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Occurrence, harmful effects and analytical determination of Ochratoxin A in coffee

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ABSTRACT

Coffee is an important stimulant and a good source of bioactive constituents with beneficial health effects. Coffee beans are readily attacked by the growth and proliferation of several fungal species from the genera *Aspergillus* and *Penicillium*. Ochratoxin A, an important toxin produced by these fungal entities, is commonly found in coffee products, from farm to cup-thus challenging the popularity of this world-acclaimed beverage. The toxin has been shown to be nephrotoxic and hepatotoxic, among other deleterious effects. This has prompted the European Commission to set limits for ochratoxin A in coffee products. In order to abide by these regulatory codes, various analytical methods have been developed and deployed in the analysis of the toxin in green coffee beans, roasted coffee and instant coffee. This review will discuss the occurrence of ochratoxin A, its harmful effects and various analytical methods that have been applied in its detection and quantification in coffee products.

INTRODUCTION

Since its introduction into Arabia across the *Bahr al-Yaman*, "Sea of Yemen" (Red Sea), many centuries ago(Miran, 2012), popularity of coffee as an important beverage has continued to grow. Apart from its organoleptic properties, coffee is a functional beverage with many beneficial effects attributed to its consumption due to the presence of some major and minor constituents with bioactive potentials (Esquivel and Jiménez, 2012; Nuhu *et al*., 2014). These chemical compounds include caffeine (Prediger, 2010), trigonelline (Dijk *et al.*, 2009; Yoshinari and Igarashi, 2010), chlorogenic acid (Moon *et al.*, 2009) and diterpenes (Sridevi *et al.*, 2011). Coffee is produced from green and roasted coffee beans of different varieties; but two main species, *Coffea arabica* and *Coffea canephora* have remained the most economically viable ones (Ky *et al.*, 2001). This cash crop is now significantly produced as an export commodity in three geographical regions of the world: America, Asia and Africa (Serra *et al*., 2005). Challenges to this important business include market shocks, extreme weather, pests, parasites and import regulations (Martins *et al*., 2003; Tucker *et al*., 2010; Burbano *et al*., 2012; Nugroho, 2014). The quality of coffee bean and its economic value are partly determined by its physical characteristics (Kathurima *et al*., 2009; Iamanaka *et al*., 2014). A daunting challenge faced by producers is how to prevent coffee products from the growth and proliferation of mycotoxin producing molds. From farm to cup, mycotoxins, especially ochratoxin A (OTA), one of the most widespread, can now be found in coffee beans and its beverage (Bucheli and Taniwaki, 2002; Duarte *et al*., 2009; Velmourougane *et al*, 2010).

This mycotoxin is produced by mainly two genera of microorganisms, *Penicillium* and *Aspergillus* (Pardo *et al.*, 2004; Perrone *et al*., 2007; Cabanes *et al*., 2010; Fernández-Baldo *et al*., 2011). Because of the risk factors associated with its presence in biological systems (Clark and Snedeker, 2006), there is a great need for sound analytical methods for its characterization and quantitative analysis. Some general reviews have been written on methods for the determination of mycotoxins in foods and drinks (Turner *et al.*, 2009; Koppen *et al*., 2010; Kaushik *et al.*, 2013). This work is particularly dedicated to methods that have been applied in the analytical determination of OTA in coffee due to its social and economic importance.

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OCHRATOXIN A

Chemistry and occurrence

Chemically, OTA (Figure 1) is a chlorophenolic compound in which a dihydroisocoumarin moiety is joined to L-phenylalanine in an amide-linkage (Khoury and Atoui, 2010).Its IUPAC designation is N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl}-L-phenylalanine. Various species of *Aspergillus* and *Penicillium* produce OTA (Anand and Rati, 2005; Castellanos-Onorio *et al*., 2011; Amezqueta *et al*., 2012; Oliveira *et al.*, 2013). Gil-Serna *et al.* (2011) suggested that due to high OTA production capacities of *Aspergillus steynii* and *Aspergillus westerdijkiae*, and the wide range of products they contaminate, these species might represent a major potential risk of OTA contamination.

