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"OCHRATOXIN A": Evaluation of Methodologies for Determination of Ochratoxin A in Food Commodities, Contamination Levels in Different Products Available in the US Market and Evaluation of Fungal Microbiota Associated with Some of the Products

Lakshmi Gompa
University of Nebraska-Lincoln, lakshmi.gompa@gmail.com

"OCHRATOXIN A"

EVALUATION OF METHODOLOGIES FOR DETERMINATION OF OCHRATOXIN A IN FOOD COMMODITIES, CONTAMINATION LEVELS IN DIFFERENT PRODUCTS AVAILABLE IN THE US MARKET AND EVALUATION OF FUNGAL MICROBIOTA ASSOCIATED WITH SOME OF THE PRODUCTS

by

Lakshmi Gompa

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“OCHRATOXIN A”

EVALUATION OF METHODOLOGIES FOR DETERMINATION OF OCHRATOXIN A IN FOOD COMMODITIES, CONTAMINATION LEVELS IN DIFFERENT PRODUCTS AVAILABLE IN THE US MARKET AND EVALUATION OF FUNGAL MICROBIOTA ASSOCIATED WITH SOME OF THE PRODUCTS

Lakshmi Gompa, M.S.

University of Nebraska, 2013

Adviser: Professor Andreia Bianchini

Abstract: Ochratoxin A (OTA) is one of the important mycotoxins that contaminate a wide range of food commodities available in the market. In this study, methodologies for analyzing OTA in commodities such as, roasted coffee, cocoa and meat were evaluated. The methods with the best recovery rates were used to analyze the incidence of OTA in these food products. Among different samples analyzed, predominantly 35% of the cocoa samples and 3% of meat samples were contaminated with OTA. Decaffeinated coffee samples showed the highest incidence of contamination (16.7%). Also as a part of this study, Veratox ^{TM} (Neogen, MI) ELISA test kit was validated for quantification of OTA in meat using a modified extraction method. Results for recovery, repeatability, cross-reactivity and robustness and linearity showed that the method was suitable for the analysis. Incidence of OTA levels in dried fruits was evaluated and among them raisins and dates showed high levels of incidence of OTA (100% and 70%, respectively). Fungal microbiota was also isolated from dried fruits was characterized and different strains of *A. niger* and *A. tubingensis* were identified.All the characterized isolates were tested for OTA production and 36.8% among them were OTA producers. Most of the OTA producers were identified as *Aspergillus niger* or *Aspergillus tubingensis*

DEDICATION

From the depth of my heart, I thank my mom and dad for the wonderful life given to me. This thesis is dedicated to my beloved dad G.V Ramana, certainly the greatest person I have met in my life.

To my loving husband, for the encouragement and support all through this journey. I couldn't make this happen without you.

To all my well-wishers for their kind words and personal time that have shared with me, at times when I'm down.

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1.1 Introduction

Mycotoxins are metabolites of fungi capable of having toxic effects in man and animals. The majority of the mycotoxins are produced by three fungal genera: *Aspergillus, Pencillium* and *Fusarium.* Toxicity syndromes caused by the intake of mycotoxins are known as mycotoxicoses. Some of the important mycotoxins are aflatoxins (B1, B2, G1 and G2), ochratoxin A, patulin, fumonisins (B1 and B1), zearlenone (ZON), T-2 and HT-2 toxins and deoxynivalenol. Mycotoxins are a cause of concern during storage, and production of the toxin depends on various factors such as: moisture content, temperature, storage period, contamination rate, broken grain and impurities, insect presence, oxygen concentration, damage during harvest, processing, and grain and seed transport (Lazzari, 1997; Scussel, 2002; Santos, 2002; Garcia et al., 2003; Scudamore, 2005). As mycotoxins cannot be removed during the milling process, it is critical to prevent grain contamination in the field and during storage by preventing the growth of the fungus.

Ochratoxin A (OTA) is one of the most important mycotoxins. It is produced by a number of fungal species from the genera, *Aspergillus* and *Pencillium* that can colonize a range of food products. The potential OTA-producing species are *A. ochraceus, A. westerdijkiae, A. steynii, A. niger* (Frisvad et al., 2004), *A. sclerotoniger, A. lacticoffeatus* (Alborch et al., 2011), *A. carbonarius* (Joosten et al., 2001), *P. viridicatum, P. verrucosum* (Pitt, 1987) and *P. nordicum* (Lund and Frisvad, 2003). Species that consistently produce OTA are *A. cretensis, A. flocculosus, A. pseudoelegans A.*

roseoglobulosus, A. carbonarius, A. westerdijkiae and *A. sulphurous*. Less consistent producers are *A. ochraceus, A. sclerotiorum, A. melleus, A. ostianus, A. petrakii*, and *A. persii* (Frisvad et al., 2004).

1.2 Biosynthesis of ochratoxin A

Ochratoxin A, the 7-(L-β-phenylalaninylcarbonyl)-carboxyl-5–chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin, is a secondary metabolite produced by some toxigenic fungi (Xiao et al., 1995). There are three types of ochratoxin: A, B and C. Ochratoxin A consists of a dihydro-isocoumarin moiety, a pentaketide synthesized through the acetate malonate pathway (Ferreira and Pitout, 1969), linked to phenylalanine through a carbonyl group (Steyn and Holzapfel, 1970) and chlorine is incorporated directly into the isocoumarin portion of the molecule (Wei et al., 1971). Ochratoxin B is the de-chloro analogue (ochratoxin β) of ochratoxin A. Ochratoxin C is the ethyl ester of ochratoxin A and is less common and the least toxic of the three (Stormer, 1992). Ochratoxin A is the most toxic of the three types. Ochratoxin A and its analogs such as ethylamide, D-phenylalanine, decarboxylated, O-methyl ether and methyl ester forms of OTA were synthesized and their crystalline structure was studied (Xiao et al., 1995). The chemical structures of three forms of ochratoxin are shown in Figure 1, 2 and 3.

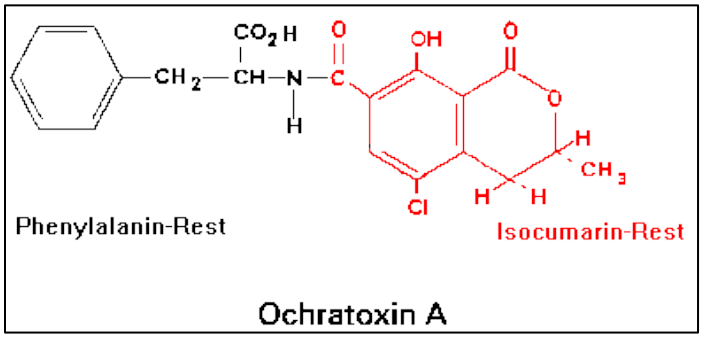

Figure 1. Chemical structure of ochratoxin A with phenylalanine and isocoumarin moieties

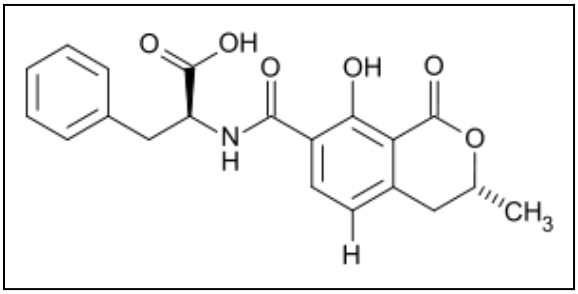

Figure 2

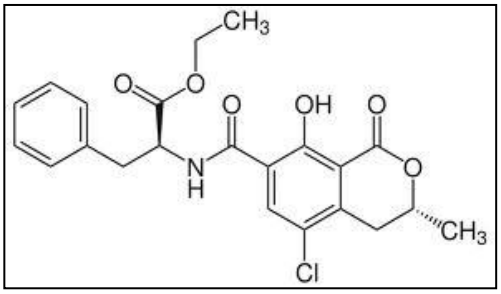

Figure 3

Figure 2: Chemical structure of ochratoxin B
Figure 3: Chemical structure of ochratoxin C

Huff and Hamilton (1979) published a possible pathway for the biosynthesis of OTA. According to their hypothesis, three distinct steps occur in OTA biosynthesis. First, mellein synthesis occurs and is followed by chlorination and carboxyl activation using polyketide synthase and chloroperoxidase. The second precursor, phenylalanine, is synthesized through the shikimic acid pathway, followed by ethyl ester activation. In the third step, linkage of the activated precursors takes place using synthetase, which generates ochratoxin C, an ethyl ester of ochratoxin A. De-esterification by an esterase is part of the last step in this postulated biosynthetic pathway (Moss, 1998).

Harris and Mantle (2001) proposed a different pathway, in which mellein and ochratoxin C play no role in ochratoxin A biosynthesis. Instead, they suggested a pathway leading from ochratoxin B (de-chlorinated form of ochratoxin A), in which the isocoumarin moiety was formed from acetate units via the pentaketide pathway and then carboxylated and chlorinated to form ochratoxin A. The final step by Harris and Mantle (2001) was the linkage of isocoumarin moiety to phenylalanine through a carboxyl group, which is catalyzed by the ochratoxin A synthetase. Ochratoxin B may be formed when chlorine concentration is low, and to some extent by dechlorination of OTA. Callaghan et al., (2003) cloned a polyketide synthase (pks) gene necessary for ochratoxin A in *Aspergillus ochraceus.*

1.3 Toxicity

Ochratoxin A is a toxic fungal secondary metabolite. Several studies have shown that OTA has nephrotoxic, immunotoxic, teratogenic, carcinogenic and possibly neurotoxic and genotoxic properties (Miraglia and Brera, 2002). Carrier mediated removal of the toxin from the blood results in a reduced burden for the animal, but at the same time leads to an increased burden on the organs of elimination, which are the kidneys and liver. For this reason, specific toxic effects can be observed in these organs such as chronic nephropathy conditions (Pfohl-Leszkowicz and Manderville, 2007). Some of the well-known diseases that have been associated with OTA are human Balkan Endemic Nephropathy (BEN), which occurred in Bulgaria and Yugoslavia and endemic kidney disease (Danish pig nephropathy) in pigs (Stoev et al., 1989). Increased incidence of

urinary tract tumors have also been linked to OTA (Fink and Gremmels, 2005).

