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Review

Recent advances in ochratoxin A-producing fungi detection based on PCR methods and ochratoxin A analysis in food matrices

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ABSTRACT

Ochratoxin A (OTA), a mycotoxin produced by various *Aspergillus* and *Penicillium* strains under diverse environmental conditions, has been found as a common contaminant of wide variety of cereals, dried fruits, spices, coffee and fermented beverages. Due to its widespread on such a large variety of agricultural commodities and the potential health risks, mainly toward humans, prompt detection is important.

To prevent OTA contamination in foodstuffs, recently several methods mostly based on PCR-based assays have been developed for identifying and quantifying OTA-producing fungi in food samples. PCR including its different formats remains the technique of choice, thanks to its ability to detect even small amounts of fungal DNA in raw materials and processed foods.

In order to meet food safety concerns and official legislated regulations, analytical techniques have been reported for OTA detection. Although most validated methods are chromatographic techniques, alternatives strategies are emerging and novel technologies using antibodies have been proposed, such as immunoassays, immunosensors and lateral-flow devices. Aptamers recently selected against OTA seem a promising tool and have been used these last years in bioanalytical methods for OTA detection, in electrochemical and optical techniques.

In this review, we discussed innovative analytical methods that have emerged in recent years for the detection of ochratoxigenic fungi and OTA in food samples.

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1. Introduction

Ochratoxin A (OTA) is a low molecular weight mycotoxin produced by certain strains of filamentous fungi of *Aspergillus and Penicillium* and detected in several food matrices. Among the OTA-producing fungi strains, most of them belong to two *Aspergillus* section: the section *Circumdati* (also called the *Aspergillus ochraceus* group) and the section *Nigri* (*Aspergillus carbonarius* and *Aspergillus niger*) (Varga, Kevei, Rinyu, Téren, & Kozakiewicz, 1996). Recently, *Aspergillus westerdijkiae* and *Aspergillus steynii*, two new species from *Aspergillus* section *Circumdati* have been split from *A. ochraceus* and reported to be stronger OTA producer than *A. ochraceus* (Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2011). Among these species, *A. carbonarius* shows higher ochratoxigenic potential, and has been found to be responsible of OTA accumulation in grapes and wines, particularly in Mediterranean region, and in coffee and cocoa (Cabañes et al., 2002; Gallo et al., 2009; Serra, Abrunhosa, Kozakiewicz, & Venâncio, 2003). In contrast with the aspergilli, which occur mainly in regions with warmer climate, the penicillia prefer lower optimum growth temperature. Two *Penicillium* species, *Penicillium nordicum* and *Penicillium verrucosum* are known to produce OTA, and have been frequently isolated from cereal crops, meat products and cheeses (Bogs, Battilani, & Geisen, 2006; Lund & Frisvad, 2003).

Because of its widespread occurrence on a large variety of agricultural commodities and the potential health risks, mainly toward humans, OTA has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (Beardall & Miller, 1994). Given the known human exposure and the abundance of toxicological data from animal studies, the European Union Scientific Committee has recommended the OTA levels below to 5 ng/kg of body weight per day (Sweeney, White, & Dobson, 2000). In the European Union, some regulatory limits have already been introduced for the levels of OTA in food products such as raw cereal grains (5 μg/kg), products derived from cereals (3 μg/kg), dried fruits (10 μg/kg), roasted coffee and coffee products (5 μg/kg), grape juice (2 μg/kg) (EC No 123/2005) and also for all types of wine (2 μg/kg) (amended Regulation EC No. 466/2001).

A dual monitoring could be considered in order to meet food safety concerns and official legislated regulations. First, the presence of fungi having the potential to produce the OTA could be checked at critical points during production of agricultural commodities as well as during the process of food and feed preparation. Early detection of these fungi could prevent OTA contamination in foodstuffs, and protect consumers from hazardous mycotoxins. Usual identification and quantification methods of food-borne fungi require time-consuming and labor-intensive morphological and physiological tests and, often mycological expertise. Moreover, discrimination between the closely related species within the *Aspergillus* genus is particularly difficult, even for expert taxonomists (Dao, Mathieu, & Lebrihi, 2005; Mulè, Susca, Logrieco, Stea, & Visconti, 2006). The current trend is toward culture-independent PCR-based methods because they overcome problems associated with selective cultivation and isolation of microorganisms, and are generally characterized by their simplicity, speed, cost-effectiveness and reliability (Lau, Chen, Sleiman, & Sorrell, 2009; Niessen, 2008).

Subsequently, OTA could be detected directly in food sample using analytical methods able to perform highly selective measurements. Since OTA is a derivative of isocoumarinic acid linked to L-phenylalanine, this toxin displays an optical activity and fluorescence proprieties (Dall'Asta, Galaverna, Dossena, & Marchelli, 2004). For this reason, chromatographic techniques have been usually taken as reference methods because of their accuracy and reproducibility (See (Monaci & Palmisano, 2004; Turner, Subrahmanyam, & Piletsky, 2009) for a review). Nevertheless they are expensive and require long procedures and skilled staff, shortcomings that limit their use for routine analysis. Alternatively, biochemical methods and particularly the immuno-based assays appear as promising techniques for OTA detection. They usually require no or simple sample clean-up such as filtration and dilution, and allow parallel analysis of multiple samples, being a powerful tool for rapid screening (Turner et al., 2009). Recently aptamers have been shown to successfully compete with antibodies as biological receptors for analytical tool development. These single-stranded oligonucleotides are selected *in vitro* in a sort time compared to the antibody production, are more stable under a wide range of conditions. Furthermore, they can be easily modified or labeled providing flexibility to develop a wide range of assessment assays (Mairal et al., 2007).

The purpose of this review is to discuss the recently developed innovative analytical methods used in the analysis and detection of OTA-producing fungi and OTA itself in food matrices (Fig. 1).

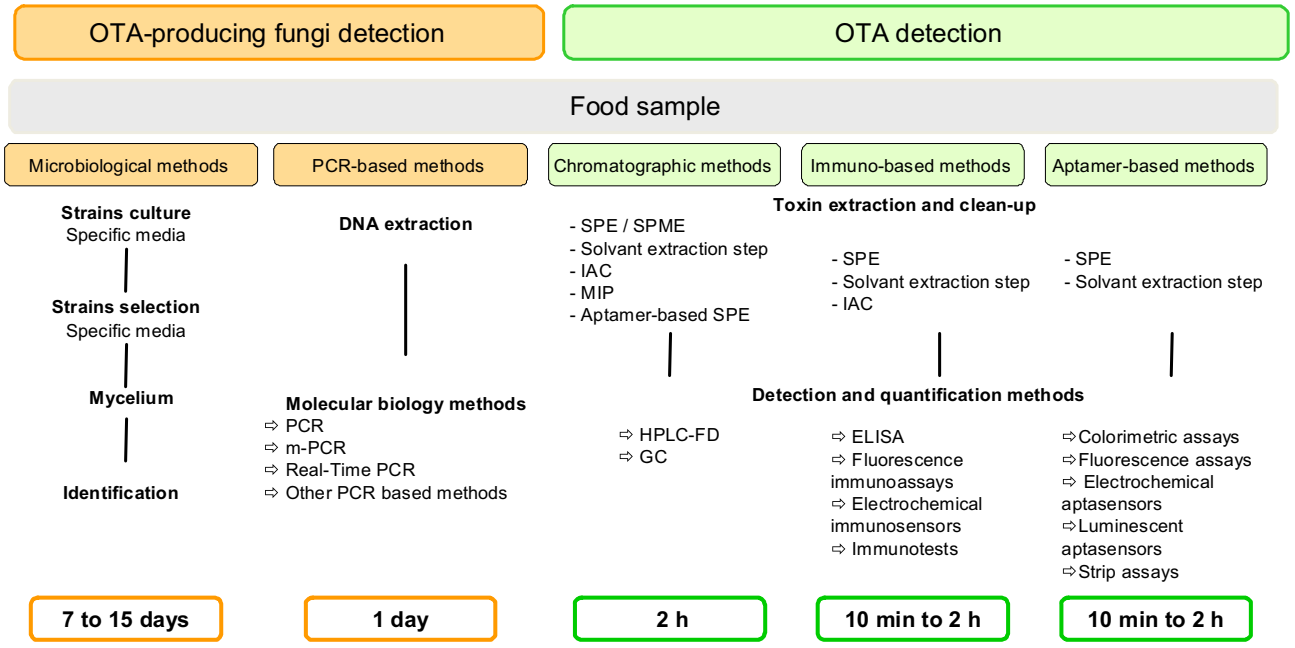

Fig. 1. Analytical methods for OTA-producing fungi and OTA detection.

2. Extraction methods applied to DNA, and OTA before analysis

Extraction and clean-up techniques are of great importance because of their influence on the sensitivity of molecular or analytical methods for OTA-producing fungi analysis or OTA determination in food matrices.