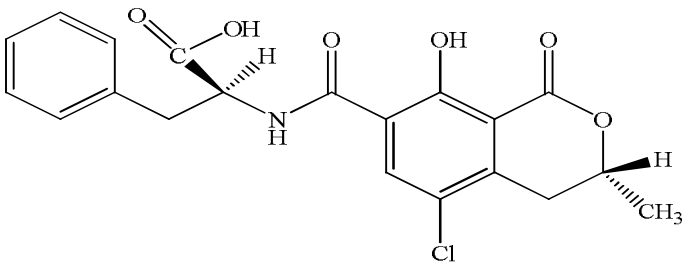

Fig. 1: Chemical structure of ochratoxin A.

OTA is found in different food and drink items (Monaci and Palmisano, 2004).Specifically, it has been detected in pork products (Bertuzzi *et al*., 2013)-even though occurrence in dairy products is generally negligible (EFSA, 2006) –chickpea (Rahimi *et al*., 2013), cocoa (Jayeola *et al*., 2011; Mounjouenpou *et al*., 2011),cereal (Aqai *et al*., 2011), and bear and wine (Gambelli and D'Addezio, 2009; Wu *et al*., 2011).

Importantly, it has been detected in coffee beans (Studer-Rohr *et al*., 1995; Palacios-Cabrera *et al.*, 2004) and in ready-to-drink coffee (Noba *et al*., 2009). Analysis of green coffee beans by Micco *et al.* (1989) showed up to 58% contamination level. Interestingly, Nehad *et al.* (2005) found that decaffeination of coffee may even encourage the growth of OTA-producing species.

Although roasting causes degradation of OTA (Romani *et al*., 2003; Castellanos-Onorio *et al*., 2011), some amount may still be found in commercial roasted coffee (Tsubouchi *et al*., 1988). Mounjouenpou *et al.* (2013) found that, compared to pure Arabica and mixed coffee brands, pure Robusta coffee was the most contaminated in the range of 0.6-18 μgKg^{-1}. This result may be worrisome considering that the maximum contaminant level set by the European Commission is 5 $\mu gKg^{-1}$for roasted coffee beans and 10 $\mu gKg^{-1}$for instant coffee (The Commission of the European Communities, EC No. 1881/2006).

Harmful effects

As a food contaminant, OTA does not only affect the physical characteristics of coffee, its presence also contributes to human exposure (Studer-Rohr *et al*., 1995; Duarte *et al*., 2011; Hennemeier *et al*., 2012).In Saudi Arabia where *Qahwah* (coffee) is a very popular beverage, high degree of OTA contamination was found (Bokhari, 2007). OTA has been detected in human milk and cord serum, and values corresponded with maternal dietary habits (Biasucci *et al*., 2010).

Its transformation into OT-α, OTA-glucosides and other metabolites may elicit some negative responses in different biological systems (Zepnik *et al*., 2003). OTA is hepatotoxic, and this effect on human hepatoma cells HepG2 is strongly dependent on concentration of proteins in the culture medium (Bösch-Saadatmandi *et al*., 2006).

Probably due to its phenolic component, OTA has a high affinity for human serum albumin at the same binding site of natural flavonoid aglycones (Poor *et al*., 2012). Cremer *et al*.(2012) have found that OTA has a strong pro-inflammatory effect on nasal epithelial cell cultures, significantly stimulating the increased release of interleukins, IL-6 and IL-8. OTA fed to gestating rabbits resulted in skeletal and soft tissues anomalies, a strong indication of its teratogenic effect (Wangikar *et al.*, 2004). Two doses of this mycotoxin (1000 and 2000 µg/kg of feed) fed to white Leghorn cockerels for seven weeks resulted in significant decrease in the counts of important hematological and serum biochemical parameters, including erythrocytes, leukocytes, hemoglobin and pack cell volume (Ahmad *et al*., 2012). In a different study, endocrine disrupting activity was displayed through steroidogenic effect after exposing H295R cells to OTA at concentration range of 0.1–1000 ng/ml, resulting in increased production of the hormone estradiol, three times that of solvent control (Frizzell *et al.*, 2013). This effect was further investigated in human placental cells JEG-3 in vitro. After 72-96 h exposure at both cytotoxic and non-cytotoxic levels, 3β-hydroxysteroiddehydrogenase/isomerase mRNA expression was increased by 281–378% (Woo *et al*., 2013).