Some studies on the nephrotoxic nature of ochratoxin A have shown a reduced glomerular filtration rate in rats after exposure to OTA (Gekle and Silbernagl, 1993). Other studies have observed thickening, as well as degeneration of the basement membrane, in chicken and rabbits (Dwivedi et al., 1984). Enzymuria and hyalinisation of glomeruli were observed in patients suffering from BEN (Pofhl-Leszkowicz and Manderville, 2007). Ochratoxin A was also capable of inducing collagen secretion in the damaged epithelial cells of the human proximal tubules, thus impairing kidney functions (Sauvant et al., 2005). In renal cultures of monkeys and rats, OTA decreased protein synthesis and DNA replication, thus increasing cellular necrosis (Kamp et al., 2005).

Ochratoxin A has various toxic effects on different biological functions. Major toxic effects include inhibition of ATP and protein synthesis and enhanced lipid peroxidation (Xiao et al., 1995). Acute and sub-acute toxicity of OTA is due to the reduction of protein synthesis as result of inhibition of phenylalanine-tRNA synthetase (Creppy et al., 1980 and 1984). In addition to inhibition of protein synthesis, it also impacts RNA and DNA synthesis (Dirheimer and Creppy, 1991; Stormer and Lea, 1995). Enzymes such as phosphoenolpyruvate carboxykinase (Meisner et al., 1983; Meisner and Krogh, 1986), succinate-cytochrome C reductase and succinate dehydrogenase (Wei et al., 1985) are also inhibited by OTA. According to Mossesso et al. (2008) there is some evidence that ochratoxin A causes increased risk of aneuploidy (an abnormal number of chromosomes)

and subsequent tumor formation. Inhibition of the enzymes linked with protein, RNA and DNA synthesis may be a reason for subsequent carcinogenicity in affected individuals.

Ochratoxin A present in human milk poses a high risk of exposure to infants. Ochratoxin A has been detected in human milk samples in different countries including Norway, Hungary, Sweden, Italy and Brazil as mentioned in various publications (Pfohl-Leszkowicz and Manderville, 2007). Ochratoxin is chemically stable and is not greatly affected by normal food processing temperatures and its half-life in the human body is 35 days (Schlatter et al., 1996; Bullerman and Bianchini, 2007) leading to average blood concentrations between 0.5 and 1 nmole/liter (Skaug, 2003; Assaf et al., 2004). Other toxic effects include cardiac and hepatic histological abnormalities, aberration of coagulation factors accompanied by hemorrhage and thrombosis in the spleen, brain, liver, kidney and heart (Albassam et al., 1987).

Lesions in the gastro-intestinal tract and lymphoid tissue in hamsters (Hagelberg et al., 1989), myelotoxicity in mice (Boorman et al., 1984; Muller et al., 1995), intestinal fragility and kidney lesions in chickens (Elling et al., 1975) were also observed. Research on poultry birds fed with contaminated feed (with 2 ppm OTA), have shown the signs of ochratoxicosis with characteristic symptoms including weight loss, decreased egg production, increased water intake, diarrhea, excessive urine excretion (renal disorder) and haematological modifications (Prior and Sisodia, 1978; Dwivedi and Burns, 1984a

and 1984b; Bailey et al, 1989; Gibson et al., 1990). At higher concentration (4 ppm), mortality was increased dramatically in poultry birds (Gibson et al., 1989 and 1990).

The International Agency for Research on Cancer (IARC, 1993) has classified OTA as a possible human carcinogen (Class 2B). In 1995, the FAO/WHO Joint Expert Committee on Food Additives established a provisional tolerable daily intake of 14 ng/kg body weight (JECFA, 1995). By 1997, eight countries had established regulations for OTA in foods ranging from 1 to 50 ng/g (FAO, 1997). In Regulation 1881/2006, the Commission of European Communities, after adopting the scientific opinion of the European Food Safety Authority (EFSA), established a tolerable weekly intake (TWI) of OTA as 120 ng/kg bw (CEC, 2006).

1.4 Regulatory limits for ochratoxin A

Many countries have established regulatory levels for OTA in various food commodities, with levels depending on the type of food matrix and also on the country imposing the limit. Some of the regulatory limits are listed in Table 1 (CEC, 2006; Heydt et al., 2011).

Table 1. Regulatory limits on OTA in different food commodities

Country	Food Matrix	Limit (μg/kg)
European Union	Unprocessed cereals	5.0
European Union	All products derived from unprocessed cereals	3.0
European Union	Dried vine fruit	10.0
European Union	Roasted coffee beans and ground coffee	5.0
European Union	Instant coffee	10.0
European Union	Wine	2.0
European Union	Grape Juice	2.0
European Union	Infant cereal based foods	0.5
European Union	Infant food for medical purposes	0.5
European Union	Cocoa and cocoa products	2.0
Canada	Raw cereal grains	5.0
Canada	Grape juice	2.0
Canada	Dried vine fruit	10.0
Italy	Meat	1.0
Romania	Meat	5.0
Denmark	Pig kidney	10.0
Denmark	Carcass condemnation	25.0

1.5 Occurrence in food commodities

Ochratoxin A is found in many plant raw materials and food products (Pohland et al., 1992). It is found in cereals and cereal derived products (Duarte et al., 2009), corn (Magnoli et al., 2007), coffee (Lombaert et al., 2002), cocoa and cocoa products (Copetti et al., 2011), figs (Iamanaka et al., 2005), chilli peppers (Thirumala-Devi et al., 2000), liquorice (Majerus et al., 2000), grape juice (Majerus et al., 2001), dried vine fruit

(MacDonald et al., 1999), wine (Otteneder and Majerus, 2000), meat (Castella et al., 2002) and many other food commodities available in the market.

Studies by various authors indicated the incidence of OTA in different food commodities. In a study in Spain, 90% (19/21) of cereal derived products were contaminated with OTA (Araguas et al., 2005). In other studies, 47% of dried figs were found contaminated with detectable levels of OTA ranging from 0.12-15.31 µg/kg (Karbancioglu-Glurer and Heperkan, 2008) and 38% of the commercial chilli powder samples purchased from an open market were contaminated with OTA (Iqbal et al., 2013). In a study in Brazil, 25% of grape juice and 28% of red wine samples were contaminated with OTA (Rosa et al., 2003).

1.5.1 Ochratoxin A in coffee

Potential ochratoxin A producers in coffee are *A. ochraceus, A. niger* and *A. carbonarius* (Joosten et al., 2001, Urbano et al., 2001, Nakajima et al., 1997, Teren et al., 1996). Other important producers are *A. sclerotoniger, A. lacticoffeatus* (Alborch et al., 2011), *A. westerdijkiae* and *A. steynii* (Frisvad et al., 2004). Optimum growth conditions for *A. niger* and *A. ochraceus* are 35-37^{o}C and 24-31^{o}C and water activities of 0.77 and 0.95-0.99, respectively (Pitt and Hocking, 1997).

According to a survey in Canada, 51% of ground and 67% of instant coffee samples were contaminated with OTA (Lombaert et al., 2002). The natural occurrence of OTA is

reportedly in the range of 0.2-360 µg/kg (Joosten et al., 2001). In Brazil, about 63%, 31% and 3% of coffee samples surveyed in a study were contaminated with *A. niger*, *A. ochraceus* and *A. carbonarius,* respectively (Taniwaki et al., 2003). Napolitano et al. (2007) reported that among samples from seven different geographic regions, Costa Rican and Indian green coffees were the most contaminated samples with 13 and 11 µg/kg, respectively, while Ethiopian coffee was the least contaminated with 3.8 µg/kg. When coffee cherries were mixed more frequently during the drying process, a significant decrease in fungal contamination was observed. A consistent reduction in OTA levels was observed after roasting of coffee beans during processing (Romani et al., 2003). The method of coffee preparation also seems to play a key role in final human exposure to OTA. After preparation, reductions of 49.8% of OTA in expresso coffee, 32.1% in mocha brewing, and 14.5% in auto drip were observed (Perez de Obanos et al., 2005).

1.5.2 Ochratoxin A in cocoa

Common ochratoxigenic species present in cocoa beans are *Aspergillus carbonarius, A. niger, A. melleus, A. westerdijkiae* and *A. ochraceus*. In a study in Brazil on cocoa beans, there was a strong positive correlation, (63% of the OTA positive samples showed the presence of *A. carbonarius*) between the presence of *A. carbonarius* and contamination with OTA in the cocoa beans (Copetti et al., 2011). In Spain, some of the roasted cocoa powder and chocolate samples surveyed in 2000 were contaminated with ochratoxin A, with the levels varying from 0.63 to 2.41mg/kg (MAPA, 2000).