2.1. DNA extraction methods of OTA-producing fungi

The DNA extraction from the sample is a crucial step in determining the sensitivity of the molecular methods. The complex nature of food matrices can strongly affect or inhibit the PCR reaction by binding or denaturing the *Taq* DNA polymerase. Moreover, the fungal cell wall is a complex structure composed of chitin, glucans, lipids and other polymers which are extremely resistant. Therefore, major challenge for good quality DNA isolation from fungi relies on the efficiency of fungal wall lysis protocols that use physical, chemical and enzymatic disruption methods (Karakousis, Tan, Ellis, Alexiou, & Wormald, 2006). No single method of cell lysis is appropriate for all fungi, as each species requires a specific method to efficiently extract DNA. Then, several techniques including laboratory methods using lysis buffer or commercial methods using DNA isolation kit for OTA-producing fungal DNA extraction have been described.

2.1.1. Isolation of DNA using laboratory extraction methods

DNA from OTA-producing fungi was often prepared with slightly modifications of previous reported method (Yelton, Hamer, & Timberlake, 1984). Fungal strains were grown on appropriate medium and mycelium was collected after 2–7 days. Then, the mycelium was frozen in liquid nitrogen, ground to a powder with mortar and pestle, and incubated with lysis buffer containing EDTA and SDS during 15–60 min at 65 °C (Bogs et al., 2006; Geisen, Mayer, Karolewiez, & Färber, 2004; Schmidt-Heydt, Richter, Michulec, Buttinger, & Geisen, 2008). This treatment with alkaline chemicals was used as a non-mechanical disruption method. Digestion enzymes such as Proteinase K (Moslem, Mashraqi, Abd-Elsalam, Bahkali, & Elnagaer, 2010), and RNAse (Atoui, Mathieu, & Lebrihi, 2007) could be added during the incubation. Alternatively, extraction could be performed using a CTAB protocol using a highly salt concentrated lysis buffer (NaCl 1.4 M) with β-mercaptoethanol, EDTA and CTAB (Sartori et al., 2006). After centrifugation, supernatant was extracted traditionally with phenol-chlorophorm and precipitated with cold 2-propanol or ethanol. The compact DNA lump was transferred and washed with ethanol (70%), the pellet was dried and dissolved in buffer.

2.1.2. Isolation of DNA using commercial DNA kit extraction

Several commercial kits are available in order to prepare pure DNA, free from contaminants and enzyme inhibitors. DNeasy Plant mini kit from Qiagen and EZNA Fungal DNA kit from OMEGA BioTek are the most frequently used to extract DNA from fungi (Gallo et al., 2009; Martínez-Culebras et al., 2009; Mulè et al., 2006) or directly from food matrix such as coffee beans (Gil-Serna, González-Salgado, González-Jaén, Vázquez, & Patiño, 2009). Sardiña et al. have optimized the protocol of the Qiagen kit to minimize the effect of polyphenols during PCR reaction, introducing 0.33% of Polyvinylpyrrolidone (PVP) into the kit buffers to eliminate the polyphenols which could interfere in PCR reaction (Sardiñas et al., 2011). In order to optimize these kits, the mycelium or the food sample could be frozen before grinding. This could lead to a more efficient break of the cell wall (Mulè et al., 2006; Sardiñas et al., 2011). Using these kits, DNA isolation is performed under 60 min without using organic solvent.

2.2. OTA extraction methods from food matrix

In general, food samples cannot be analyzed without some preliminary sample preparation, because contaminants are too diluted and the matrix is rather complex. Extraction and clean-up methods are of critical parts in analytical procedures, because they determine the length of the analytical procedure, accuracy, recovery, and achievable detection limits. Although most of the methods for OTA analysis used liquid–liquid extraction (LLE), solid–liquid extraction (SLE) has been developed as alternative,

owing to its simplicity and economy in terms of time and solvent needs. However cleaning effect could be insufficient for the complexity of some food matrices. During the past few years, new techniques have been developed for clean-up process (Monaci & Palmisano, 2004; Turner et al., 2009).

2.2.1. Solid-phase extraction (SPE)

Conventional SPE is generally performed by passing aqueous samples through a solid sorbent in a column. The analyte (OTA) is eluted from the solid medium with an appropriate organic solvent and a washing step is realized with an organic solvent to remove the interferences. One highly important aspect in SPE is the selection of the sorbent. Conventional solid phase materials, such as the surface-modified bonded silica C-18 are frequently used for wine analysis (Kuang et al., 2010; Tessini et al., 2010; Zezza, Longobardi, Pascale, Eremin, & Visconti, 2009). Commercial Sep-Pak RP-18 cartridges have also been used for cereals analysis (Vega et al., 2009).

2.2.2. Solid-phase microextraction (SPME)

In the early 1990s, the solid-phase microextraction (SPME) method for sample extraction was introduced. Several types of SPME have been extensively developed (coated fibers, stir bars, and coatings inside tubes or needles). These sampling devices are portable and easy to use, providing good potential for on-site applications of SPME-based techniques (Ouyang & Pawliszyn, 2006). SPME methods have been successfully applied for direct extraction of OTA in wine samples, with a LOD of 0.07 ng/mL (Aresta, Vatinno, Palmisano, & Zambonin, 2006) and also in more difficult or heterogeneous matrix such as green coffee beans (LOD = 0.3 ng/g) (Vatinno, Aresta, Zambonin, & Palmisano, 2008) or cheese (LOD = 1.5 ng/mL) (Zhang, Cudjoe, Vuckovic, & Pawliszyn, 2009).

2.2.3. Immunoaffinity columns (IAC)

Antibody-based IAC is the more efficient method for the clean-up of complex matrices like coffee, beer, and wine that can be directly loaded onto an IAC column. All the compounds except the target are washed off by water or aqueous buffer and the toxin was eluted by methanol or methanol buffer. Based on this principal, many commercials IACs have been developed for OTA (Table 1) and IACs have been used as a sample clean up tool for OTA determination in numerous methods (Fabiani, Corzani, & Arfelli, 2010; González-Peñas et al., 2004; Heurich, Kadir, & Tothill, 2011; Scott & Trucksess, 1997; Visconti, Pascale, & Centonze, 2001). For solid samples, the purification step could be preceded by a solvent extraction step.

Table 1
Commercial immunoaffinity columns for sample clean-up prior to analysis.

Company	Product name	Suitable for	Time of clean-up
LC Tech (Germany), www.lctech.de	OtaCLEANTM	HPLC-FLD or LC-MS.	nd[a]
Libios (France), www.libios.fr	Puri-Fast OTA IAC	HPLC	nd
R-biopharm AG (Germany), www.r-biopharm.com	OchraprepTM	HPLC	20 min
	RIDA® Ochratoxin A	HPLC or ELISA	nd
Romer Labs® (Austria), www.romerlabs.com	OchraStarTM	HPLC	nd
	OchraStarTMFIT	HPLC	nd
Vicam Science (USA), vicam.com	OchraTestTM	fluorometer or HPLC	15 min
	OchraTestTMWB	HPLC, UPLC or LC/MS/MS	10 min

HPLC-FLD: High performance liquid chromatography with fluorescence detection; LC-MS: Liquid chromatography-mass spectrometry; ELISA: Enzyme-linked immunosorbent assay; UPLC: Ultra Performance Liquid Chromatography, LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry.

[a] nd, not determined.

2.2.4. Molecularly imprinted polymer (MIP)

Attempts to replace the antibody by a synthetic counterpart, molecularly imprinted polymers (MIPs) have been proposed. MIP is a polymer that was formed in the presence of a molecule that is extracted afterward, thus leaving complementary cavities behind. These polymers show a certain chemical affinity for the original molecule and can be used for separation. The first application of MIP in OTA analysis from food extracts has been performed by Zhou in 2004. MIP particles were synthesized from *N*-phenylacrylamide (PAM) and slurry-packed into a microcolumn for selective SPE of OTA. Both binding and desorption of OTA were successfully achieved under optimal conditions (Zhou, Lai, & Miller, 2004). MIP was also prepared using pyrrole (Py) as the functional monomer and was synthesized in the presence of carbon nanotubes in order to enhance the binding capacity of the newly synthesized MIP. With LOD of 0.08 ng/mL and a total analysis time of 30 min for one sample of red wine, the method was very competitive as compared to the SPME method (Yu & Lai, 2006, 2007). Alternatively, a two-dimensional SPE clean-up protocol on C18-silica and a target-selective MIP material has been proposed and tested with red wine samples. Method evaluation with spiked (0.033–1.0 ng OTA/mL) and commercial red wines provided recoveries >90%, with LOD of 0.01 and 0.033 ng/mL (Maier, Buttinger, Welhartizki, Gavioli, & Lindner, 2004). MIP seem also to be very promising and cheaper alternative to IAC sorbents for clean-up and preconcentration of OTA, as recently demonstrated for the clean-up of wheat samples. The developed and validated method shows satisfactory linearity, precision and accuracy (Ali et al., 2010).