OTA may hinder the normal functioning of kidney by decreasing the production of vascular endothelial growth factor (Stachurska *et al*., 2011). Elsewhere, Abdu *et al.* (2011) have observed several histopathological changes in the renal corpuscles of rats exposed to OTA which might ultimately lead to renal failure. Previously, Stoev (2010) has examined the carcinogenic effects of OTA in various internal organs of Plymouth Rock chicks. In this study, only liver and kidney were the target organs for OTA carcinogenicity, even though many degenerative effects were observed in brain, kidney, liver and lymphoid organs. A site-specific response was observed when gpt-δ rats were exposed to carcinogenic doses of OTA for 13 weeks, leading to induction of karyomegaly and apoptosis at the outer stripe of the outer kidney medulla (Hibi *et al.*, 2011). OTA forms covalent adduct with DNA (Mantle *et al*., 2010) and causes direct or oxygenic DNA damage (Ali *et al*., 2011). The key to these cytotoxic and carcinogenic effects of OTA might be its perturbation of mitotic processes through inhibition of histone acetyltransferases (Czakai *et al.*, 2011).

Table 1: Summary of analytical methods for the determination of ochratoxin A in coffee.

Matrix	Clean-up	Separation/Detection	LOD	Reference
Green coffee	-	FTEIA[a]-Chemiluminescence	8 ng/g	Sibanda *et al.*, 2001
Roasted coffee	SP,IAC	LC-FLD	<0.2 ng/g	Entwisle *et al*., 2001
Green coffee	IAC	TLC-FLD	<0.2 ng/g	Pittet *et al*., 2002
Green coffee	IAC	TLC-FLD/Densitometry	0.5 µg/kg	Santos and Vargas, 2002
Green/Roasted coffee	SP	HPLC-FLD/MS	0.1 ng/g	Ventura *et al.*, 2003
Green coffee	IAC	HPLC-FLD	0.16 ng/g	Gollucke *et al*., 2004
Green coffee	IAC	LC-FLD	<0.12 ng/g	Vargas *et al*., 2005
Green coffee	LL	TLC-FLD	5 µg/kg	Ventura *et al.*, 2005
Green coffee	-	ELISA-FLD	3.75 ng/g	Fujii *et al*., 2006
Green, Roasted, Instant coffee	IAC	HPLC-FLD	0.80 ng/g	Fujii *et al*., 2007
Green coffee	IAC	Biochip-Chemiluminescence	≤2.1 µg/kg	Sauceda-Friebe *et al.*, 2011
Instant coffee	IAC	HPLC-FLD	0.05 µg/kg	Vecchio *et al*., 2012
Roasted coffee	IAC	HPLC-FLD	0.01 ng/g	Graziani *et al*., 2012
Roasted coffee	IAC	LC/ESI/MS/MS	1.0 ng/g	Bandeira *et al.*, 2012a
Green coffee	IAC	LC-MS/MS	1.2 ng/g	Bandeira *et al.*, 2012b
Instant coffee	-	Biosensor-CRET[b]	0.5 ppb	Mun *et al*., 2013
Ground roasted coffee	IAC	HPLC-FLD	<0.03 µg/kg	Mounjouenpou *et al*., 2013

[a] Flow-through enzyme immunoassay, [b] Chemiluminescence resonance energy transfer

Extraction and Clean-Up Methods for ota in Coffee

Detection of OTA in coffee can be done without going through sample preparation steps of extraction and sample clean-up. In many instances, however, these steps are necessary in order to remove interferences and improve analytical sensitivity. To this end, liquid-liquid extraction (LLE) and solid-liquid extraction (SLE) of both soluble and solid coffee using suitable solvents can be encountered(Mun *et al*., 2013; Ventura *et al*., 2005). In order to expedite desorption of analytes from their parent matrixes, these processes are sometimes driven or facilitated by ultrasonic agitation(Fujii *et al*., 2007)or microwave heating(Graziani *et al*., 2012). A mixture of methanol and sodium bicarbonate is commonly applied as extraction solvent for OTA in different matrixes (Bandeira *et al*., 2012a; Santos and Vargas, 2002; Vargas *et al*., 2005). Arguably, the most commonly applied clean-up techniques are liquid-liquid (LL),solid-phase (SP), and immunoaffinity column (IAC) procedures.I n some cases, both extraction and clean-up procedures are carried out simultaneously(Sibanda *et al*., 2002).