1.5.3 Ochratoxin A in meat

Animal food products, meat and edible tissues can contribute to the total OTA intake through a "carry-over effect" (Gareis, 1996). Accumulation of toxin in the animal tissue after the intake of contaminated feed is due to the carry-over effect. Ochratoxin A can also be produced by molds growing on pork products during the ripening process, which are known to give a characteristic flavor to the final product (Gareis, 1996). Many fungal species produce OTA in meat products and in particular *P. nordicum* has been mainly isolated from proteinaceous foods, such as cheeses and fermented meats (Castella et al., 2002; Lund and Frisvad, 2003). Additionally, *P. nordicum* has a proven ability to grow on meats (Battilani et al., 2003; Sorensen et al., 2008). Some animal products reported to be contaminated with OTA in the European Union, with an average concentration of 0.052 µg/kg (Jorgensen, 2005). In a survey evaluating the occurrence of OTA in meat products from a German market, Gareis and Scheuer (2000) reported a maximum concentration of 0.141 g/kg and also contamination of 68%, 67% and 77.2 % of the liver, bologna and blood sausage samples, respectively. According to Ostry (2001), favorable conditions for the production of mycotoxins in meat and meat products are: a) the presence of oxygen, b) a temperature between 4^{o}C to 40^{o}C, c) a pH value between 2.5 and 8, d) minimum water activity of 0.80 and e) maximum salt concentration of 14%.

1.5.4 Ochratoxin A in dried fruits

Dry vine fruits and nuts are used as ingredients in cereal based foods such as cereal bars, biscuits, puddings, cookies and breads. Black Aspergilli are regarded as common spoilage fungi of these commodities and prominent among them are *Aspergillus niger, A. carbonarius, A. aculeatus, A. ellipticus, A. heteromorphous* and *A. japonicus*. Although *A. niger* and *A carbonarius* are the most common among them, *A. carbonarius* is most likely the potential OTA producer in dried fruits (Romero et al., 2007). Morphological differentiation between the two species is difficult and hence molecular based techniques are required for their identification (Abraca et al., 1994; Schmidt et al., 2004).

Several surveys have been conducted in many countries to evaluate the natural occurrence of OTA in dry vine fruits and also to estimate the capability of black Aspergilli (*Aspergillus* section *nigri*) isolates to produce ochratoxin A. In a survey done in Brazil, 15% of black Aspergilli isolates from dry fruits were found to produce OTA. Among the dry fruits analyzed, high incidence levels of OTA, 26.3% and 33% were observed in dried figs and black Sultana, respectively (Iamanaka et al., 2005). In a survey in Argentina, *A. carbonarius* was the major OTA producer (82.6%) in dried vine fruits (Magnoli et al., 2003). In a survey conducted in the United Kingdom, 88% of the dry vine fruit samples had detectable levels of OTA and the highest level found was 53.6 µg/kg (MacDonald et al., 1999).

1.6 Ochratoxin A detection and quantification

Several analytical methods for determining OTA have been reported. These methods generally involve extraction, cleanup and detection of the toxin. Several solvents are used in toxin extraction from the sample, such as mixtures of dichloromethane–citric acid (Barna-vetro et al., 1996), acetonitrile–water (Eskola et al., 2002), methanol–phosphoric acid (MacDonald et al., 1999), and methanol–sodium chloride (Abdulkadar et al., 2004). A clean up procedure is frequently used and it usually employs solid-phase extraction columns such as anion-exchange (SAX) (Pelegri et al., 1997), silica (Eskola et al., 2001), C^{18} (Scudamore et al., 1999) or immunoaffinity columns (IACs) (Eskola et al., 2002, Zimmerli et al., 1995, Soleas et al., 2001).

The solid phase extraction (SPE) columns mentioned in the literature are usually referred to as “catch and release” columns. With this type of column, different types of sorbent beds are used depending on the type of food matrix and subsequent analytical method used for detection and quantification, such as High Pressure Liquid Chromatography (HPLC) or mass spectrometry. Some of the sorbent beds used are made of silica gels that are matrixed with phenyl or quaternary amine or octadecyl functional groups. The retention mechanism may be ionic, polar or non-polar. SPE interactions are relatively non-selective, reversible and are not based on specific chemical reactions between compounds of interest and functionalized sorbent.

In these “catch and release” SPE columns, the sorbent “catches” the compound of interest

along with some impurities. Washing the column with solvents will remove the impurities leaving the compound of interest on the column for subsequent "release" with appropriate solvent (Biotage, 2013). The "catch and release" mechanism is shown in Figure 4. Immunoaffinity cleanup (IAC) columns have monoclonal antibodies specific for the toxin of interest that are attached to supporting beads for the retention of the compound to the column. Upon washing of the column and release of the toxin, the process concentrates the toxin into a few millilitres of appropriate solvent (Vicam, 2013)

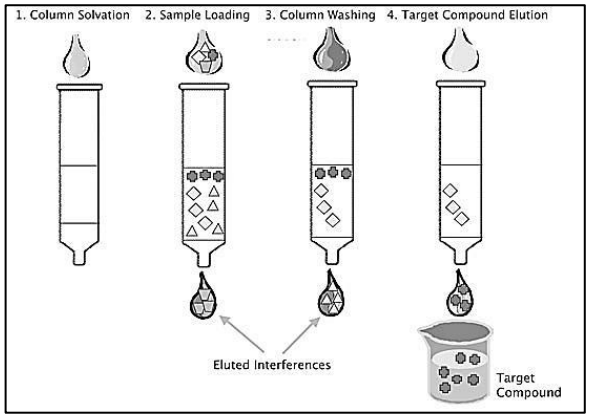

Figure 4. "Catch and Release" mechanism for solid phase extraction columns

Once the toxin has been extracted, purified and concentrated, a combination of chromatographic procedures with various detection methods can be used for detection and quantification. Some among these procedures and detection methods are: thin layer chromatography with densitometry detection (Santos and Vargas, 2002), gas chromatography with mass spectrometry detection (Soleas et al., 2001), liquid chromatography with fluorescence detection (LC–FLD) (Visconti et al., 1999; Eskola et

al., 2001 and 2002), liquid chromatography with mass spectrometry (LC–MS) (Richard et al., 1999) and tandem mass spectrometry (LC–MS–MS) detection (Jorgensen and Vahl, 1999).

Rapid detection and determination is performed by immunochemical methods such as enzyme linked immunosorbent assay (ELISA) (Barna-vetro, 1996). “Rapid methods” for analysis of mycotoxins refers to a method faster than the respective reference method in terms of time, with similar sensitivity and specificity. Even though HPLC, along with fluorescence detection, is one of the most important methods for the analysis of ochratoxin A in different food commodities, sample preparation is time-consuming, since it involves a series of cleanup and extraction processes.

Advanced methods for detection of mycotoxins, including fluorescence polarization immunoassay (FPIA) (Shim et al., 2004; Zezza et al., 2009) and electron spray mass spectrometry coupled with HPLC have been used for quantitative determination of ochratoxin A in coffee, wine and blood (Lau et al., 2000). However, these methods involve complicated and time-consuming sample preparation. Therefore, rapid methods with little sample preparation are preferred. Some of them are enzyme linked immunosorbent assay (ELISA), membrane based immunoassays and lateral flow devices.

Mostly indirect and sandwich type ELISA test kits are used for detection of the compound of interest. Figure 5 shows the procedure for each of the method. The indirect

ELISA test method has the following steps, 1) compound of interest is attached to a microtiter plate; 2) addition of primary antibody followed by a washing step; 3) addition of secondary antibody conjugated with an enzyme; and 4) addition of substrate that will change color upon reaction with the enzyme. The higher the amount of the primary antibody that attaches to the sample, the stronger is the color. The sandwich ELISA test procedure has the following steps: 1) a capture antibody specific for the antigen (compound of interest) is attached to a microtiter plate; 2) addition of antigen containing sample followed by a washing step; 3) addition of second antibody specific to the antigen often conjugated with enzyme (antigen is sandwiched between two antibodies); and 4) addition of substrate that changes color upon reaction with enzyme (Lequin, 2005). De Saeger et al., (1999) used a membrane based flow through device for OTA detection in wheat that used anti-OTA immunoglobulin and horseradish peroxidase enzyme in the formulation of the device.