2.2.5. Aptamer solid phase extraction affinity columns

Aptamers, a new class of molecules, appear as promising recognition tools for OTA extraction from complex food matrices. Recently, the *in vitro* selection procedure, called SELEX (Systematic Evolution of Ligands by EXponential enrichment), has permitted the identification of DNA aptamer that bind with high affinity and specificity to OTA (Cruz-Aguado & Penner, 2008a). This aptamer has been immobilized by covalent binding on agarose support to develop aptamer-based affinity columns that was used for OTA analysis in wheat samples. OTA concentration was determined either fluorometrically (Cruz-Aguado & Penner, 2008b) or using HPLC (De Girolamo, McKeague, Miller, DeRosa, & Visconti, 2011). The reported aptamer-based columns could be reused five times without affecting the binding affinity of the aptamer toward OTA.

In recent studies, aptamers were covalently immobilized on activated sepharose or on magnetic nanospheres for beer (Rhouati, Paniel, Meraihi, & Marty, 2011), red wine (Chapuis-Hugon, du Boisbaudry, Madru, & Pichon, 2011) and others various food samples analysis (Wu, Duan, Wang, & Wang, 2011). Comparison with commercial IACs showed the high selectivity brought by the oligosorbents and confirmed that the use of aptamers constitutes a good alternative to antibodies due to the numerous advantages of aptamers, such as their proprieties of reusability and their cost of development.

3. PCR-based methods for fungi detection in food samples

Nowadays, PCR has replaced the cumbersome microbiological analysis by amplification of specific genomic markers rather than growing the living organisms under study (Abdin, Ahmad, & Javed, 2010; Niessen, 2008). Due to the strong specificity and high degree of sensitivity, PCR-based methods appear as a good tool to provide early detection of OTA producing in order to control or reduce

fungal mass and toxin production at early and critical stages of the food chain (Dao et al., 2005; Niessen, Schmidt, et al., 2005).

Within the last years, PCR-based methods have been set up for the detection, differentiation and the identification of OTA-producing fungi. A brief description of those methods reported to analyze the OTA-producing fungi in food samples is given below and summarized in Table 2. Specific primers used in these methods are given in Table 3.

3.1. Target of PCR-based methods

One of the most important factors in the set up of PCR-based methods is the targeted DNA sequence of interest organism. PCR systems for the detection of OTA-producing fungi target in general genes involved in the OTA biosynthetic pathway, such as the polyketide synthase gene (*pks*). This gene is supposed to be exclusively present in fungi potentially producing OTA. Besides this gene, also the internal transcribed spacer sequences (ITS1 and ITS2) of the rDNA unit could be used as target.

3.1.1. Characterization of pks genes in OTA-producing fungi

Chemically, OTA consists of an isocoumarin polyketide that is amide-linked to the amino acid L-phenylalanine. The biosynthetic pathway for OTA has not yet been completely established in any fungi. However, the isocoumarin group is formed from acetate and malonate *via* a polyketide synthesis pathway, involving a polyketide synthase (PKS) that is considered as key enzyme for the OTA biosynthesis (Niessen, Schmidt, et al., 2005; O'Callaghan, Caddick, & Dobson, 2003). They are multifunctional proteins with several domains encoded by one gene (Bingle, Simpson, & Lazarus, 1999). Since the KS domain is the most conserved domain among different PKSs, some pairs of degenerated primers targeting this domain have been designed and used to amplify the corresponding DNA fragment from different types of *pks* genes of OTA-producing fungi.

Among the two Aspergillus section of concern, five different *pks* genes have been identified in *A. ochraceus* (Edwards, O'Callaghan, & Dobson, 2002; Varga, Rigó, Kocsubé, Farkas, & Pál, 2003), nine different in *A. westerdijkiae* NRRL 3174 (Atoui, Phong Dao, Mathieu, & Lebrihi, 2006; Bacha, Atoui, Mathieu, Liboz, & Lebrihi, 2009; Dao et al., 2005) as well as five different in *A. carbonarius* 2Mu134 = CBS 120167 (Atoui et al., 2006; Gallo et al., 2009). Some *pks* genes have also been identified in *P. nordicum* (Geisen et al., 2004) and *P. verrucosum* (Schmidt-Heydt et al., 2008).

3.1.2. ITS region primers-based

The sensitivity of PCR-based assay can be considerably improved when multi-copy sequences, such as ribosomal DNA regions, are used. ITS1 and ITS2 regions are present at 100 to 300 copies per haploid fungal genome and are considered high variable regions. This high variability is particularly useful when it is necessary to discriminate among closely related species or at intraspecific level (Abdin et al., 2010; Accensi, Cano, Figuera, Abarca, & Cabañes, 1999). ITS regions, had been used to carry out phylogenetic and population studies in filamentous fungi and to develop specific PCR assays to identify important mycotoxigenic species affecting commodities such as *Fusarium* or *Aspergillus* (González-Jaén, Mirete, Patiño, López-Errasquín, & Vázquez, 2004; Henry, Iwen, & Hinrichs, 2000). Species-specific primers designed on the basis of ITS sequence comparisons have been developed for the identification at the species level of *A. carbonarius, A. ellipticus, A. heteromorphus, A. japonicus*, and *A. niger* (González-Salgado, Patiño, Vázquez, & González-Jaén, 2005; Oliveri, Torta, & Catara, 2008; Patiño, González-Salgado, González-Jaén, & Vázquez, 2005; Perrone, Susca, Stea, & Mulè, 2004). Both ITS1 and ITS2 regions have been

Table 2
PCR, m-PCR and qPCR methods for detection of OTA-producing species in food matrices.

Producer fungal species	Gene target	PCR-based method	Food sample	References
A. carbonarius	*pks* gene	PCR	Grapes	Gallo et al. (2009)
	pks gene: KS domain	PCR	Vineyards	Spadaro et al. (2010)
	pks gene and *ITS*	m-PCR	Wines	Rossi et al. (2011)
	pks gene: AT domain	qPCR	Grapes	Atoui et al. (2007)
	pks gene: KS domains	qPCR	Wine grapes	Selma et al. (2008)
	pks gene: KS and AT domains	qPCR	Fruits, wine	Selma et al. (2009)
	ITS region of the rDNA	PCR	Wine grapes	Patiño et al. (2005), Oliveri, et al. (2008)
		PCR	Chilli, paprika	Sardiña et al. (2011)
		m-PCR	Coffee beans	Sartori et al. (2006)
		qPCR	Grapes	González-Salgado et al. (2009)
	Calmodulin gene	PCR	Grapes	Perrone et al. (2004)
		qPCR	Grape berries	Mulè et al. (2006)
A. niger	*pks* gene: AT domain	PCR	Vineyards	Spadaro et al. (2010)
	pks gene	qPCR	Wines, corn, soya	Castellá and Cabañes (2011)
	ITS region of the rDNA	PCR	Chilli, paprika, Wine grapes	Oliveri et al. (2008), Sardiñas, et al. (2011)
			Grapes, wheat	González–Salgado et al. (2005)
		m-PCR	Coffee beans	Sartori et al. (2006)
A. ochraceus	*pks* gene	PCR	Foodstuffs	Dao et al. (2005)
	ITS region of the rDNA	PCR	Coffee beans, grapes, chilli, paprika	Patiño et al. (2005), Moslem, et al. (2010), Sardiñas, et al. (2011)
		PCR	Grapes, coffee	Gil-Serna et al. (2009)
		m-PCR	Coffee beans	Sartori et al. (2006)
		qPCR	Coffee beans, grapes	Gil-Serna et al. (2009)
A. steynii	ITS region of the rDNA	PCR	Chilli, paprika	Sardiñas et al. (2011)
		PCR	Grapes, coffee	Gil-Serna et al. (2009)
A. westerdijkiae	ITS region of the rDNA	PCR	Grapes, coffee	Gil-Serna et al. (2009)
		PCR and qPCR	Coffee beans, grapes, chilli, paprika	Sardiñas et al. (2011), Gil-Serna et al. (2009)
P. nordicum	*pks* gene	PCR	Cured meats	Bogs et al. (2006)
		qPCR	Wheat	Geisen et al. (2004)
P. veruscosum	*pks* gene	PCR	Cured meat	Bogs et al. (2006)
		PCR and qPCR	Wheat	Schmidt-Heydt et al. (2008)

AT: Acyltransferase, KS: Ketosynthase, ITS: Intergenic Transcribed Spacer.

Table 3
Primers used for PCR, m-PCR and qPCR assays for OTA-producing species detection.