Immunoaffinity column clean-up methods

In the sample preparation of OTA for analysis, IAC is used for the clean-up of extracts from green coffee beans, roasted coffee beans and soluble or instant coffee (Pittet *et al.*, 1996; Diaz *et al*., 2004).IAC, as a clean-up system for OTA (at concentration > 1.2 ng/g), is officially recommended by the Association of Official Analytical Chemists (Entwisle *et al*., 2001; Ahmed *et al.*, 2007). After washing the column with water, the prepared extract is usually diluted in phosphate buffered saline (PBS) and then run through the column at an appropriate flow rate; the column is then rinsed with the PBS, dried under vacuum and the OTA content eluted with a suitable solvent such as methanol (Mounjouenpon *et al*., 2013).

IACs use antibodies that are specific for OTA (Lobeau *et al*., 2005; Vargas *et al.*, 2005), thus creating an antigen-antibody complex that is the basis for this clean-up procedure. Columns, such as Afla-Ochra-Zea (Roswitha and Klaus, 2004), OchraPrep (Vanesa and Ana, 2013), OchraTest (Graziani *et al*., 2012; Vincenzo *et al*., 2012) and Afla OchraTest (Trucksess *et al*., 2008) that meet required specifications are commercially available. Recoveries for extracts of green coffee beans, roasted coffee and instant coffee-spiked at OTA concentrations between 0.5 and 6.5 µg/kg- were between 59.0 and 108.8% (Sugita-Konishi *et al*., 2006; Bandeira *et al*., 2012a; Vecchio *et al*., 2012). Recoveries above 100% can be attributed to three types of interferences, i.e. formation of open-ring OTA during alkaline extraction, isomerization of OTA during roasting, and the presence of non-chlorinated analogue of OTA known as OTB (Tozlovanu and Pfohl-Leszkowicz, 2010).

Solid-phase clean-up methods

For some time now, various solid phase media have been employed in the extraction and sample clean-up of many different organic compounds, including OTA in coffee (Lindenmeier *et al.*, 2011; Kathurima *et al*., 2012; Caprioli *et al*., 2013; Fan *et al*., 2013). The type of sorbent material used partly determines the performance of solid-phase extraction (SPE) and clean-up methods. In most cases, a sorbent with high affinity for the analyte in mobile phase is used to effect separation and clean-up. However, Lobeau *et al.*(2005) have developed a tandem assay column having a layer for adsorbing interfering substances in the sample. To prevent false positives in both high performance liquid chromatography (HPLC) and flow-through enzyme immunoassay (FTEIA), aminopropyl clean-up was used to restrict cross-reacting compounds while allowing the chromatographic elution of OTA (Sibanda *et al*., 2002).

In a different method, a polymeric multifunctional column (MFC) which possessed reversed-phase and anion exchange functionalities was used for clean-up of OTA following its extraction with 1% sodium bicarbonate (Ventura *et al*., 2003). High specificity of molecularly imprinted polymer (MIP) and simplicity of micro-solid phase extraction (µ-SPE) were combined in the method explained by Lee *et al.*(2012a). In this method, a porous membrane-protected µ-SPE column, loaded with MIP, was

employed in the extraction of OTA; desorption was subsequently performed under the influence of ultrasonication. Selection of the type and amount of mobile phase or elution solvent also significantly affect the performance of this approach. Sibanda *et al*.(2002) have found that the use of a mixture of methanol and 5% aqueous sodium bicarbonate, both as extraction solvent and as mobile phase, achieved a high partition coefficient for the extraction and clean-up of OTA. Although the introduction of SPE has resulted in a relative decrease in solvent consumption compared to LLE procedures, there is still a need for a greener approach. This is provided by the µ-SPE method explained by Lee *et al*.(2012b)in which only 400 µL of methanol was used to desorb OTA from Zeolite Linde Type L sorbentunder 5 min ultrasonication,.

Detection and Quantification of ota in Coffee

A study conducted by Vecchio *et al.* (2012) has revealed a high frequency of OTA contamination in instant coffee samples. Out of 50 commercial coffee products tested for different mycotoxins, 36% were positive for OTA (Vaclavik *et al*., 2013). The daily consumption of OTA from coffee in Europe is 2-3 ng/kg bw (Mounjouenpou *et al*., 2013), and Pittet *et al*.(1996) have found higher than normal figures of OTA in green coffee beans purchased in East European countries. Using a certified material and spiked samples, 69% of coffee beans, ground roasted coffee and soluble coffee products imported by Argentina were found contaminated by OTA (Vanesa and Ana, 2013). About 0.46 ng/g of this toxin was also found in sound Brazilian green coffee beans destined for exports (Gollucke *et al*., 2004). Considering the importance of these findings vis-à-vis health implications of OTA, and to assist in complying with stipulated maximum limits, it becomes extremely necessary to continue to deploy sensitive, fast and accurate analytical methods for its detection in coffee.