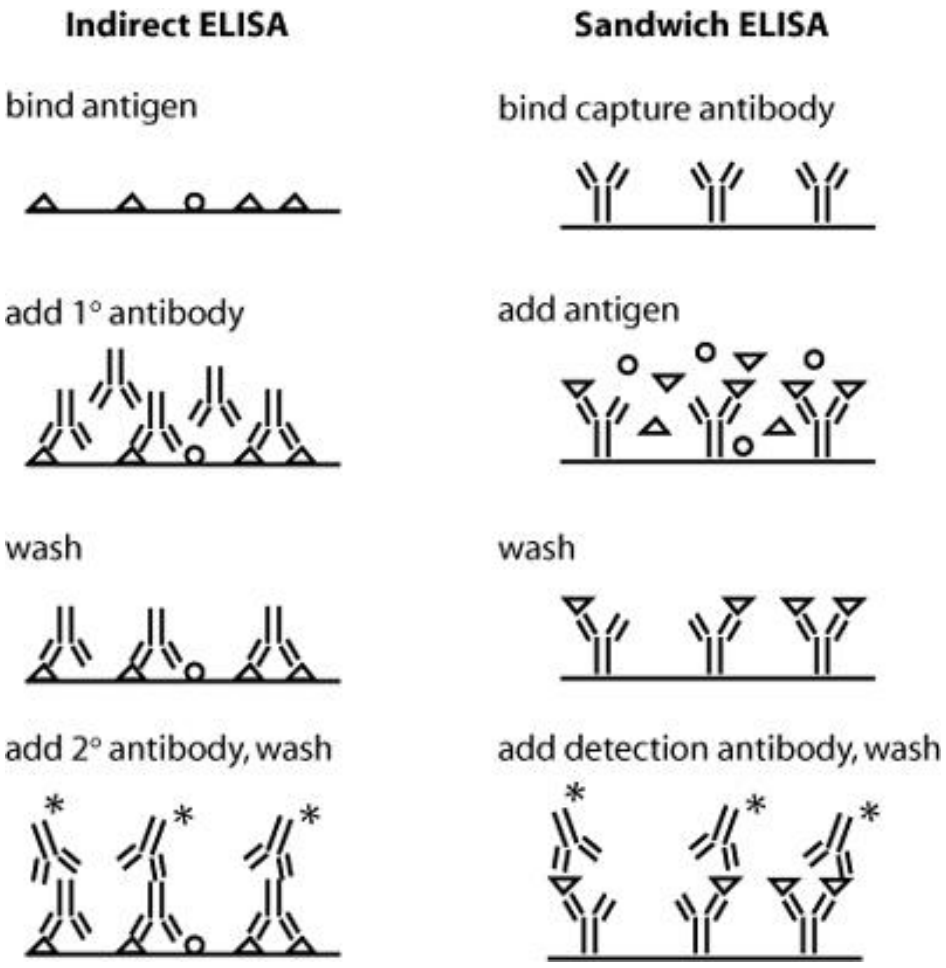

Figure 5. Schematics steps for Indirect (left) and Sandwich (right) ELISA (ocw.mit.edu)
Triangle indicate the compound of interest; * indicates an antibody conjugated with an enzyme

ELISA test kits are one of the most commonly used rapid methods for detection of mycotoxins in different food commodities, as they are rapid, simple, sensitive and portable. They require minor cleanups in addition to the high specificity and sensitivity. ELISA test kits are available for OTA analysis in cereals, cereal products, coffee, cocoa, wine, tea, beans, potatoes, maize and wheat flour (Abouzied et al. 2002, Zheng et al., 2005). In a survey in Brazil, Fujii et al., (2006) used indirect competitive ELISA test kits to detect OTA in green coffee.

Validation studies of any analytical procedure require that certain parameters be determined. These parameters include, but are not limited to extraction efficiency, precision, sensitivity, specificity and robustness. Extraction efficiency is a measure of efficiency of the method to isolate the analyte of interest and is expressed as the percentage (%) of analyte recovered. Ideally, the recoveries should range between 70%-120%. Precision describes the intravariation between replicates assayed at different concentrations and is expressed as percentage of coefficient of variation (% CV). Sensitivity is commonly defined as the lowest dilution of mycotoxin that can be detected by the method. Cross-reactivity of the antibodies, interferences and matrix effects affect the specificity. Repeatability and reproducibility are normally chosen to estimate interlaboratory performance of the method. Repeatability usually provides the smallest value of variation because the results were obtained by the same operator, with the same equipment and within short intervals of time. Reproducibility, on the other hand, provides the largest expected variation because it is obtained by varying the factors like using two operators (Boque et al, 2002).

Therefore, before rapid tests can be used for research or for evaluation of compliance of commodities with regulations, they need to be validated. Validation is important because the antibodies used in the test kit may present cross-reactivity to compounds similar to the mycotoxins of interest and also, the food matrix may interfere with the method of

analysis and affect its efficiency. Therefore, validation studies on the precision and accuracy of ELISA methods are essential and critical.

Validation of ELISA test kits for OTA in food commodities have been done by various authors, based on the criteria of accuracy, precision, ruggedness, limit of detection and repeatability. Zheng et al. (2005), validated AgraQuant ELISA test kits for corn, milo, barley, wheat, soybeans and green coffee for stability, accuracy, precision, ruggedness and limit of detection. It was concluded that these test kits were effective in detecting ochratoxin A and B in all the commodities analyzed. Another validation study was done on RIDASCREEN® OTA kit for dessert wines (Alcaide and Aguilar, 2008) and the study concluded that the ELISA test under evaluation was effective for measuring OTA ranging from 0.25 to 9 μg/ml in dessert wines.

1.6.1 Methods used for analysis of coffee samples

Different studies of OTA in coffee have used different methods for mycotoxin extraction and cleanup, all followed by detection and quantification by HPLC coupled with a fluorescence detector. These different methods include a combination of various extraction solvents, cleanup methods and mobile phases. A summary of the different methods used for the detection of OTA in coffee by HPLC according to different authors is provided in Table 2. For many methods mentioned in Table 2 either Ochraprep™ (r-Biopharm, Darmstadt, Germany) or Ochratest™ (Vicam, MA, USA) were used for the immunoaffinity cleanup. For all methods, excitation and emission wavelengths used with

the fluorescence detector were around 333 nm and 460 nm, respectively.

Table 2. Methods used for HPLC detection of OTA in coffee

Extraction solvent	Mobile phase	Cleanup	Reference
1% aqueous sodium bicarbonate	Methanol: acetonitrile: Sodium acetate (29:29:42)	MAX catridge	Ventura et al., (2003)
Methanol: 3% aqueous sodium bicarbonate solution (1:1)	Acetonitrile: 4 mM sodium acetate (0.5% acetic acid solution) (42:58)	Immunoaffinity columns	Taniwaki et al., (2003)
Methanol: 3% aqueous sodium bicarbonate solution (1:1)	Methanol: acetonitrile: Sodium acetate (29:29:42)	Solid phase and immunoaffinity columns	Lombaert et al., (2002),
Methanol: 3% aqueous sodium bicarbonate solution (1:1)	Acetonitrile: 2% acetic acid (42:58)	Solid phase and immunoaffinity columns	Abdulkar et al., 2004
1% aqueous sodium bicarbonate	Acetonitrile: 2% acetic acid (44:56)	Solid phase and immunoaffinity columns	Vatinno et al., 2008
Methanol: 3% aqueous sodium bicarbonate (1:1)	Water: acetonitrile: glacial acetic acid (51:48:1)	Immunoaffinity columns	Suarez-Quiroz et al., 2004
3% aqueous sodium bicarbonate	Acetonitrile: water: acetic acid (40:60:1)	Solid phase and immunoaffinity columns	Mantle and Chow, 2000

Validated ELISA test kits are also available to measure OTA from 2 to 40 ppb in corn, milo, barley, wheat, soybeans and green coffee. Accuracy and precision of ELISA test kits are comparable to HPLC within this range (Zheng et al., 2005). Validation studies were also done for the RIDASCREEN® (R-Biopharm AG, Germany) for the detection of OTA kit in dessert wine with LOQ of 0.062 μg/L (Alcaide and Aguilar, 2008). ELISA test kits for different mycotoxins have been validated for various food commodities.

These ELISA test kits are from different manufactures such as R-Biopharm, AgraQuant and Neogen (Zheng et al., 2005)

1.6.2 Methods used for analysis of cocoa samples

Most of the methods include extraction followed by immunoaffinity cleanup and HPLC analysis at excitation and emission wavelengths around 333 and 443 nm, respectively.

As discussed for coffee samples, analysis of OTA in cocoa also uses different types of cleanup procedures depending on the nature of the sample. These include most commonly solid phase and immunoaffinity cleanup, even though some studies have used a partition method. Similar to the method variations observed for coffee, combinations of different mobile phases and extraction solvents have been used. In independent studies, Bonvehi (2004) and Amezqueta et al. (2008) used methanol and 3% aqueous sodium bicarbonate solution (50:50) as the extraction solvent, Ochraprep™ immunoaffinity columns as the cleanup step, and acetonitrile: water: acetic acid as the mobile phase. In another study, 1% aqueous sodium bicarbonate, Ochraprep™ immunoaffinity columns and acetonitrile: water: acetic acid (51:47:2) were used as extraction solvent, cleanup method and mobile phase, respectively, to quantitate OTA (Copetti et al., 2011). Lobeau et al. (2007) used Ochratest™ columns along with ederol filters for screening of OTA in samples of cocoa powder. Brera et al. (2003) used a combination of sodium bicarbonate (0.1%) and polyethylene glycol (0.3%) as an extraction solvent.

1.6.3 Methods used for analyses of meat samples

Most of the methods described in the literature for OTA analysis in meat and edible tissues include multiple steps like a) extraction followed by back extraction of the toxin, b) dilution and immunoaffinity cleanup and c) HPLC analysis. A method used by Ceci et al., (2007) consists of homogenization of tissue with 1M phosphoric acid followed by extraction and back extraction with ethyl acetate and sodium hydrogen bicarbonate; columns were used for immunoaffinity cleanup of the extract followed by HPLC analysis. In a study in Denmark on pig meat samples by Jorgensen and Petersen (2002), the extraction solvent used was dichloromethane: ethyl acetate (1:3), with immunoaffinity cleanup, followed by HPLC analysis using a mobile phase of acetonitrile: water: acetic acid (50:49:1). Guillamont et al. (2005) followed a different procedure that consists of homogenization of tissue with chloroform: ortho-phosphoric acid 85% (100: 4), extraction by partitioning using sodium bicarbonate solution, and immunoaffinity cleanup followed by HPLC analysis.