Fungal species	Primer pairs	Primer sequences (5′-3′)	Amplicon size (bp)	References
A. carbonarius	CAR1	GCATCTCTGCCCCTCGG	420	Patiño et al. (2005), Oliveri, et al. (2008), González-Salgado et al. (2005)
	CAR2	GGTTGGAGTTGTCGGCAG		
	AcPKSF1	AGCATCTATGCTGGCCAATC	186	Spadaro et al. (2010)
	AcPKSR1	AATGTACTCTCGCGGGCTAA		
	$OPX7F_{809}$	AGGCTAATGTTGATAACGGATGAT	809	Fungaro et al. (2004), Sartori, et al. (2006)
	$OPX7R_{809}$	GCTGTCAGTATTGGACCTTAGAG		
	PKac2for	TGATCCTCGAGGTGGTGTA	nd	Gallo et al. (2009)
	PKac2rev	TTCGACGTAGGTGGTATCGA		
A. niger	ITS1	TCCGTAGGTGAACCTGCGG	420	González-Salgado et al. (2005), Sardiñas, et al. (2011)
	NIG2	AAAGTCAATCACAATCCAGCCC		
	$OPX7F_{372}$	CAGTCGTCCAGTACCCTAAC	372	Sartori et al. (2006)
	$OPX7R_{372}$	GAGCGAGGCTGATCTAAGTG		
A. ochraceus	OCRA1	CTTCCTTAGGGGTGGCACAGC	400	Patiño et al. (2005)
	OCRA2	GTTGCTTTTCAGCGTCGGCC		
	OCRAF	CTTTTTCTTTTAGGGGGCACAG	430	Gil-Serna et al. (2009)
	OCRAR	CAACCTGGAAAAATAGTTGGTTG		
	OCA-V	ATACCACCGGGTCTAATGCA	260	Sartori et al. (2006)
	OCA-R	TGCCGACAGACCGAGTGGATT		
A. steynii	STEYF	CCACGCGGCCGCCGGGGGAG	315	Gil-Serna et al. (2009), Sardiñas, et al. (2011)
	STEY2	CGGGGGGGACGAGGACCCAAC		
A. westerdijkiae	WESTF	CTTCCTTAGGGGTGGCACAG	430	Gil-Serna et al. (2009), Sardiñas, et al. (2011)
	WESTR	CAACCTGATGAAATAGATTGGTTG		
P. nordicum	otapksPVfor	TACGGCCATCTTGAGCAACGGCACTGC	500	Bogs et al. (2006)
	otapksPVrev	ATGCCTTTCTGGGTCCAGTA		
P. veruscosum	otapksPVfor	CCGCAGGAAGGATCACGAAGAA	415	Schmidt-Heydt et al. (2008)
	otapksPVrev	TCCTACTTCCCCTGCTGCAAATCA		
	otapksPV1for	TTGCGAATCAGGGTCCAAGTA	51	Schmidt-Heydt et al. (2008)
	otapksPV1rev	CGAGCATCGAAAGCAAAAACA		

successfully used to detect and identify toxigenic *Aspergillus* species using PCR assays (Dao et al., 2005; Patiño et al., 2005).

3.2. PCR methods for OTA-producing fungi detection

PCR is a technique for amplifying DNA sequences *in vitro*. Primers (short DNA fragments) containing sequences complementary to the target region along with *Taq* DNA polymerase are key components to enable selective and repeated amplification.

PCR-based methods have been exploited for the identification of *A. carbonarius*, one of the main black *Aspergilli* OTA-producer in coffee, cocoa and grapes. Spadaro et al. have designed a set of primers toward the DNA sequence of a *pks* gene of a strain isolated from a vineyard (Spadaro et al., 2010). The reported method was successfully tested on different species of *Aspergillus* and *Penicillium* present in vineyard or able to produce OTA. Recently, a new *pks* gene (*ACpks*), which appears to contain sequence corresponding to both KS and acyltransferase (AT) regions was identified from an *A. carbonarius* strain isolated from grapes. Specific primers were designed and applied to a preliminary PCR screening of *Aspergillus* isolates (Gallo et al., 2009). A PCR method has also been developed for the differentiation and the detection of two *Penicillium* species, *P. nordicum* and *P. verrucosum*, responsible of OTA occurrence in cereals products and fermented meats, respectively. The reported PCR which is targeted against two genes of the OTA biosynthetic pathway is a very reliable method to distinguish those two very similar *Penicillium* species (Bogs et al., 2006). These last years, Gil-Serna group has described specific PCR protocols based on the multi-copy ITS region to detect and discriminate among the main ochratoxigenic species of *Aspergillus* in foodstuffs within section *Circumdati*: *A. westerdijkiae*, *A. ochraceus* and *A. steynii* (Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2009; Patiño et al., 2005). 39 *Aspergillus* strains isolated from diverse food matrices (e.g. grapes, coffee, spices, cereals) were successfully tested. More recently, 93 samples of paprika and chilli collected in Spain were tested for PCR amplification using a set of species-specific primers for seven *Aspergillus* species (Sardiñas et al., 2011). The most common OTA-producing Aspergilli were *A. niger* aggregate (67.7%), followed by *A. carbonarius* and *A. steynii*.

All these reported methods demonstrate that identification based on PCR amplification of targeted genes is considered to be a reliable technique. The developed species-specific PCR strategies have been validated and could be routinely used to identify OTA-producing fungi.

3.3. m-PCR detection for OTA-producing fungi detection

The term “multiplex PCR” (m-PCR) refers to simultaneous amplification of many targets in a single reaction by using more than one pair of primers. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require more reagents and several times. The primer design must be rigorous in order to find a compromise between the annealing temperatures and durations of optimal elongation of each PCR reactions. This choice is not easy, especially as other constraints exist on the nature of the locus to be amplified.

The first m-PCR for the detection of *A. carbonarius, A. niger* and *A. ochraceus* in food sample was developed in 2006, using three species-specific primer pairs designed from ITS regions of rRNA genes (Sartori et al., 2006). The reported method was tested with inoculated, and naturally contaminated coffee bean samples, and rendered results in 48 h, saving reasonable time and effort in comparison to classical methods. Recently, a m-PCR based assay for the detection of *A. carbonarius* and *A. niger* has been developed and applied to berries of some grape varieties used in Italy for producing wine (Rossi et al., 2011). Two primers pairs were used targeting the *Acpks* gene sequence of both species and the ITS of the rDNA units of *A. carbonarius* leading to a dual signal only for *A. carbonarius.* The mPCR-based assays described showed high sensitivity and effectiveness in detecting *A. carbonarius* from grape DNA.

Hence, the two m-PCR techniques developed are useful to rapidly identify several isolates and represents a contribution to the early detection of fungal contamination in important food matrices.

3.4. Real time-PCR-based detection for OTA-producing fungi detection

The real-time PCR (*qPCR)* combines the sensitivity of conventional PCR with the generation of a specific real-time fluorescent signal throughout the reaction, thus allowing immediate analysis of the reaction kinetics and quantification of specific DNA targets. The amplification of the PCR product can be detected using either no-specific fluorescent dyes that intercalate with any double-stranded DNA, such as SYBR Green dye or labeled probes, such TaqMan hydrolysis probe. Both strategies have been used in monitoring and quantification of OTA fungal producer.

Detection of *A. carbonarius* isolated from grapes samples have been performed using qPCR assays based on sequences of *pks* gene (Atoui et al., 2007) or on conserved regions of the *A. carbonarius* calmodulin gene (Mulè et al., 2006). According to the positive correlation found between *A. carbonarius* DNA content and OTA concentration in these studies, it can be consider that *A. carbonarius* DNA content lower than 10 pg DNA/mg of berry could assure good security. Selma et al. (2009) have developed a duplex qPCR system that combines the *A. carbonarius*-specific β-ketosynthase domain assay and the amplification of the acyltransferase (AT) domain of the species belonging to the *A. niger* aggregate using SYBR Green and TaqMan qPCR approaches (Selma, Martínez-Culebras, Elizaquível, & Aznar, 2009). Both procedures were successfully used to monitor, in a single reaction, the presence of both OTA-producing species in grapes. Recently, a new qPCR procedure has been developed for the rapid and specific detection and quantification of *A. niger* aggregate using as a target the *pks* gene (Castellá & Cabañes, 2011). Among a total of 91 strains assayed in this study, 40 were isolated from foods samples such as wine grape, corn, soya and feedstuffs. Other examples of qPCR system targeting a *pks* gene were developed for monitoring growth and OTA production of *P. nordicum* from wheat (Geisen et al., 2004) and of *P. verrucosum* also from wheat (Schmidt-Heydt et al., 2008).

In order to enhance the sensitivity and the specificity of qPCR assays, multi-copy sequences can be used to design specific primers. González-Salgado et al. have developed two qPCR assays, based on the ITS sequence, using SYBR Green dye and TaqMan probe for the detection and quantification of *A. carbonarius* (González-Salgado, Patiño, Gil-Serna, Vázquez, & González-Jaén, 2009). These protocols allowed detecting at least 10^6 spores without incubation, or a minimum of 0.4 pg DNA/g grape berries. In the same time, Gil-Serna et al. (2009) have developed a qPCR assay also based on the use of ITS region to detect and quantify *A. ochraceus* and *A. westerdijkiae* contamination in grapes and green coffee beans (Gil-Serna, Gonzales-Salgado, et al., 2009). According to the experiments of the reported studies, the lower cost of qPCR based on SYBR Green is an advantage of this method for detection and quantification protocols used in routine analyses of commodities.