While polymerase chain reaction (PCR) has been deployed as an important tool for the identification of OTA producing fungal species (Schmidt *et al*., 2004; Morello *et al*., 2007), methods are most importantly directed toward detection of the toxin in different food items. The bulk of these methods that have been applied for the quantification of OTA in coffee are by liquid chromatography (LC) in combination with several detection systems, such as fluorescence detection (FLD) or mass spectrometry (MS). These methods can be very selective and often have low limits of detection (LOD) that are sufficient for the sensitive detection of OTA in samples. Other applications in this regard include enzyme-linked immunosorbent assay (ELISA), and deoxyribonucleic acid (DNA) and peptide-based biochips. These applications are summarized in Table 1.

Methods based on liquid chromatography/High performance liquid chromatography

Techniques of LC are applied for the separation of components of a matrix between a stationary phase and a liquid mobile phase. This liquid phase varies with the type of application. The technique is sub-divided into two main forms, column and planar, based on the form and design of the supporting material for the liquid or solid stationary phase. The liquid mobile phase is commonly driven by gravity or capillarity. In some instances, a low to moderate pressure is applied by mechanical means. Where high pressure is applied, the technique is referred to as HPLC. LC is also divided into normal and reversed-phase modes based on the polarity of the development solvent or mobile phase. The technique is commonly used for the analytical separation of mixture of organic compounds for both preparative and quantitative applications.

Methods based on the column type, in combination with various types of detection systems, were applied in the determination of OTA in coffee (Vargas *et al*., 2004; Sugita-Konishi *et al*., 2006; Bandeira *et al*., 2008). Ventura *et al*.(2003) have developed a sensitive method (LOD, 0.1 ng/g) for the determination of OTA in 20 different coffee samples from different sources using a narrow-bore reversed-phase HPLC analysis with fluorescence detector and acetonitrile/water (0.1% formic acid) (40:60) mobile phase; results were confirmed by single quadruple-MS with ESI source. The official AOAC method for detection of OTA is IA-HPLC with FLD (Ahmed *et al*., 2007), and this was applied in the determination of OTA in coffee at excitation wavelength (λ) of 332 nm and emission λ of 476 nm (Vargas *et al*., 2005). A similar method was developed and validated by Diaz *et al*.(2004) for the analysis of OTA in soluble coffee and green coffee samples with respective limit of quantitation (LOQ) of0.6 µg/kg and 0.3 µg/kg. Previously, a value comparable to that of soluble coffee was obtained for roasted coffee beans following a clean-up procedure with sequential phenylsilane and IAC (Entwisle *et al*., 2001). During roasting of coffee beans, about 90% of OTA may be degraded, and two of its degradation products, one as a result of isomerization (14-(R)-OTA) and the other as result of decarboxylation (14-decarboxy-OTA), were quantified by HPLC-MS/MS (Cramer *et al*., 2008).

The dearth of certified reference material (CRM) has been a major setback in the analysis of OTA. To overcome this challenge, an LC method with tandem-MS detection (LC/MS/MS) was proposed for the production of CRM that would be suitable for application in the determination of OTA in food samples (Bandeira *et al*., 2008). This was achieved by the development of the European Reference Material for the determination of OTA in roasted coffee (ERM-BD475)(Koch *et al*., 2011). Another LC/MS/MS was described for OTA determination in coffee using a SynergiHydro C_{18} column, a mixture of 0.05% of trifluoroacetic acid and methanol (20:80, v/v)as mobile phase, and Selective Reaction Mode with positive electrospray ionization (SRM-ESI+) for detection (Bandeira *et al*., 2012a); for the quantification of OTA, $[M+H]^+$ m/z 239 and m/z 358, fragmentation productsof a pseudo-molecular precursor ion, $[M+H]^+$ m/z 404, were used. Argon and high purity nitrogen were employed as collision gas and nebulizer gas, respectively, and matrix-matched calibration curve was linear between OTA concentrations of 3.0 and 23.0 ng/g (Bandeira *et al*., 2012b).