1.6.4 Methods used for analysis of dried fruits

Methodology used for analysis of OTA in dried fruits includes immunoaffinity columns for the separation of toxin from the sample extract, and a combination of liquid chromatography and fluorescence detection for the quantification step (Roemero et al, 2005).

Commercial ELISA test kits have also been used for the detection and quantification of OTA in different dried fruits (Zheng et al., 2005). In a study in Greece, 6% of *A. niger* and 78% of *A. carbonarius* isolated from raisins produced high amounts (>25 ppb) of OTA (Tjamos et al., 2004). Veratox^{TM} ochratoxin test kits were used in this research.

1.7 Identification of fungal microbiota

Ochratoxin A contamination in different food commodities is linked with the presence of *Aspergillus* species, especially species belonging to the black Aspergilli group. Among the black Aspergilli, *Aspergillus carbonarius* is reported to have the highest ochratoxigenic potential (Cabañes et al., 2002; Battilani et al., 2003; Martínez-Culebras and Ramón, 2007). The early detection of *A. carbonarius* in various food commodities is important to prevent contamination with OTA. Therefore the identification of the fungal microbiota associated with certain foods is very important.

Detection and quantification of toxigenic fungi is traditionally done using plating techniques and selective and semi- selective media (Pitt and Hocking, 1997). The two species *A. carbonarius* and *A. niger* are very closely related and the most notable morphological difference is the production of large spores *by A. carbonarius*. (Leong et al., 2006; Romero et al., 2007). Therefore, skilled personnel are required to identify each fungal isolate under a microscope and this is a laborious process. Due to the lack of a specific selective medium for *A. carbonarius*, the identification procedure by morphology becomes laborious, time consuming and expensive (Pollastro et al., 2006). Some media

have showed a restricted germination of *A. carbonarius* conidia resulting in underestimated colony forming units (CFU) (Pollastro et al., 2006). So, morphological identification of *A. carbonarius* and *A. niger* is difficult and not highly reliable as it requires manual observation.

Several DNA based methods have been developed for rapid, sensitive and specific identification of *A. carbonarius*. The most important of them is the Polymerase Chain Reaction (PCR), is used for making multiple copies of a desired fragment of DNA. However, before the PCR step, DNA should be isolated and purified from the fungal mycelium or spores. Isolation of DNA from mycelium or spores involves many steps and currently many DNA isolation kits are available such as the DNeasy Plant Mini Kit (Qiagen, Germany) and the EZNA Genomic DNA isolation kit (Omego Bio Trek). Once isolated, the DNA should be stored at -20^{o}C until PCR reaction.

The PCR step requires the following components in the reaction mixture: Taq DNA polymerase, dNTP's (deoxy nucleotides), PCR grade water and primers (for amplification of desired gene). The amplified DNA fragment further allows for detection and identification of the species. The primers used for PCR should be specific for identification of a species and usually these target the genes responsible for producing toxin or some specific biochemical function. Some of the primers used for identifying *A. carbonarius* are ITS1, ITS4, QCARBOI1 and QCARBO2 targeting the internal sequence of rRNA genes (Gonzalez-Salgado, 2009). A very sensitive PCR assay was also

developed for the detection of *A. carbonarius*, which can provide a positive result even when the initial amount of DNA is as low as 12.5 pg (Niessen et al., 2005). Selma et al. (2008) developed a q-PCR based method for the detection and quantification of *A. carbonarius* in grapes. In this study, two specific primers (AcKS10L/AcKS10R), and a probe were designed for targeting the β-ketosynthase domain of a polyketide synthase.

Real time PCR (q-PCR) is one of the more promising DNA tools for identification and quantification of fungal species. The principle is that, a DNA-binding dye binds to double-stranded (ds) DNA in a PCR reaction, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity, which is measured at each cycle of the PCR reaction, thus allowing target DNA concentrations to be quantified. However, some dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as primer dimers). This can potentially interfere with, or prevent, accurate quantification of the intended target sequence. Therefore, a newer method with fluorescent reporter probes has been developed to detect only the DNA containing the probe sequence. The use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification.

Summary:

Ochratoxin A is an important mycotoxin that contaminates many food commodities available in the market. Acute and chronic toxicity of the toxin pose a risk to people that may consume contaminated products. Other countries including the European Union, Canada and Italy have imposed regulatory limits on OTA in different food commodities. To date, in the US, no national surveys have been conducted on high-risk products to determine if OTA is a health concern for the general public. Part of this thesis, provides quantitative data on coffee, cocoa and meat samples purchased from different regions of the US. This study also includes the evaluation of methods for the analysis of OTA in coffee, cocoa and meat samples.

The validation of rapid methods such as ELISA is necessary before it can be considered for research or evaluation of commodities regarding compliance with regulatory limits. Cross-reactivity and other matrix components may interfere with the test kit, thus decreasing its reliability. Other important parameters include extraction efficiency, precision, sensitivity and robustness. Part of this thesis describes the validation of an ELISA test kit for the analysis of OTA in meat samples, as no current validation studies using this matrix have been done.

The presence of toxigenic fungi in food samples indicates serious risk for mycotoxin contamination, as improper storage conditions may lead to production of high amounts of toxin. Therefore, evaluation of the fungal microbiota associated with certain products and

OTA content is important for better understanding the proper storage conditions for these products. Part of the study described in this thesis focused on isolation and characterization of fungal microbiota from dried fruit samples and the quantification of OTA in those samples by ELISA test kits.

Objectives:

1) To evaluate the methodology for detection and quantification of ochratoxin A in roasted coffee, cocoa and meat by HPLC;
2) Survey of ochratoxin A levels in roasted coffee, cocoa and meat samples purchased from different states in the US, using the methodologies evaluated under objective 1;
3) Validation of an ELISA test kit for quantification of ochratoxin A in meat samples;
4) Detection of ochratoxin A in dried fruit samples by ELISA;
5) Evaluation of the fungal population in dried fruit samples and evaluation of fungal isolates obtained from samples for their potential to produce OTA.

Chapter 2: MATERIAL AND METHODS

2.1 Evaluation of HPLC methods for analysis of ochratoxin A

2.1.1 Coffee and cocoa

The AOAC Official Method 2000.09, with a few changes, was used to analyze the ochratoxin A content in coffee and cocoa samples. The method includes extraction of the toxin, cleanup of the extract and quantification by HPLC analysis. To evaluate the efficacy of the extraction step, different extraction solvents, acetonitrile (Fisher, Ltd): water (60:40 v/v) and different combinations of methanol (Fisher, Ltd): 3% aqueous sodium bicarbonate (Acros Organics, Ltd) (50:50, 70:30, 60:40 and 40:60 v/v) were used to extract the toxin from the sample. Different cleanup procedures were also evaluated that included different immunoaffinity columns, Ochratest^{TM} (Vicam, Ltd) and Ochraclean^{TM} (Pickering, Ltd).

2.1.1.1 Spiking levels for coffee and cocoa

To evaluate the performance of the different extraction solvents and cleanup procedures, samples were spiked with OTA and the recovery rates obtained by the different procedures were compared. European and Italian limits of ochratoxin A in roasted coffee and cocoa are 5 and 2 µg/kg, respectively. Considering those limits, coffee samples were spiked with 10 and 5 µg/kg of OTA to represent a high and low contamination level. For cocoa samples, 4 and 2 µg/kg of OTA were used as high and low levels, respectively. A set of 5 replicates for each spiking level was prepared. To spike the samples, standards were prepared in 100% methanol from ochratoxin A purchased from Sigma-Aldrich.

2.1.1.2 Ochratoxin A determination

Cocoa samples were used without further preparation; while roasted coffee samples were ground using a coffee grinder (Burr Mill, Ltd). For both commodities a 15 g sample was weighed in an 500 ml erlenmeyer and 150 ml of extraction solvent was added to the flask. The erlenmeyer was kept on a shaking device for 30 minutes, followed by filtration of its contents through filter paper (Whatmann #4). An aliquot of the filtrate (10 ml) was diluted with 10 ml of extraction solvent and passed through a solid phase extraction (SPE) column (J.T Baker, Europe). The column has been previously conditioned by passing methanol (10 ml) and 3% aqueous sodium bicarbonate (5 ml) solutions through the column. After passing the extract, the columns were washed with wash reagents, methanol: 3% aqueous sodium bicarbonate (1:3) and 1% aqueous sodium bicarbonate. The column was then air-dried by passing the volume equivalent through a 10 ml syringe (BD company). The toxin was finally eluted using the elution reagent (methanol: water, 7:93 (v/v)). During the entire procedure for the SPE column, the flow rate did not exceed 5 ml/min. The eluent from the SPE column was diluted with 30 mL of PBS buffer (pH 7.4) in preparation for the next step of the cleanup procedure.

Immunoaffinity columns (IAC) (Ochratest™, Vicam (Ltd) and Ochrclean™, Pickering, (Ltd)) were used for further cleanup and concentration of the toxin into a few milliliters of methanol. Procedures suggested by the manufacturers were followed. For the Ochratest™ (Vicam), diluted eluent from the SPE column was passed through the IAC at a flow rate not >5 mL/min. Later the column was washed with 10 mL of water and air

dried for 10-20 seconds. The toxin attached to the absorbent bed of the column, was eluted using methanol (4 ml) into a vial. The solvent was then evaporated under a stream of air leaving the toxin with some extraction residue remaining in the vial.