By using qPCR, a positive correlation between OTA content and gene expression has been indicated for *P. nordicum, P. verrucosum, A. ochraceus* and *A. carbonarius* (Atoui et al., 2007; Geisen et al., 2004; Mulè et al., 2006; Schmidt-Heydt et al., 2008). These studies demonstrated that the production of OTA in relation to the DNA content is dependent either on environmental conditions or the physiological activity of the species analyzed. Moreover, a positive correlation has been found between *A. carbonarius* DNA content and OTA concentration in food samples, allowing for the estimation of the potential risk from OTA contamination. qPCR appears to be a highly suitable method for identifying and quantifying OTA-producing fungi.

3.5. Other PCR-based methods for OTA-producing fungi detection

3.5.1. Restriction fragment length polymorphism–PCR

Nowadays, Restriction fragment length polymorphism (RFLP) of total genomic DNA could be coupled to PCR allowing differentiation and also identification of microbial isolates. The ITS rDNA fragments of 173 *A. niger* strains isolated from grapes were amplified by PCR and their amplicon were digested by the endonuclease *Rsa*I (Accensi et al., 1999; Bau, Castellá, Bragulat, & Cabañes, 2006). The reported method allowed to screen the *A. niger* aggregate strains and to classify the OTA-producing strains in type N (characterized by two fragments of 519 and 76 bp) and the non OTA-producing strains in type T (characterized by one fragment of 595 bp). RFLP-PCR has also been applied to the identification of *A. niger*, *A. tubingensis*, *A. carbonarius* and *A. aculeatus* isolated from grapes using specific-species ITS primers, and *HhaI*, *NlaIII*, and *RsaI enzymes* (Martínez-Culebras & Ramón, 2007), and to the screening of OTA-producing isolates of the *A. niger* aggregate targeting ITS, Intergenic Spacers (IGS) and β-tubulin gene portion, and *Rsa* I, *and Hinf* I enzymes (Zanzotto, Burruano, & Marciano, 2006).

3.5.2. Amplified fragment length polymorphism

The Amplified fragment length polymorphism (AFLP) technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. Results obtained with AFLP analysis by Schmidt et al. indicate that no clear correlation between genetic similarity of the *A. carbonarius*, and *A. niger* strains studied and their potential to produce OTA has been found (Schmidt, Taniwaki, Vogel, & Niessen, 2004). AFLP was also employed for the study of the intraspecific variability of 17 *Aspergillus* strains isolated from berry and allowed to verify the presence of mixed populations in the same vineyard (Oliveri et al., 2008). AFLP analysis might be appropriate to discern isolates at intraspecific level.

3.5.3. Random amplification of polymorphic DNA

The Random Amplification of Polymorphic DNA (RAPD) method produces distinctive sets of DNA fragments when genomic DNA is subjected to PCR primed by short arbitrary oligonucleotide primers. 25 strains of *A. ochraceus* collected from coffee beans were subjected to RAPD analysis. The isolates were resolved into 2 distinct groups, each containing both OTA producing and non-producing strains, indicating that there is no association between molecular genotypes and the ability to produce the toxin (Fungaro et al., 2004). A RAPD analysis with *Penicillium* strains isolated from ham was carried out (Bogs et al., 2006). From the 62 strains tested, 11 which produce OTA had the same pattern than the control strain of *P. nordicum*. None of the other strains isolated produce OTA, and most of them had a RAPD pattern characteristic to *Penicillium nalgiovense*, indicating the congruence of all data.

4. Biochemical methods for OTA analysis from food samples

As mycotoxin contamination occurs in trace amount, highly sensitive and accurate methods are required for OTA detection. Biochemical methods have been emerged as a powerful alternative to classical methods in recent years. The different biochemical methods that could be used for OTA analysis are described below.

4.1. Immunology-based methods

Immunoassays are based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific toxin from other molecules. High specificity and sensitivity of antibodies permit sample dilution that reduces matrix effects from samples. Immunoassays are promising tools for routine detection and

quantification, due to the high sample throughput and relative low cost. Moreover, they require neither sophisticated equipment nor skilled personnel. Due to the small size of OTA (MW 403.8), sandwich assay schemes are not possible. All these methods are based on the competition strategy and have the potential for rapid qualitative or semi-quantitative measurements. Immunological methods developed for OTA detection are briefly described below and those applied to food sample analysis are summarized in Table 4.

4.1.1. Enzyme-linked immunosorbent assays (ELISA)

The first immunologic method developed for OTA determination was an enzyme-linked immunosorbent assays (ELISA) (Kawamura et al., 1989). Since then, a number of ELISA formats have been performed and validated for OTA analysis. ELISA is well established as a high throughput assay with low sample volume requirements, and often has less sample clean-up procedures compared to conventional HPLC methods.

The basic principal of the assays is the immobilization of biomolecules (antibody or antigen) onto the wells of microtiter plates, followed by a competitive process, between free OTA and enzyme labeled biomolecules. In the direct competitive ELISA format, the 96-well ELISA plate was coated with antibodies. After blocking step, the competition was allowed to proceed between free OTA from sample and enzyme-labeled OTA. Several washing steps were then performed to remove the unbound OTA. Finally, substrate solution was added and the absorbance was read using appropriate instrument. In the direct competitive ELISA format, OTA derivates, conjugated to bovine serum albumin (BSA) were adsorbed onto the wells of the microtitre plate, followed by blocking step. Then, the competition was allowed to proceed, by adding free OTA from sample and primary anti-OTA antibody. Secondary enzyme-labeled anti-IgG antibody was added and allowed to react. Washing steps were applied between each reaction step to remove unbound compounds. Finally, substrate solution was added and the absorbance was read.

Our group has recently reported a direct-competitive ELISA using an OTA–horseradish peroxidase synthesized conjugate for determination of OTA in wines samples (Radoi, Dumitru,

Table 4
ELISA, immunosensors and non-instrumental immunotests developed for OTA detection in food matrices.

Method	Matrix	Extraction and clean-up method	LOD μg/L	References
ELISA	Chicken meat, wheat flour	nd	0.025	Kawamura et al. (1989)
	Roasted coffee	Aminopropyl clean-up	4 μg/kg	Sibanda, De Saeger, Barna-Vetro, & Van Peteghem (2002)
	Natural sweet wine	IAC clean-up	0.054	Alcaide et al. (2008)
	Must and wine	IAC clean-up	0.005	Flajs et al. (2009)
	Corn sample	Methanol extraction	1.7 μg/kg	Giray, Atasayar, & Sahin (2009)
	Red and white wine	IAC clean-up	0.1	Fabiani et al. (2010)
	Wine	Dilution in water	0.003	Giesen et al. (2010)
	Wine	IAC clean-up	0.1	Radoi et al. (2009)
	Cereal	Methanol extraction	0.55	Zhang et al. (2011)
	Cereal flour	Methanol extraction	1.7 μg/kg	Folloni et al. (2011)
	Cereal	nd	0.01	Yu, Vdovenko, Wang, & Sakharov (2011)
Fluorescence polarization immunoassay	Barley	Methanol extraction	3	Shim et al. (2004)
	Cereal	Methanol extraction	0.02	Huang, Tao, Shi, Tang, & Jin (2006)
	Red wine	SPE clean-up	0.7	Zezza et al. (2009)
	Cereal	Methanol extraction	0.05	Huang et al. (2009)
Enzyme-linked electrochemical immunosensor	Wine	nd	0.18	Alarcon et al. (2004)
	Wheat	Acetonitrile extraction	0.06	Alarcon et al. (2006)
	Wine	PVP clean-up	0.3	Prieto-Simon et al. (2008)
	Corn	Methanol extraction	0.008	Liu et al. (2009)
	Wheat	Acetonitrile extraction	0.2	Bonel et al. (2010)
	Wheat	Acetonitril extraction	0.10	Vidal, Bonel, Duato, & Castillo (2011)
	Wine	IAC clean-up	0.05	Heurich et al. (2011)
	Apples	nd	0.05 μg/kg	Fernández et al. (2011)
	Red wine	nd	0.008	Perrotta et al. (2012)
Label free immunosensor	Wine grapes	Methanol extraction	0.02	Fernández et al. (2010)
	Food samples	Acetonitrile extraction and Mycosep clean-up	0.1	Van der Gaag et al. (2003)
	Barley and wheat flour	Acetonitrile extraction	0.5	Adanyi et al. (2007)
	Cereals and beverages	Methanol extraction	0.058	Yuan et al. (2009)
	Wine	nd	0.94	Zamfir et al. (2011)
Array biosensor	Cereals, coffee and wine	Methanol extraction	3.8 μg/kg	Ngundi et al. (2005)
	Cereals	Methanol extraction	150 μg/kg	Ngundi et al. (2006)
	Green coffee	IAC clean-up	0.3	Sauceda et al. (2011)
Non instrumental immunotest based on visual detection	Wheat	nd	0.4	De Saeger et al. (1999)
	Cereal and beverage	Methanol extraction	1	Wang, Liu, Xu, Zhang, & Wang (2007)
	Red wine and coffee	Methanol extraction	1	Saha et al. (2006)
	Colored herbs and spices	Methanol extraction	nd	Goryacheva, De Saeger, et al., 2007
	Spices	Methanol or chloroform extraction	10	Goryacheva, De Saeger, Delmulle, et al. (2007)
	Coffee	nd	5	Liu et al. (2008)
	Beer	nd	0.2	Goryacheva, Basova, et al. (2007)
	Cereals and soybean	Methanol extraction	10	Lai et al. (2009)
	Red wine	Clean-up column	2	Rusanova et al. (2009)
	Red wine	Clean-up layer	2	Beloglazova et al. (2010)
	Red wine, red pepper	Clean-up adsorbent layer	2	Goryacheva et al. (2010)
	Maiz and barley	Methanol extraction	5	Urusov, Kostenko, Sveshnikov, Zherdev, & Dzantiev, 2011b