Thin layer chromatography

Thin layer chromatography (TLC) is an example of the planar LC in which the stationary phase material is thinly spread on a planar solid support such as glass. Development can be one-dimensional or two-dimensional. Detection of components of a sample is commonly through visual inspection. Applications of TLC are routinely found in analytical determinations of organic compounds.

One-dimensional TLC method, in both normal and reversed-phase modes, was developed for OTA determination in green coffee using visual and densitometric analyses, and results were positively correlated ($r > 0.99$) with those obtained from HPLC analysis (Santos and Vargas, 2002).No false positive or false negative was observed in the TLC screening method described by Pittet and Royer (2002) for the detection of OTA in green coffee at control level of 10 µg/kg. Ventura *et al.*(2005) have developed a cheap, rapid and easy method, based on normal two-dimensional TLC, for the determination of OTA in different coffee samples from different origins; toluene-methanol-formic acid (8:2:0.03, v/v/v) and petroleum ether-ethylacetate-formic acid (8:10:1, v/v/v) were used as the first and second dimension development solvents, respectively, and OTA content was estimated by visual inspection under UV lamp at 365 nm.

Other methods

Researchers are deploying a lot of efforts and resources in the development of new methods for the detection of OTA in coffee. Experiments with ELISA and biochips are showing promising results. A method based on indirect competitive ELISA that uses a monoclonal antibody (mAb) for the detection OTA in freshly harvested coffee was described (Fujii *et al*., 2006). Result obtained by this method showed positive correlation to that obtained by HPLC analysis ($r= 0.90$), and no cross-reaction was observed between the antibodies and two analogs of OTA (OTB and OTα). Due to possibly the presence of matrix effect in ELISA, higher values of OTA were recorded compared to HPLC results, with the highest calculated ELISA/HPLC values for green coffee, roasted coffee and instant coffee as 1.46, 1.11, and 1.82, respectively. In order to match results of ELISA with findings from stable isotope dilution assay (SIDA), therefore, IA chromatography was performed as an extract clean-up before ELISA (Lindenmeier *et al*., 2011). Sibanda *et al.*(2001) had earlier developed an FTEIA-based method which did not display any false positives or negatives during screening trials performed on green coffee bean samples.

Combining a flow-through reagent, a stand-alone automated Munich Chip Reader 3 (MCR3) platform, and chemiluminescence detection, a fast method of indirect competitive immunoassay was developed by Sauceda-Friebe *et al.* (2011)for the screening of OTA in green coffee extracts. The biochip was constructed by contact spotting immobilization of a peptide-functionalized OTA analog on a glass plate. Recently, Mun *et al.* (2013) have described a method with the shortest reported total assay time of 10 min for the detection of OTA in coffee. In this method called chemiluminescence resonance energy transfer (CRET), a biosensor was constructed using a single-stranded DNA, and this can detect as low as 0.5 parts per billion (ppb) concentration of OTA in coffee without cross reaction to other mycotoxins. A highly stable colorimetric aptamer sensor-based method was described for the detection of OTA at a very low concentration of about 1nM(Lee *et al*., 2014). This detection limit could be further lowered to 0.75 pM by using mythelene blue as a redox indicator for a two-level cascaded signal amplification strategy (Yang *et al*., 2014).Apart from very low detection limits, another advantage of using such specific aptamers is their high selectivity for OTA to the detriment of its analogs such as N-acetyl-l-phenylalanine and zearalenone (Lv *et al*., 2014).

CONCLUSIONS

OTA is an important mycotoxin produced, as a secondary metabolite, by primarily two genera of fungus, *Aspergillus* and *Penicillium*. Research findings have indicated its nephrotoxicity and hepatotoxicity among other deleterious effects. Occurrence of this toxin in coffee, a world-acclaimed beverage and a source of important chemicals, is a serious source of concern to both producers of coffee beans and to consumers of coffee products alike. This has prompted the European Commission to stipulate limits for the level of OTA in roasted coffee beans and in instant coffee. To assist in abiding by these regulatory codes, different analytical methods, including chromatographic applications, and peptide and DNA biochips, have been developed and applied to the determination of OTA in coffee products. There is, however, a continuous need for improvement in these methods in order to ultimately cut down the total analysis time and to improve sensitivity of determination. This measure will aid in safeguarding consumer health and ensuring quality of coffee as an important stimulant and a good source of bioactive compounds with many important health benefits.

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