods for special medical purposes intended specifically for infants	0.50
11.	Spices, including dried spices	
	Piper spp (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	15
	Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika)	20
	Mixtures of spices containing one of the abovementioned spices	15
12.	Liquorice (Glycyrrhiza *glabra, Glycyrrhiza inflate* and other species)	
	12.1. Liquorice root, ingredient for herbal infusion	20
	12.2. Liquorice extract, for use in food in particular beverages and confectionary	80
13.	Wheat gluten not sold directly to the consumer	8.0

Table 2. Recent publications including SPE for determination of OTA

Analyte	Sorbent	Analytical technique	LOD	LOQ	Matrix	Ref.
Immunoaffinity columns (IACs)						
OTA, OTB, OTC, MeOTA	Ohraprep	HPLC-FLD	0.16 ng/L (OTA), 0.32 ng/L (OTB), 0.27 ng/L (OTC), 0.17 ng/L MeOTA	0.5 ng/L	Red wine	(Remiro et al., 2010)
OTA	Ochraprep	HPLC-FLD	n.a.	0.66 ng/g	Cocoa Beans	(Roberts & Chang-Yen, 2014)
OTA	OchraStar	HPLC-FLD	0.08 µg/kg	0.25 µg/kg	Roasted and instant coffees	(Galarce-Bustos et al., 2014)
OTA	OchraTest	HPLC-FLD	0.06 µg/kg	0.12 µg/kg	Pork ham, dry-	(Pietri et al., 2011)

					cured ham	
OTA	Ochraprep	HPLC-FLD	0.1 µg/kg	0.3 µg/kg - 0.4 µg/kg**	Raw ham muscle, salami and dry-cured ham	(Dall'Asta et al., 2010)
OTA	OchraTest	HPLC-FLD	0.2 µg/kg	0.5 µg/kg	Spices	(Zhao et al., 2014)
OTA	Ochraprep	HPLC-FLD	0.004 - 0.100 µg/kg**	0.01 - 0.3 µg/kg**	Plant and animal food stuffs	(Skarkova et al., 2013)
OTA AFB_1, AFB_2, AFG_1, AFG_2	AflaOchra HPLC	HPLC-PCD-FLD	0.042 - 0.053 ng/g**	Mean: 0.16±0.02 **	Cocoa products	(Turcotte et al., 2013)
OTA AFB_1, AFB_2, AFG_1, AFG_2, ZEA	AOZ HPLC	HPLC-PCD-FLD	0.05 ng/g	0.2 ng/g	Rice, oat, maize, barley, wheat	Rahmani et al., 2010

OTA, AFB_1, AFB_2, AFG_1, AFG_2, ZEA	AOZ HPLC	UPLC-PCD-FLD	13 ng/kg	150 ng/kg	Barley	Ibáñez-Vea et al., 2011
OTA, AFB_1, AFB_2, AFG_1, AFG_2, ZEA, DON, FB_1, FB_2, FB_3, T-2, HT-2.	AOFZDT2	HPLC-PCD-(OPA)-DAD-FLD	0.187 ng/g	0.750 ng/g	Rice, wheat, maize flakes	Soleimany et al., 2011
OTA	OchraTest	LC-MS/MS	0.06 µg/kg	0.19 µg/kg	Pork	Duarte et al., 2013
OTA	OchraTest	LC-MS/MS	0.01 µg/kg	0.08 µg/kg**	Korean fermented soybean paste	Ahn et al., 2016
OTA	OchraTest	LC-MS/MS	0.001 - 0.008	0.005-0.025	Dietary products	Tam et al., 2011

			ng/g**	ng/g**		
OTA, AFB_1, AFB_2, AFG_1, AFG_2, ZEA	AOZ HPLC	LC-MS/MS	0.01 µg/L *	0.03 µg/L *	Wheat, oat, rice and corn	Liao et al., 2011
OTA, AFB_1, AFB_2, AFG_1, AFG_2,, FB_1, FB_2, ZEA, DON, Nivalenol (NIV), T-2, HT-2	Myco6in	LC-MS/MS	n.a.	1 µg/kg	Cereals and derived products	Lattanzio et al., 2014
OTA, AFB_1, AFB_2, AFG_1, AFG_2, DON,	Myco6in	UHPLC-MS/MS	0.1 µg/kg	0.25 µg/kg	Barley, maize breakfast cereals, peanuts	Vaclavikova et al., 2013