LOD: Limit of detection; nd: not determined; ELISA: Enzyme-linked immunosorbent assay; IAC: Immunoaffinity column; SPE: Solid phase extraction; LLE: liquid–liquid extraction; PVP: Poly (vinylpyrrolidone).

Barthelmebs, & Marty, 2009). Different ELISA formats have also been reported for detection of OTA in chicken meat, barley, wheat, maize, and wine samples (Flajs, Domijan, Ivić, Cvjetković, & Peraica, 2009; Kawamura et al., 1989). Using this technique, some commercial companies have developed and marketed ELISA microtiter plate kits which have well defined applicability, analytical range and validation criteria. Some of them are particularly well adapted for OTA determination in cereals, dried fruits, coffee, cocoa, tea, beer, wine and grape samples (Alcaide & Aguilar, 2008; Fabiani et al., 2010; Giesen, Jakubowski, Panne, & Weller, 2010; Zhang et al., 2011). Although ELISA tests showed detection limit in the range of 0.005–0.55 μg/L, but the possibility of false positive and negative results requires confirmatory LC tests. Moreover, ELISA methods are still time-consuming. The performance of one to four working steps such as washing, blocking, sample incubation, and staining requires a total time of 30 min to 3 h to obtain the test results.

4.1.2. Fluorescence immunoassay

Fluorescence methods are less expensive than other immunoassay, and are more suitable for automation. The first fluorescence competitive immunoassay for OTA determination was developed by Shim et al. in 2004 (Shim et al., 2004). When the method was validated with contaminated barley samples, a matrix effect was observed. Later on, a fluorescence method has been successfully applied to 154 naturally contaminated red wine samples (Zezza et al., 2009). However, the LOD range (0.02–3 μg/L) for fluorescence assay is higher as compared to those obtained with conventional ELISA. Furthermore, labeling of biomolecules with fluorochrome is required to perform fluorescence detection.

4.1.3. Enzyme-linked electrochemical immunosensors

Immunosensors are analytical devices, which utilize the sensitivity and selectivity of antibody closely connected to or integrated within a physical transducer (e.g., electrochemical, mass, optical, thermal) and coupled to a data acquisition and processing system. Among the different transduction methods, electrochemical techniques were selected to combine the high sensitivity of this method to the high selectivity of antibodies. Immunosensors present several advantages over traditional antibody-based ELISAs such as the ability to perform faster analysis and continuous monitoring, and the feasibility of miniaturisation making them portable.

The first immunosensor reported for OTA detection was based on a direct competitive enzyme-linked assay using polyclonal antibodies immobilized on carbon screen printed electrodes. Differential pulse voltammetry was used as the electrochemical method for detection and the sensor was applied to the analysis of wheat samples, obtained after one-step extraction procedure (Alarcón, Micheli, Palleschi, & Compagnone, 2004). Since then, electrochemical immunosensors based on disposable carbon screen printed electrodes have been developed for the detection of OTA in wine samples by our group (Prieto-Simón, Campàs, Marty, & Noguer, 2008) and in wheat samples (Alarcón et al., 2006; Bonel, Vidal, Duato, & Castillo, 2010). Since immobilization surface is of great importance in immunosensor development, gold screen printed electrode surface modified with a self assembled monolayer of 1,6-hexanedithiol, 4-nitrophenyl diazonium salt and carboxymethylated dextran has also been used to detect OTA in corn and wine samples (Heurich et al., 2011; Liu et al., 2009; Radi, Muñoz-Berbel, Cortina-Puig, & Marty, 2009). Recently an enzyme-linked electrochemical immunosensor combining magnetic beads as solid phase for antibody immobilization and screen printed electrodes for electrochemical detection has been developed. The sensor showed very lower LOD (0.008 μg/L) as compared to already developed electrochemical immunosensor, in addition to its potential for field applications (Perrotta, Arévalo, Vettorazzi, Zón, & Fernández, 2012).

The acceptable sensitivity, specificity and selectivity achieved by the described immunosensors prove their suitability to analyze complex samples for food applications.

4.1.4. Label free immunosensor

Because of the complexities involved using labeled reagents, it is certainly preferable to avoid their use and instead monitor the binding reaction directly. Efforts are now focused on developing alternative label-free strategies with convenient detection techniques. Recently, the development and characterization of an electrochemical method using square wave voltammetry combined with the use of modified magnetic nanoparticles has been reported (Fernández-Baldo, Bertolino, Messina, Sanz, & Raba, 2010). This label free method allowed a rapid and sensitive determination of OTA in wine grapes and is proposed to be suitable for the detection and quantification of OTA in apparently healthy fruits post-harvest for assuring safety and quality of food. Competitive immunosensors based on surface plasma resonance (SPR) and optical waveguide lightmode spectroscopy have been developed for OTA detection in cereals and beverages without a clean-up step (Adányi et al., 2007; Van der Gaag et al., 2003; Yuan, Deng, Lauren, Aguilar, & Wu, 2009; Zamfir et al., 2011). Recently an immunoanalytical system based on SPR detection amplified by the use of colloidal gold immunoconjugate has been reported (Urusov, Kostenko, Sveshnikov, Zherdev, & Dzantiev, 2011a). These immunoassay techniques may be suitable for cheep and portable equipments.

Electrochemical impedance microscopy is a powerful technique that can be used to study the electrical properties of the device interface and trace the reaction occurring on it. Our group has recently used this technique to develop an immunosensor for the sensitive detection of OTA. The OTA antibody was immobilized making use of the carbodiimide chemistry on gold surface screen printed electrodes (Radi, Muñoz-Berbel, Lates, & Marty, 2009). Other impedimetric immunosensors based on nanocrystalline bioactive TiO_2-chitosan, chitosan/polyaniline, and acacia gum modified surfaces for antibody immobilization have been reported for OTA detection (Khan & Dhayal, 2008, 2009; Khan, Dey, Hazarika, Saini, & Dhayal, 2011). Nanoparticles have also been used to increase the electroactive surface area for the immobilization of antibody thus enhancing electron transfer to electrode surface and increasing the sensibility of the system. Chitosan-fumed silica nanoparticles and chitosan-iron oxide nano partiles films have been used to co-immobilize antibody and BSA for OTA detection (Kaushik, Solanki, Ansari, Ahmad, & Malhotra, 2008, 2009). The LOD values observed with label free detection (0.02–0.94 μg/L) was comparable to the above described methods. Label-free detection methods are particularly interesting to prevent conjugation protocols and to avoid disturbances from conjugated markers. Up to now, all the reported label-free immunosensors have not been tested with real samples. Although, immunosensors based of transducer techniques, such as SPR or EIS are a direction in which innovation can be expected.

4.1.5. Array immunosensors

Array biosensors are mainly used to detect multiple analytes simultaneously on a single substrate. They are rapid, simple to use and require no sample pretreatment prior to analysis. Ngundi et al. reported an array biosensor for detection of OTA in cereals and beverages (Ngundi et al., 2005). Later on, same group demonstrated the versatility of the array biosensor for the detection of mycotoxins OTA, DON, AFB1 and FB simultaneously (Ngundi, Shriver-Lake, Moore, Ligler, & Taitt, 2006). Recently, a regenerable immunobiochip using an automated microarray chip reader has been reported for OTA determination in green coffee extract (Sauceda-

Friebe et al., 2011). The developed sensor with chemiluminescence detection showed a LOD of 0.3 μg/L, many folds lower than the other array biosensors for OTA detection. This method without any sample clean-up showed a rapid and inexpensive screening for OTA in green coffee extract.