For the HPLC analysis, the residual material in the vial was re-dissolved into 1 mL of mobile phase (water: acetonitrile: acetic acid (51:48:1)) by vortexing for 1 min. The solution was then filtered using 0.2 µm (nylon membrane) filter discs (Pall, Acro Discs) into another vial for subsequent HPLC analysis. A set of standards- 20, 10, 5 and 1 ng of OTA /ml were used to prepare a standard curve. Samples and standards (100 µl) were injected into the HPLC system. A Dionex Ultimate 3000 series with a C18 column (30 x 2.1 mm, 1.8 µm, CA, USA) was used for the HPLC analysis. A fluorescence detector, set of excitation and emission wavelengths of 333 nm and 460 nm, respectively was used for detection and quantification of ochratoxin A. The mobile phase used was a combination of water: acetonitrile: acetic acid (51: 48: 1 v/v) at 1 ml/min flow rate.

Recovery rates were calculated based on the amounts of the OTA spiked into the sample and the amount quantified by the HPLC method as described in the Equation 1. Statistical analysis was performed for the 5 replicates in the recovery experiments for both coffee and cocoa using different extraction solvents. SAS 9.3 (SAS, Inc, Cary, NC, USA) and ANOVA analysis was performed for the recovery experiments. Significance of treatments was considered when the significance level of the test was at least 5% ($p \leq 0.05$)

$$\text{Recovery (\%)} = \frac{\text{[OTA] in the sample as determined by HPLC}}{\text{[OTA] spiked into the sample}} \times 100$$

Equation 1. Calculation of recovery rate (%)

2.1.2 Meat

A method suggested by Jurgensen and Petersen (2002) was used for the analysis of ochratoxin A in pork and ham. The method includes extraction, immunoaffinity cleanup and HPLC analysis. A mixture of dichloromethane: ethyl acetate (HPLC grade) (1:3) was used as the extracting solvent for the analysis. Some hazardous solvents, like chloroform and 100% dichloromethane, have been suggested in the literature as extraction solvents but their use in this project was discontinued due the to safety reasons, as they emit carcinogenic vapors.

2.1.2.1 Spiking levels for meat

To evaluate the performance of the extraction solvent, samples were spiked with OTA and the recovery rates compared. The proposed Italian limit of ochratoxin A in meat is 1 µg/kg. Considering that limit, 1 and 2 µg of OTA /kg of sample was spiked to represent low and high spiking levels, respectively.

2.1.2.2 Procedure

The method suggested by Jorgenson and Petersen (2002) includes extraction of the toxin from the sample, isolation of toxin using an immunoaffinity column and subsequent HPLC analysis. 100 ml of extraction solvent (dichloromethane: ethyl acetate (1:3)) was added to a 25 g sample and blended (Waring Professional Blender) for 1 min. After blending the mixture was filtered using filter paper (Whatmann #4). An aliquot of the filtrate (10 ml) was evaporated to dryness and the residue was re-dissolved in 2 mL methanol and 30 mL PBS buffer (pH 7.3). After dissolving the residue, the solution was filtered to remove any suspended fat using filter paper (Whatmann #4). The filtered solution was then passed through the immunoaffinity column. Later the column was washed using 20 ml of water and the toxin was eluted from the column using 4 ml of methanol. The extract was evaporated under a stream of air leaving the toxin with some extraction residues. The residue was re-dissolved in 1 ml of mobile phase (water: acetonitrile: acetic acid (51: 48:1) and filtered using 0.2 µm (Nylon membrane) filter discs (Pall, Acro Discs). Samples (100 µl) were injected into a HPLC system for analysis. The parameters used for the HPLC analysis were the same as the ones used for coffee and cocoa analysis. The recovery rates were calculated for the 5 replicates performed in the recovery experiment of meat using Equation 1, previously mentioned.

2.1.3 Limit of detection and limit of quantification

Once the methods for OTA analysis were chosen, their limits of detection (LOD) and limit of quantification (LOQ) were determined in coffee, cocoa and meat using the

method suggested by the European Pharmacopoeia 5.0. The formula for calculating signal-to-noise (S/N) ratio and required ratios for LOD and LOQ are shown in Table 3. Figure 6 explains the terms used in the formula for calculating signal-to-noise ratio. The LOQ and LOD were calculated based on the signal-to-noise ratio of 3 different OTA standards, 5, 10 and 20 ng/ml and averaged.

Table 3. Formula for S/N, LOQ and LOD

S/N = 2H/h
S/N =10 ≅ LOQ
S/N =3 ≅ LOD

H= height of the peak corresponding to the component of interest in the chromatogram measured from the maximum of the peak to the extrapolated baseline of the signal observed

h= range of the background noise in a chromatogram obtained after injection application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution

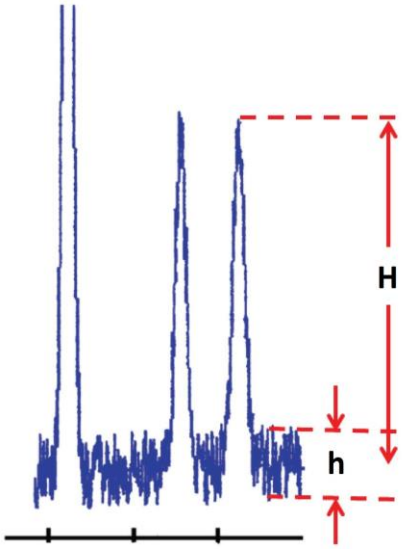

Figure 6. Chromatogram with H and h terms

2.2 Ochratoxin A quantification in market samples

The methods chosen for OTA quantification in coffee, cocoa and meat were then used for determination of this toxin in samples obtained in the US market.

2.2.1 Coffee

Coffee samples were obtained from small and big chain grocery stores from Nebraska, Minnesota, California, Texas and Illinois. The coffee samples purchased for the analysis of ochratoxin A were segregated into groups depending on the type of farming, roasting and origin of beans. Table 4 describes the sampling plan of coffee and also the number of samples analyzed per group. From each market area, around 40 samples were obtained. These coffee samples were segregated into groups based on the criteria mentioned in Table 5. Then, 3 samples per group for each location were randomly selected and analyzed for OTA. A total of 142 samples were analyzed and each onewas extracted in duplicate.

Table 4. Sampling plan for coffee samples

Criteria	**Number of samples analyzed**
Origin of beans	
South and Central American	41
Unknown	90
Type of Farming	
Conventional	70
Organic	71
Type of processing	
Dark roast	58
Light roast	55
Decaffeinated	30

Table 5. Different combinations for grouping coffee samples

Combination criteria for analyzing coffee samples
Decaffeinated/ conventional
Decaffeinated/ organic
Dark roasted/unknown origin/ conventional
Dark roasted/ unknown origin/ organic
Dark roasted/ S. A* origin/ conventional
Dark roasted/ S. A origin/ organic
Light roasted/ unknown origin/ conventional
Light roasted/ unknown origin/ organic
Light roasted/ S. A origin/ conventional
Light roasted/ S. A origin/ organic

* S. A= South and Central America

2.2.2 Cocoa

Cocoa samples were obtained from small and big chain grocery stores from Nebraska, Minnesota, California, Texas and Illinois. The cocoa samples purchased for the analysis of ochratoxin A were segregated into two groups depending on the type of processing, Dutch or regular. From each group, 2 samples from each location were randomly selected and analyzed for OTA. A total of 20 cocoa samples were analyzed and each one was extracted in duplicate.

2.2.3 Meat

Meat samples were obtained from small and big chain grocery stores from Nebraska, Minnesota, California and Texas. The meat samples purchased were divided into two groups, pork and ham samples. A total of 40 samples were analyzed and each was extracted in duplicate.

2.3 Validation of ELISA test kit for quantification of ochratoxin A in meat

Ochratoxin A ELISA test kits (Veratox, Neogen Ltd) were validated for the quantification of OTA in meat. The parameters tested for the validation study were extraction efficiency, linearity, cross-reactivity, robustness, and repeatability.

Performance of the extraction solvents suggested by the manufacturer, 50% and 70% methanol in water, was tested. Meat samples spiked with 20 ppb of OTA were used for calculating the efficacy of the extraction solvents. A set of 5 replicates was done for each extraction solvent. The preliminary results obtained with the two solvents were low, with an average of 33.3% and 26.7% for 50% methanol and 70% methanol, respectively. The meat matrix may have interfered with the extraction, lowering the recovery rates. Therefore, another solvent, dichloromethane: ethyl acetate (1:3) (used for the OTA analysis in meat samples using HPLC), along with a modified procedure was used for testing the efficacy of ELISA test kit for quantification of OTA in meat.

The modified procedure included the following steps, a) addition of 100 ml of extraction solvent to 25 g of sample and blending (Waring Professional Blender) for 1 min, b) filtering through filter paper (Whatmann #4), c) evaporation of an aliquot (10 ml) of the filtrate using a stream of air leaving the toxin and extract residues in the vial and d) re-dissolving residue in 2ml methanol and 30 ml PBS (pH 7.3) buffer followed by filtering through filter paper (Whatmann #4). The filtrate was used for the analysis of ochratoxin A using ELISA test kits following the protocol provided with the kit. Interestingly, this solvent showed 91.8% of recovery. Therefore, dichloromethane: ethyl acetate (1:3) was used for the validation study.

2.3.1 Recovery experiment

For the recovery experiments meat samples were spiked with 20 ppb of OTA and then evaluated according to the protocol described above. The procedure was done 5 times to evaluate the extraction efficiency of the solvent, dichloromethane: ethyl acetate (1:3)

2.3.2 Linearity

Linearity of the standard curve was calculated using standards provided by the manufacturer and others that were prepared using the ones provided with the kit. The set of standards used for evaluation of linearity of the standard curve were 0, 5, 10, 15, 20 and 25 ppb. All the standards were tested in duplicate.