ZON, HT-2, T-2, FB_1, FB_2, FB_3						
OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1	AflaOchra HPLC	UHPLC-MS/MS	2-9 ng/kg**	6-25 ng/kg**	Cereal based, cereal and milk based baby-food, baby milk, raw milk	Beltrán *et al*., 2011
OTA	Monoclonal antibody produced from a stable hybridoma cell line (4H10)	UHPLC-MS/MS	0.5-1 µg/kg**	0.2-0.2 µg/kg**	Cereals and feeds	Meng et al., 2013
OTA,	AflaOhrapr	LC-MS/MS	n.a.	0.25	Cereals,	Desmarcheli

AFB_1, AFB_2, AFG_1, AFG_2, AFM_1	ep			µg/kg	cocoa, oil, spices, infant formula, coffee and nuts	er et al., 2014
OTA, AFB_1, AFB_2, AFG_1, AFG_2	ToxinFast	LC-MS/MS	0.01 µg/kg	0.5 µg/kg	Bee Pollen	Xue et al., 2014
	OchraTest					
OTA	OchraStar	HPLC-FLD	0.0106 µg/L	n.a.	Red wine	Fabiani et al., 2010
OTA AFB_1, AFB_2, AFG_1, AFG_2, ZEA	AOZ HPLC	HPLC-PCD-FLD	0.05 ng/g	0.2 ng/g	Rice, corn, wheat, oat, barley	Rahmani et al., 2011
Molecularly imprinted polymers (MIPs)						
OTA	Synthesized	MISPE-FLD	1.2	n.a.		Vidal et al.,

	MIP		ng/mL*			2012
OTA	Synthesized MIP	HPLC-FLD	0.075 ng/mL	0.225 ng/mL	Red wine	Giovannoli et al., 2014
OTA	Affinimip SPE OTA	HPLC-FLD	n.a.	n.a.	Wheat	Ali et al., 2010
OTA	Affinimip SPE OTA	HPLC-FLD	0.025 g/mL*	0.08 ng/mL*	Beer, wine and grape juice	Cao et al., 2013
Oligosorbents (OSs)						
OTA	Aptamer	HPLC-FLD	n.a	n.a	Red wine	Chapuis-Hugon et al., 2011
OTA	Aptamer	HPLC-FLD	0.2 ng/mL*	n.a	Beer	Rhouati et al., 2011
OTA	Aptamer	TRF	n.a.	0.5 µg/kg	Wheat	De Girolamo et al., 2012
OTA	Aptamer	HPLC-FLD	23 pg/g	77 pg/g	Wheat	De Girolamo et al., 2011

OTA	Aptamer	UHPLC-FL	0.5 µg/kg	1.5 µg/kg	Ginger powder	Yang et al., 2014
OTA	Aptamer	HPLC-FLD	n.a	n.a	Wheat	Ali & Pichon, 2014
OTA	OTA-binding hexapeptide	HPLC-FLD	0.45 µg/L*	1.5 µg/L*	Wine	Giovannoli et al., 2015
Other specific sorbents						
OTA	MycoSep 229 Ochra	HPLC-FLD / ELISA	0.0109 µg/L / 0.1000 µg/L	n.a.	Red wine	Fabiani et al., 2010
OTA	MycoSep 229 Ochra MycoSpin	HPLC--FLD	MycoSep: 0.12-0.27 µg/L, 0.30-0.99 µg/kg** MycoSpin: 0.14-	MycoSep: 0.40-0.48 µg/L, 1.00-3.31 µg/kg** MycoSpin: 0.90-2.95	Wine, Beer roasted coffie, chili	Prelle et al., 2013

			0.88 µg/L, 2.12-3.03 µg/kg**	µg/L, 7.08-10.12 µg/kg**		
OTA	MycoSep 229 Ochra	HPLC-FLD and ELISA	HPLC-FLD: 0.0109 µg/L ELISA: 0.1000 µg/L	n.a.	Wine	Fabiani et al., 2010
OTA	MycoSep 229 Ochra	HPLC-FLD	1.0 µg/kg	3.0 µg/kg	Red paprika and black pepper	Bononi et al., 2010
OTA, AFs, NIV, DON, T-2, HT-2, FB_1, FB_2, FB_3, PAT, ZEA	MultiSep 229 Ochra	LC-MS/MS	0.01 µg/kg	0.04 µg/kg	Corn grits	Tamura et al., 2014
OTA	ISOLUTE	UHPLC-	0.13	0.41 µg/L	Red wine	Mariño-