4.1.6. Non instrumental immunotests based on visual detection

Rapid disposable membrane-based assays have been developed in multiple formats, such as membrane-based test strips and flow-through tests. Those tests are based on a direct visual reading. They are simple and powerful tools for the preliminary screening of OTA-contaminated samples. Apart from their cost-effectiveness, they are characterized by the simple and fast analysis, which moreover can be performed at the point of sampling (Krska & Molinelli, 2009).

Membrane-based test strips, also called a lateral-flow devices (LFD) are based on the capillary migration of the sample along the strip. If OTA is present in the sample, the toxin bind to anti-OTA antibody conjugated to colloidal gold particles. Otherwise, those labeled antibodies will bind to OTA immobilized in the test line, allowing color particles to concentrate and form a visible line. The intensity of the test line will be inversely correlated to OTA concentration in the sample. The assay is validated in the control line, where anti-antibody will capture charged and uncharged conjugated antibody. LFD are used for the specific qualitative or semi-quantitative detection of OTA. LFD kits are commercially available (ELISA Technologies, USA) and allow a rapid screening of OTA in wine under non-laboratory environments in conformity with the EU regulations. However, membrane-based immunoassays result often in false-positive responses, particularly in the analysis of red wines, due to matrix interferences.

Flow-through membrane-based immunoassays are comparable with LFD in rapidity and ease of use. Anti-OTA antibody was coated onto a membrane and competitive immunoassay was performed by dropped successively the following reactants: anti-OTA antibodies, the sample (with or without OTA), OTA-HRP conjugate and finally the HRP substrate for color reaction. Washing step was performed between additions of each compound. The possibility of using this device for the detection of OTA has been explored in wine samples (Saha, Acharya, & Dhar, 2006). However, Rusanova et al. have demonstrated that major restrictions arise from the red wine matrix sample, which negatively affects both the selectivity and the sensitivity of the membrane-based test (Rusanova et al., 2009). These authors have investigated a sepharose gel-based flow though assay, combined with a clean-up column to analyze red wine samples. Comparison of this method with HPLC-FLD validated the proposed assay. This method was optimized for a simultaneous detection of OTA and 2,4,6,-tricholorophenol in wine samples (Beloglazova et al., 2010). Recently, Goryacheva et al. have proposed a purification method of highly colored food product for the immunoassay based on visual detection (Goryacheva, Rusanova, Beloglazova, Voronov, & De Saeger, 2010), and applied the approach to OTA analysis in red wine samples. Many tests have also been developed for the detection of OTA in grains, roasted and green coffee, high colored herbs, spices and wheat samples (De Saeger & Van Peteghem, 1999; Goryacheva, De Saeger, Delmulle, et al., 2007; Goryacheva, De Saeger, Nesterenko, Eremin, & Van Peteghem, 2007; Lai, Fung, Yang, Renrong, & Xiong, 2009; Saha et al., 2006). All the immunostrip tests employed in the analysis of OTA have their advantages and limitations. The main shortcoming of immunostrips is their limited sensitivity with the detection limit in the range from 0.4 to 10 μg/L. Nevertheless, in most cases the achieved sensitivity is enough to use them as inexpensive and fast screening tools, complementary to more accurate techniques.

4.2. Aptamer-based assays

Aptamers are short single-stranded oligonucleotides engineered through an *in vitro* selection procedure, also called SELEX. In this screening process, a random sequence oligonucleotide library, usually consisting of approximately 10^{14}–10^{16} oligonucleotide strands with a random sequence of defined size, is incubated with the target of interest, such as OTA. Sequences that bind to the target are separated from the unbound species using a suitable partitioning method, and then, the sequence of these candidates is amplified using PCR. This population of selected sequences represents a mixture of oligomers with variable affinity toward the target analyte. The single-strand population obtained after the purification step is incubated with a fresh sample of the target for the next round of selection. Iteration of the above protocol results in the isolation of a pool of nucleotide sequences displaying sequential motifs, which after 8–15 iterative SELEX runs converge to one or a few binding sequences. Once the sequence is identified, an aptamer is produced by chemical synthesis (Stoltenburg, Reinemann, & Strehlitz, 2007). The SELEX process has been applied for the screening of DNA aptamers against OTA (Barthelmebs, Jonca, Hayat, Prieto-Simon, & Marty, 2011; Cruz-Aguado & Penner, 2008a).

Aptamers hold significant advantages over antibodies. As they are chemically synthesized, their production does not require the use of animal and is therefore less expensive and tedious. Aptamers can also be easily labeled with a wide range of reporter molecules such as fluorescent dyes, enzymes, biotin, or aminated compounds, enabling the design of a variety of detection methods (Jayasena, 1999). Furthermore, the function of immobilized aptamers can be easily regenerated and aptamers can be reused. Due to these advantages, aptamers can thus be considered as a valid alternative to antibodies or other bio-receptors. The various analytical techniques based on aptamers selected against OTA, developed these last years are described below and summarized in Table 5.

4.2.1. Aptamer-based colorimetric assay

Along with the rapid progress in modern analytical technologies, more and more aptamer-based colorimetric assays have been developed to replace immunoassay. Aptamer –base colorimetric assays have attracted particular attraction because of the versatility in the immobilization of aptamer. Recently, three methods were performed by our research group to develop aptamer-based colorimetric assays for OTA detection. Barthelmebs et al. has investigated competitive enzyme-linked aptamer assays (ELAA) for OTA determination in wine and compared results to the classical ELISA. The direct format has been successfully applied for the detection of very low amounts of OTA in wine sample (1 ng/ml) in less than 125 min (Barthelmebs, Jonca, et al., 2011). Yang et al. presented an aptamer-based colorimetric assay for OTA using unmodified AuNPs as indicator. The aptamer could easily be adsorbed on AuNPs, thus enhancing the stability of the nanoparticles, even in phosphate buffer. Addition of OTA induced a change in the conformation of aptamer that lost the ability to protect AuNPs, leading to salt-induced AuNPs aggregation. This phenomenon is indicated by a color modification from red to blue that could easily be observed by the naked eye. The developed assay requires only 5 min for a measurement, but a preconcentration step should be added for real sample analysis due to the high LOD obtained (8 μg/L) (Yang, Wang, Marty, & Yang, 2011). The same author reported another aptasensor based on horseradish peroxidase-mimicking DNAzyme and the OTA specific aptamer sequence. The developed aptasensor was validated with wine samples and showed a linear relationship with OTA concentration

Table 5
Aptamer-based assays and sensors developed for OTA detection in food samples.

Method	Matrix	Extraction and clean-up method	LOD μg/L	References
Enzyme-linked aptamer assay	Wine	PVP clean-up	1	Barthelmebs et al. (2011)
	Wine	Toluene extraction	1	Yang et al. (2011)
Aptamer-based fluorescence assay	Wheat	nd	2	Cruz Aguado and Penner (2008b)
	Maize	Chloroform extraction	0.011	Duan et al. (2011)
	Red wine	PVP clean-up	7.48	Sheng et al. (2011)
	Beer	nd	9.64	Guo et al. (2011)
	Corn	Methanol extraction	0.8	Chen et al. (2012)
Enzyme-linked amperometric aptasensor	Red wine	SPE clean-up	0.030	Kuang et al. (2010)
	Wine	PVP clean-up	0.11	Barthelmebs et al. (2011)
	Wheat	Acetonitrile extraction	0.07	Bonel et al. (2011)
	Red wine	nd	0.2	Tong, Zhao, Zhang, Xu, & Chen (2012)
Label free aptasensors	Wheat starch	Acetonitrile extraction	0.001	Tong et al. (2011)
Luminescent aptamer biosensor	Wheat	Methanol extraction	0.007	Wang et al. (2010)
	Maize	nd	0.0001	Wu et al. (2011)
Aptamer-based strip assay	Red wine	SPE clean-up	0.001	Wang et al. (2011)

LOD: Limit of detection; PVP: Poly (vinylpyrrolidone); SPE: Solid phase extraction; nd: Not determined.

upto 4 μg/L, with a LOD of 1 μg/L (Yang, Lates, Prieto-Simón, Marty, & Yang, 2012).

4.2.2. Aptamer-based fluorescence assay

Fluorescent detection is widely used in the fabrication of aptamer-based assays due to the ease of labeling aptamers with different fluorescent dyes, fluorophores and quenchers. Several strategies have been employed to convert aptamers into fluorescent signal probes. A fluorescence polarization assay, based on the displacement of a fluorescently labeled oligo from the aptamer by OTA has been performed (Cruz-Aguado & Penner, 2008b). The presented method provided an advantage over fluorophore-quenching systems and other steady-state fluorescence approaches in that no modification of the aptamer or the target is required. Duan et al. developed an assay based on the fact that when the immobilized aptamer bonds to the OTA, it can induce the conformation change of aptamer, finally leading to the fluorescence signal change. The developed technique has been applied successfully for OTA determination in maize samples (Duan, Wu, & Wang, 2011). Some research groups have also reported assays based on the quenching fluorescence of free aptamer by different kinds of quenchers for OTA detection in red wine and beer samples. Nevertheless, the detection limit obtained in both cases was upper the regulatory limits of the European Union, preventing the use of the reported aptasensors to food control (Guo, Ren, Wang, & Wang, 2011; Sheng, Ren, Miao, Wang, & Wang, 2011). Recently, Chen et al. have developed a target-induced structure-switching aptasensor with fluorescence detection. The developed assay system showed a wide linear detection range (1–100 μg/L) and a LOD of 0.8 μg/L was achieved. The biosensor displayed good recovery values (83–106%), when tested with corn samples (Chen, Fang, Liu, & Zeng, 2012).