2.3.3 Cross-reactivity

The cross reactivity of the antibodies used in the ELISA test kit was tested for ochratoxin A and ochratoxin B. For this evaluation the solvent was spiked with 10 ppb of OTA. The procedure was repeated 5 times for each toxin.

2.3.4 Robustness

The robustness of the ELISA test kit was tested by changing the incubation times in the protocol. There are two incubation steps in the protocol, 1) 10 min after adding the mixture of sample and conjugate to antibody coated wells and 2) 10 min after adding substrate for color development. The incubation times tested are listed in Table 6. For this evaluation the solvent used in the re-dissolving step was spiked with 15 ppb of OTA and 5 replicates were done for each combination of incubation times.

Table 6. Changed incubation times on ELISA protocol for testing robustness of the kit

Trial	1st Incubation time	2nd Incubation time
10/10	10 min	10 min
10/12	10 min	12 min
12/10	12 min	10 min
12/12	12 min.	12 min

2.3.5 Repeatability

The repeatability of the method for ochratoxin A analysis was studied by comparing the recovery levels from the spiked samples obtained by different operators on different days.

Samples were spiked with 0, 5 and 10 ppb levels of OTA. Two lab operators did the experiment independently in duplicate for each spiked level in 5 consecutive days. After spiking, samples were extracted as previously described for the analysis by ELISA and the extracts were quantified using the ELISA kits according to the manufacturer's instructions.

2.3.6 Statistical evaluation

Statistical analysis was done for the recovery, cross-reactivity, robustness and repeatability experiments by performing ANOVA using SAS 9.3 (SAS. Inc, Cary, NV, USA). For the recovery experiments, statistical analysis was done to determine any significant difference between the average recovery rates of different extraction solvents. For the cross reactivity, ANOVA was performed for any significant difference in cross reactivity showed by the toxin in different solvents. The effect of incubation times on the robustness of the method was also tested by statistical analysis. Finally, any significant difference in the average recovery rates by two independent operators (at two spiking levels) was evaluated and ANOVA was performed for each spiking level.

2.4 Detection of ochratoxin A in dried fruits using ELISA test kits

Veratox^{TM} (Neogen, MI USA) ochratoxin A ELISA test kit was used for the detection of OTA in dried fruits, which included samples of raisins, dates, apricots and figs. The

procedure includes extraction, followed by quantification by ELISA as directed by the kit's manufacturer.

2.4.1 Sampling of dried fruits

Dried fruit samples including dates, apricots, raisins and figs, were purchased in small and big chain grocery stores in Lincoln and Omaha, NE. Samples were segregated into groups depending on the type of farming. Table 7 describes the sampling plan for dried fruits.

Table 7. Sampling plan for dried fruits

Type of dry fruit	**Type of farming (number of samples)**	
Apricot	Conventional (6)	Organic (5)
Dates	Conventional (5)	Organic (5)
Raisins	Conventional (7)	Organic (11)
Figs	Conventional (6)	Organic (5)

2.4.2 Extraction

According to the instructions provided by the kit manufacturer, a 25 g sample was weighed in a blending jar and 100 ml of extraction solvent (50% methanol) was added. The mixture was blended for 3 min and filtered using filter paper (Whatmann #1). The filtrate was used for quantification by ELISA according to the kit's protocol.

2.4.3 ELISA test protocol

The procedure suggested by the manufacturer for the ELISA test was followed and it consisted of the following steps: a) 100 µl of conjugate provided by the kit was added to the mixing wells, b) 100 µl of standards or samples were added to corresponding mixing wells for thorough mixing, c) 100 µl from the mixing wells was transferred to the antibody coated wells and incubated for 10 min, d) after incubation, liquid from wells was discarded and the wells were washed with deionized water, e) 100 µl substrate from the reagent bottle provided by the kit was added to antibody wells and incubated for 10 min, f) 100 µl red stop solution was added to the antibody wells and absorbance was recorded at 650 nm.

2.5 Isolation and identification of fungi from dried fruits

The incidence of fungal contamination was evaluated in the samples of dried fruits. Also, the fungal microbiota of the samples was evaluated and representative organisms were isolated from the dried fruits using a selective medium. Isolates were identified based on their DNA sequences and OTA production was tested for all the fungal isolates.

2.5.1 Fungal incidence

The method suggested by Gonzalez-Salgado (2009) was followed for the evaluation and isolation of fungal microbiota from dried fruits. From each sample 5 fruit pieces were directly plated on DRBC agar (Oxiod, Hampshire, UK) and incubated at 25^{o}C for 7 days.

All the Samples were plated in duplicate. After incubation, the presence of contamination was determined by the number of pieces showing fungal growth compared to the total pieces plated.

2.5.2 Isolation of Fungal microbiota from dried fruits

From the plates used for direct plating of dried fruits, different molds representing the population observed were isolated and purified using PDA (Sigma-Aldrich) and incubated at 25^{o}C for 7 days. Once the fungal isolates had been purified, they were stored at 4^{o}C for further evalution.

2.5.3 Fungal preparation for DNA extraction

All the fungal isolates stored at 4^{o}C were grown on Sabouraud (Fluka, Ltd) broth for mycelial production and subsequent DNA extraction. For this procedure, 25 ml of sabouraud broth was placed in an erlenmeyer and inoculated with loop of fungal spores. The flask was incubated at 25^{o}C on a shaking incubator for 2 days. After 2 days, the fungal mycelium was harvested by filtration through filter paper (Whatmann #1) and stored at -80^{o}C. The stored fungal mycelia were freeze-dried using a lyophilizer (-52^{o}C and 0.09 torr) for 2 days and then stored again at -80^{o}C.

2.5.4 Characterization of fungal isolates using PCR and genome sequencing

The stored freeze-dried cultures were used for DNA extraction. Qiagen DNeasy Plant extraction mini kit was used for the DNA extraction and the procedure suggested by the manufacturer was followed. After extraction all the DNA samples were stored at -20^{o}C. The internal transcribed spacer regions (ITS) of fungal ribosomal RNA, non-coding and variable, were used as primers to identify and also for measuring the phylogenetic relationships in fungi. For this study, ITS1F and TW13 (Invitrogen, Ltd), forward and backward primers, respectively, were used in the PCR reaction to amplify the DNA previously extracted.

The PCR amplification protocol is described in Table 8. The amplified DNA products were detected by running 1% agarose gel for 1 hr at 40V. The amplified PCR products were stored at -20^{o}C and sent for bidirectional sequencing at the genomics core of the Research Technology Support Facility at Michigan State University.

Table 8. PCR amplification protocol

Initialization step	4 min and 94^{o}C
Denaturation step	30 sec and 94^{o}C
Annealing step	30 sec and 52^{o}C
Elongation step	1min and 72^{o}C
The complete cycle was repeated 29 times for the amplification of the DNA fragment	

2.5.5 Phylogenetic analysis of fungal isolates

Phylogenetic trees were developed with the fungal isolates from each variety of dried fruit. Only forward sequences were used to develop the phylogenetic trees as well as for identifying the species. Independent phylogenetic trees were prepared for each variety of dried fruits, dates, apricots, figs and raisins. The software Mega 5 was used for building phylogenetic trees.

2.5.6 Evaluation of potential for ochratoxin A production by the fungi isolated form dried fruits

All fungal isolates were streaked on YES medium so their potential for ochratoxin A production would be tested. All the streaked plates were incubated at 25^{o}C for 7 days. After incubation, three 6 mm discs were cut from three different places of a fungal colony from each plate. The agar plugs were combined with 1 ml methanol and allowed to extract for 1 h, followed by filtration through 0.2 µm filter disc (Pall, Acro Discs). An aliquot (100 µl) of the filtrate was injected into an HPLC system for OTA detection. The parameters used for the HPLC analysis were similar to the ones used for evaluation of OTA in coffee, cocoa and meat, except that no quantification was performed.

Chapter 4. RESULTS AND DISCUSSION

3.1 Evaluation of HPLC methods for analysis of ochratoxin A

Recovery experiments were used to evaluate the performance of the extraction and cleanup steps in the OTA analysis by HPLC. Tests were done for each commodity of interest using different spiking levels. High and low spiking levels were determined based on regulatory limits for OTA on the products of interest as set by either European Commission or Italy. For each spiking level 3-5 replicates were analyzed and the amount of OTA recovered from the samples used to determine the best conditions for extraction and cleanup.

3.1.1 Coffee

The evaluation of HPLC methods for OTA analysis in coffee started with the AOAC official method 2000.09, to which modifications were applied. Preliminary recovery studies showed that the solvent acetonitrile: water (60:40) did not perform well, since it provided recovery rates below 14%. Also cleanup with Ochraclean™ (Pickering, CA) columns, when manufacture's recommendations were followed, did not produce recovery rates above 23%. The solvents and cleanup columns that showed promising results in the preliminary tests were then evaluated in three replicates with the best ones repeated 5 times. Therefore, based on recovery rates from the preliminary results, two solvents were evaluated: 3% sodium bicarbonate: methanol (50:50) and 3% sodium bicarbonate: methanol (40:60). The recovery rates provided by the solvents were used to determine the best one. All extractions evaluated were followed by cleanup procedures using

Ochratest™ (Vicam, MA, USA) as the immunoaffinity column. Table 9 shows the results of recovery rates obtained for different solvents when analyzing OTA in coffee samples.