	Myco	MS/MS	µg/L			Repizo et al., 2015
Non-specific sorbents						
OTA, AFM_1, ZEA, α-zearalenol	Oasis HLB	LC-MS/MS	0.001 - 0.005 µg/kg**	0.003 - 0.015 µg/kg**	Raw milk, liquid milk and milk powder	Huang et al., 2014
OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2	Oasis HLB	LC-MS/MS	CCα ≤ 0.59 µg/kg** CCβ ≤ 1.21µg/kg **	≤1 µg/kg**	Swine muscle, liver, kidney and fat), bovine and sheep (muscle), chicken (liver), fish (muscle and skin),	Chen et al., 2012

					eggs, milk	
OTA, bisphenol A, AFB_1,	Oasis HLB	LC-MS/MS	n.a.	1 µg/L for juice 0.08 µg/kg for cereals and 0.15 µg/kg for butter peanut	Butter peanut, cereal-based baby food, fruit juice (apple and grape)	Song & Moezzi, 2013
OTA, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, neosolaniol,	Oasis HLB	LC-MS/MS	n.a.	0.2 ng/L	Bottled water	Mata et al., 2015
OTA, AFB_1, AFB_2,	Oasis HLB	LC-MS/MS	0.1-0.3 µg/kg**	0.3-1 µg/kg**	Dried fish products, entrails,	Sun et al., 2015

AFG_1, AFG_2, ZEA, T-2, HT-2, DON					muscle	
OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, PAT, DON, T-2, HT-2, ZEA, FB_1, FB_2, FB_3	Oasis HLB + Multisep 229 Ochra	UHPLC-MS/MS	0.06 µg/L	0.2 µg/L	Wine	Tamura et al., 2012
OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, ZEA, ergocornine	Oasis HLB	LC-MS/MS	0.11 µg/L	0.36 µg/L	Wine	Pérez-Ortega et al., 2012

, Pesticides						
OTA	C18	HPLC-FLD	0.06 µg/L	n.a.	Wine	Ocnaru et al., 2014
OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1	Strata C18-E (on-line SPE)	UHPLC-MS/MS	0.01-0.03 µg/kg**	0.05-0.09 µg/kg**	Dried fruits	Campone et al., 2015
OTA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, PAT, DON, NIV, T-2, HT-2, ZEA, FB_1, FB_2, FB_3	C18	UHPLC-MS/MS	n.a.	1.0 ng/mL	Beer-based Drinks	Tamura et al., 2011
OTA, OTB, DON, 3-	Silica gel, florisil and	UHPLC-MS/MS	0.03 -- 0.17	0.09 -- 0.51	Rice, buckwhea	Jin et al., 2010

acetyl-DON, 15-acetyl-DON, NIV, fusarenon X, moniliformin, ZEA, zearalanone,	kieslguhr		µg/kg** (OTA) 0.04 -- 0.20 µg/kg** (OTB)	µg/kg** (OTA) 0.12 -- 0.61 µg/kg** (OTB)	t, broomcorn, bean, mung bean, rice, millet	
OTA	Anion-exchange SPE column	HPLC-FLD	0.01 µg/L	0.03 µg/L	Wine	Zhong et al., 2014
OTA	NH_2	FP Immunoassay	0.8 µg/kg	250 µg/kg	Wheat	Pascale et al., 2014
OTA	IAC + aminopropyl SPE	Spectrofluorimeter after deconvolution of fluorescence spectra	0.2 ng/mL	n.a.	Wine	Longobardi et al., 2013
OTA,	Mixed-	UHPLC-	0.3-1	0.46-2	Green,	Nielsen et

fumonisins (B_2, B_4, B_6)	mode reversed phase-anion exchange	MS/MS	µg/kg**	µg/kg**	roasted, and instant coffee	al., 2015
OTA, ZEA, AFB_1, AFM_1	Mixed macropore-silica gel SPE on-line	UHPLC--MS/MS	1 ng/L -- 5 ng/kg**	n.a.	Liquid milk and milk powder	Wang & Li, 2015

*In pure solvent standard.

**Values depending on the matrix.

n.a. Not available.