4.2.3. Labeled amperometric aptasensor

Aptamers due to their flexible single-stranded chains have the ability to fold into well defined three-dimensional structures upon binding to the target molecule. This behavior facilitates the aptamer–target complex formation by probing the electron transfer features of the redox moieties. Based on this strategy, Kuang et al. developed an ultrasensitive electrochemical platform for the detection of OTA in red wine. In this method, three single-stranded DNA molecules, including the aptamer have been used. The first one (DNA1), complementary to the aptamer has been immobilized on the surface of an electrode. The second one (DNA3), complementary to the aptamer has been functionalized with a gold nanoparticule (AuNP). Methylene Blue (MB) has been used as electrochemical probe. The target OTA competed with both DNA1 and DNA3 to combine with the aptamer, inducing lost of DNA from the surface of the electrode and reducing the redox current of MB (Kuang et al., 2010). The increasing interest on the use of magnetic particles has also favored their combination with aptamers. Last year, our group has reported the development of a novel electrochemical aptasensor, based on disposable screen printed electrodes, using magnetic particles as solid support (Barthelmebs, Hayat, Limiadi, Marty, & Noguer, 2011). This strategy of immobilization was shown to improve the performance of the assay because magnetic beads provide a relatively large numbers of binding sites for biochemical reactions, which offer higher efficiency of interactions between the samples and reagents, resulting in faster assay kinetics for the sensitive detection. The reported aptasensor was successfully used for OTA analysis in wine samples. Magnetic beads were also used as solid support for the development of electrochemical competitive aptasensors for the detection of OTA in wheat samples. The developed assays showed a very low limit of detection and mid point values (Bonel, Vidal, Duato, & Castillo, 2011). The sensitivity and limit of detection (in the range of 0.03–0.8 μg/L) of the reported aptasensors is higher to that obtained using amperometric immunosensors with antibodies to OTA, that validated the application of electrochemical aptasensors for OTA detection.

4.2.4. Label free aptasensor

The main problem associated with electrochemical aptasensors is the labeling of aptamer or biomolecule to perform electrochemical detection. To overcome this limitation, an impedimetric aptasensor based on covalently immobilized aptamer onto mixed PANI-SA LB films was developed by Prabhakar et al. The developed aptasensor showed high stability, sensitivity and specificity for OTA detection, and could be tested with real samples in future (Prabhakar, Matharu, & Malhotra, 2011). Tong et al. reported an exonuclease-catalysed electrochemical aptasensor based on immobilization of a DNA probe and the complementary aptamer on a gold electrode. The developed method allowed very sensitive detection of OTA (0.001 μg/L) in wheat starch samples due to introduction of exonuclease into the system (Tong, Zhang, Xu, & Chen, 2011).

4.2.5. Luminescent aptasensor

Luminescent aptasensensing has become an important and promising method among the biosensors. Wang et al. developed an electrochemiluminescent biosensor for the diction of OTA using a DNA aptamer as the biological recognition element and N-(4-aminobutyl)-N-ethyl-isoluminol as the signal producing compound. The aptasensor has been fabricated by immobilizing

Table 6
Comparison of the different PCR-based methods used for OTA-producing fungi detection.

	Advantages	Limitations
PCR	High specificity and sensitivity, Easy to perform, Simple thermocycler	Detection of a single target DNA, Detection of live and dead fungi, No quantitative, Contamination problem and false results.
m-PCR	High specificity and sensitivity, Detection of several target DNA, Easy to perform, Simple thermocycler, Rapid detection for pathogen identification directly from culture	No quantitative, Detection of live and dead fungi
qPCR	Accurate and sensitive quantification of target DNA, Highly sensitive, specific and reproducibility, Rapid analysis time, Possibility of routine analysis	Need for expensive equipment, Strict operational procedure, Detection of live and dead fungi, Inhibitor sensitivity
RFLP-PCR	Fast and robust identification method, Simultaneous screening of numerous samples, Simple equipment	Analysis by comparison with reference species, Require a large amount of DNA, Expensive
AFLP	Fast identification method, Analysis of intraspecific variability, Simple equipment, High reproducibility, Do not require sequence information	Analysis by comparison with reference species, DNA quality must be very high, Need high technology specially for results analysis
RAPD	Fast identification method, Simple equipment, Do not require sequence information, Less expensive, Small amount of DNA	Analysis by comparison with reference, Low reproducibility and co-migration problem, DNA quality must be very high

Table 7
Comparison of the different analytical parameters for OTA detection methods.

Analysis methods	Advantages	Limitations
Enzyme-linked colorimetric assay	Less sample volume, Less clean-up procedure, Portable, Highly specific, Easy to use	Single use, Time consuming, Less sensitive. Need labels for color development
Fluorescence based assay	Less expensive, More suitable for automation	Less sensitive, Need fluorchrome for detection
Enzyme-linked amperometric biosensor	Highly selective and sensitive, Possibility of the regeneration and reuse, Rapid analysis time, Continuous monitoring and easy miniautorization	Need for label to perform electrochemical detection
Luminescent biosensor	Superior analytical performance, High detectability, Short assay time, Less sample and reagents consumption	Variability in the rate of enzyme catalyzed luminescence reaction, Temperature dependant
Label free biosensor	Label free detection, Cost effective, Faster analysis, Portable equipments, Simple sensing protocol, Good sensitivity	Less specific, High matrix effect
Array biosensor	Simultaneous detection of multianalytes, Rapid and simple to use, No sample pretreatment	Low sensitivity, Less specific
Non instrumental assay based on visual detection	Easy to use, Simple and powerful tool, cheap, Very short analysis time, highly suitable for field analysis	Limited sensitivity, Interferences from the sample matrix

aptamer on the surface of gold nanoparticle modified gold electrode. The purposed biosensor has been tested with wheat samples and then validated by an official method (Wang, Duan, Hun, & Wu, 2010). Wu et al. reported a luminescent bioassay based on the aptamer-conjugated magnetic nanoparticles as recognition element and upconversion nanoparticles as signal labels. The method has been used to detect OTA at extremely low level (0.0001 μg/L) in maize samples (Wu, Duan, Wang, & Wang, 2011).

4.2.6. Aptamer-based strip assay

An aptamer-based test strip format has also been proposed for OTA determination in red wine samples. The assay is based on the competition for the conjugated aptamer-gold nanoparticules between OTA and complementary DNA probe (Wang, Ma, et al., 2011). The same author reported a fluorescent strip sensor based on aptamer-quantum dots technology for OTA detection (Wang, Chen, et al., 2011). The LOD for the qualitative detection by visual inspection was as low as 1 μg/L. For both sensors, detections could be achieved in less than 10 min, indicating aptamer-based strip as a powerful tool for OTA detection.

5. Conclusion

In the last decade, there has been a wide interest in the use of the PCR-based methods (Table 6) to replace traditional culture-based detection methods to develop new strategies for controlling OTA contamination in food. However, it should be kept in mind that DNA-based enumeration is not comparable to culture-based enumeration owing to unspecific amplification of both viable and dead microorganismes in PCR. Although in our opinion PCR screening is the most effective in allowing easier and quicker assessments of OTA-producing fungi species in food matrices, further efforts might be done to perform identification without previous fungal DNA extraction or from various matrices. In addition, the multiplex detection of more species and the qualitative and quantitative screening of genes involved in the metabolic pathway of OTA production should improved the effectiveness of screening.

A broad range of emerging methods (Table 7) for practical analysis and detection of OTA in food samples appears in the last years especially focus on the developing fast screening assays. Among the immuno-based assays, non-instrumental immunotests provide simple, easy-to-use, cost-effective tools that compete with ELISA methods and are complementary to more sophisticated techniques. Immunosensors and array immunosensors combine the high affinity of the biochemical interactions, resulting in high sensitivities and low limits of detection, with the possible miniaturisation, portability and automation of the devices. At this stage, aptamers appear as alternative recognition tools to replace antibodies as binding reagents in diagnostic assays. Consequently, a new trend toward the development of aptamer-based assays is in progress. In all probability, aptamers will be applied in standardized diagnostic test kits in the near future but further investigations are needed with respect to the effects of sample matrices on aptamer performance in order for this promising binder to truly compete with antibodies.

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