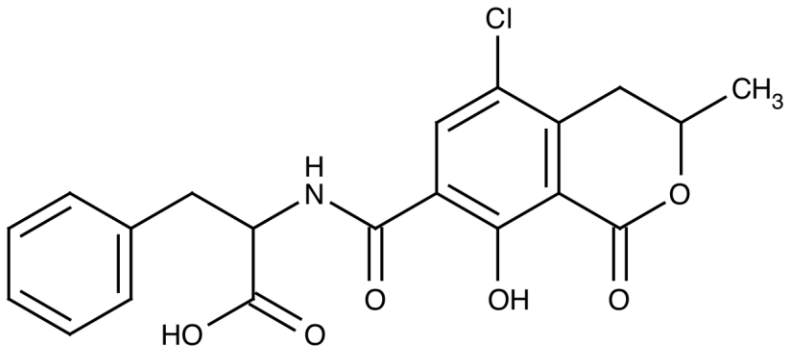

Figure 1. Ochratoxin A

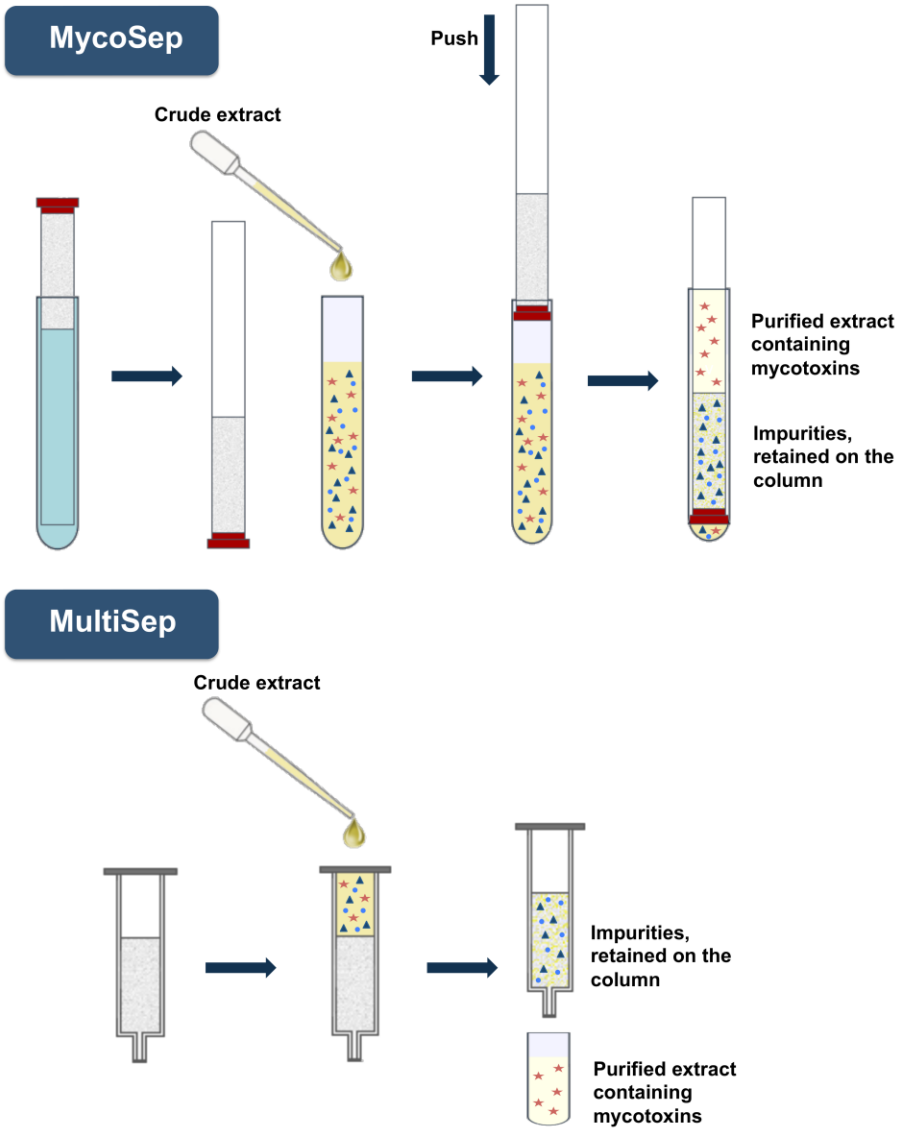

Figure 2. MycoSep and MultiSep working principle (adapted from

http://www.romerlabs.com/en/products/mycotoxins/mycosep-multisep/)