Table 9. Recovery results obtained with different solvents when analyzing OTA in coffee

Extraction solvent	Spiking level	% Recovery			
		Rep1	Rep2	Rep3	Average± Std dev
SB[1]: M[2] (50:50)	5 µg/kg	83	87.6	83.6	80.6±5.93[a]
SB: M (40:60)	5 µg/kg	64.06	45.55	43.64	51.1±11.28[b]

Average recoveries with different superscript showed statistical difference by ANOVA ($p<0.05$)
SB[1] = 3% aqueous sodium bicarbonate
M[2] = 100% methanol

Statistical analysis by ANOVA (Appendix A) indicated that extraction with 3% sodium bicarbonate: methanol (50:50) provided the highest extraction efficiency while the other solvent tested showed a significantly lower recovery rate. Therefore, further extractions were performed with that solvent at the 5 µg/kg spiking level and also at 10 µg/kg. Table 10 shows the recovery rates for all extractions done using 3% sodium bicarbonate: methanol (50:50) at both spiking levels of 5 µg/kg and 10 µg/kg.

The recovery rates obtained with 3% sodium bicarbonate: methanol (50:50) from spiked coffee samples ranged from 64.5% to 97.7%. These results are similar to the recovery results of 64-89% obtained by Lombaert et al. (2002) and Pardo et al. (2004) in coffee samples. Both research groups used the same AOAC 2000.09 method, extraction solvent and mobile phase as described here for detection and quantification of OTA.

Table 10. Recovery rates for analysis of OTA in coffee samples using 3% sodium bicarbonate: methanol (50:50) as extraction solvent

	Recovery rate (%)					
Spiking level	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Average+ Std dev
5 µg/kg	74.7	74.1	83	87.6	83.6	80.6±5.9
10µg/kg	97.4	64.5	89.2	97.7	80.1	85.8±13.9

3.1.2 Cocoa

The evaluation of HPLC methods for OTA analysis in cocoa also started with the AOAC official method 2000.09, to which modifications were applied. Preliminary recovery studies showed that the solvent acetonitrile: water (70:30) did not perform well, since it provided the recovery rates below 33%. Also cleanup with glass filter papers (Sigma-Aldrich, MO) did not produce recovery rates above 60.0% The solvents and cleanup columns that showed promising results in the preliminary test were then evaluated in three replicates with the best ones evaluated 5 times. So, based on the preliminary results, two solvents were evaluated: 3% sodium bicarbonate: methanol (50:50) and 3% sodium bicarbonate: methanol (60:40). The recovery rates provided by the solvents were used to determine the best one. All extractions evaluated were followed by cleanup procedures using Ochratest^{TM} (Vicam, MA, USA) as the immunoaffinity column. Table 11 shows the recovery results of different solvents when analyzing OTA in cocoa samples.

Table 11. Recovery results obtained with different solvents when analyzing ochratoxin A in cocoa

Extraction solvent	Spiking level	Recovery rate (%)			
		Rep 1	Rep 2	Rep 3	Average ± Std dev
SB[1]: M[2] (50:50)	2 µg/kg	59.9	99.9	107.5	74.8±24.5[a]
SB: M (40:60)	2 µg/kg	36.9	43.4	38	39.43±3.4[b]

Average recoveries with different superscript showed statistical difference by ANOVA ($p<0.05$)
SB[1] = 3% aqueous sodium bicarbonate
M[2] = 100% methanol

Statistical analysis by ANOVA (Appendix B) indicated that extraction with 3% sodium bicarbonate: methanol (50:50) provided the highest extraction efficiency while the other solvent tested showed a significantly lower recovery rate. Therefore, further extractions were performed with that solvent at the 2 µg/kg spiking level and also at 4 µg/kg. Table 12 shows the recovery rates for all extractions done using 3% sodium bicarbonate: methanol (50:50) at both spiking levels of 2 µg/kg and 4 µg/kg.

The recovery results obtained with the 3% aqueous sodium bicarbonate: methanol (50:50) for the extraction of OTA from spiked cocoa samples ranged between 45-107%. This range was similar to the extraction efficiency of solvents used in different studies by Brera et al. (2003) (78-96%) and Amezqueta et al. (2004) (88%). These studies used HPLC based methods; however, different extraction solvents were used as part of the analysis procedure.

Table 12. Recovery rates for analysis of OTA in cocoa samples using 3% sodium bicarbonate: methanol (50:50) as extraction solvent

Spiking level	Recovery rate (%)					
	Rep1	Rep2	Rep3	Rep4	Rep5	Average ± Std dev
4 µg/kg	90	80.9	78.2	95.3	108.8	90.5±10.7
2 µg/kg	59.9	99.9	107.5	44.2	62.5	74.8±24.5

3.1.3 Meat

To determine the best method for OTA analysis in meat samples the method suggested by Jorgensen and Petersen (2002) was the first method to be evaluated. This method uses dichloromethane: ethyl acetate (1:3) as the extraction solvent. Based on the good levels of recovery obtained with this method in preliminary evaluations and the challenges imposed by a meat matrix, this method was chosen for further evaluation. Other studies used an extraction procedure that included a series of steps: homogenization of the tissue with phosphoric acid, extraction using ethyl acetate, and back extraction with 0.5 M sodium bicarbonate solution (Bozzo et al., 2008; Losito et al., 2004). In this method, the aqueous extract obtained was later cleaned using immunoaffinity columns. This method requires a particular Ultra Turrax T25 homogenizer, and the vapors from the solvent pose a safety hazard. Other solvents tested in preliminary experiments were chloroform and dichloromethane, but their usage was discontinued because of the safety hazards, such as potential inhalation of hazardous and carcinogenic vapors. Table 13 shows the recovery results of OTA in meat using the method proposed by Jorgensen and Petersen (2002). The recovery rates obtained with dichlormethane: ethyl acetate (1:3) for the extraction of

OTA from meat samples ranged between 82.8%-111.0% and these recoveries were better than the recovery rates (74%-86%) reported by Monaci et al., 2004.

Table 13. Recovery rates fro analysis of OTA in meat samples using dichloromethane: ethyl acetate (1:3) as extraction solvent

Spiking level	Recovery rate (%)					
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Average ± Std dev
1 µg/kg	101.3	131.5	119.6	100.9	101.8	111.0±12.45
2 µg/kg	69.8	85.9	86.7	99.1	72.6	82.8±10.62

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

Limit of detection (LOD) and limit of quantification (LOQ) of OTA in coffee, cocoa and meat were calculated as suggested by the European Pharmaeopoeia 5.0. The calculated values for coffee were 0.47 and 1.6 µg/kg; in cocoa were 0.47 and 1.6 µg/kg; and for meat 0.22 µg/kg and 0.75 µg/kg for LOD and LOQ, respectively. In coffee samples, the LOD value obtained was less sensitive than the ones mentioned by different authors, 0.2 µg/kg (Lombaert et al., 2002) and 0.1 µg/kg (Pardo et al., 2006). For cocoa, the obtained LOD values were within the range of 0.01-1.06 µg/kg as mentioned by different authors (Brera et al., 2003; Amezqueta et al., 2004; Marina et al., 2010). The obtained LOD value obtained for meat was also less sensitive than the range mentioned by different authors (0.02-0.15 µg/kg) (Guillamont et al., 2005; Bozzo et al., 2008; Toscani et al., 2007).

3.2 Ochratoxin A quantification in market samples

3.2.1 Coffee

The purchased samples were segregated as mentioned in the sampling plan as mentioned in the sampling plan (Section 2.2.1). Random samples to represent each product category were taken and analyzed using 3% sodium bicarbonate: methanol (50:50) as the extraction solvent. A total of 142 samples were analyzed and each sample was extracted and analyzed in duplicate. Out of the total samples analyzed, 9.4% (14/142) showed OTA above detectable levels and 3.4% (5/142) above quantifiable limits. Among those samples that could be quantified for OTA, the contamination levels ranged between 0.5 µg/kg to 6.5 µg/kg. Only one sample (6.5 µg/kg) out of 142 samples showed contamination with OTA above European regulatory limits. Table 14 shows the results obtained for analysis of OTA in the different coffee samples.

All the samples analyzed in this study were roasted coffee samples. Among the dark and light roasted samples analyzed none showed any quantifiable level of OTA. Only decaffeinated samples showed quantifiable levels of OTA, with 33.4% (10/30) of the samples above detectable levels and with 16.7% (5/30) of the samples above the quantifiable limit. When only the origin of the coffee beans was considered, the samples with unknown origin showed an incidence of 5.6% (5/90) of OTA; while beans from South and Central America did not shown any contamination. Samples of coffee beans from conventional farming showed an incidence of 10.0% (7/70) of OTA at detectable levels; while beans from organic farming showed an incidence of 2.8% (2/71). Figure 7

shows the incidence of samples contaminated with OTA for each category of coffee samples.

Canadian and Brazilian surveys of OTA in retail coffee and coffee cherry samples, respectively, indicated the presence of the toxin in the product. A range of contamination of 0.1-3.1 µg/kg was observed in the Canadian samples; while 0.2-109 µg/kg was observed in Brazilian samples. The survey in Brazil indicated that 7% of the total samples were contaminated with OTA. The samples from Canada showed a higher incidence (59%) of contamination with OTA, since 42 (out of 71) of the samples surveyed had OTA levels above the quantifiable level (Lombaert et al., 2002; Taniwaki et al., 2003).

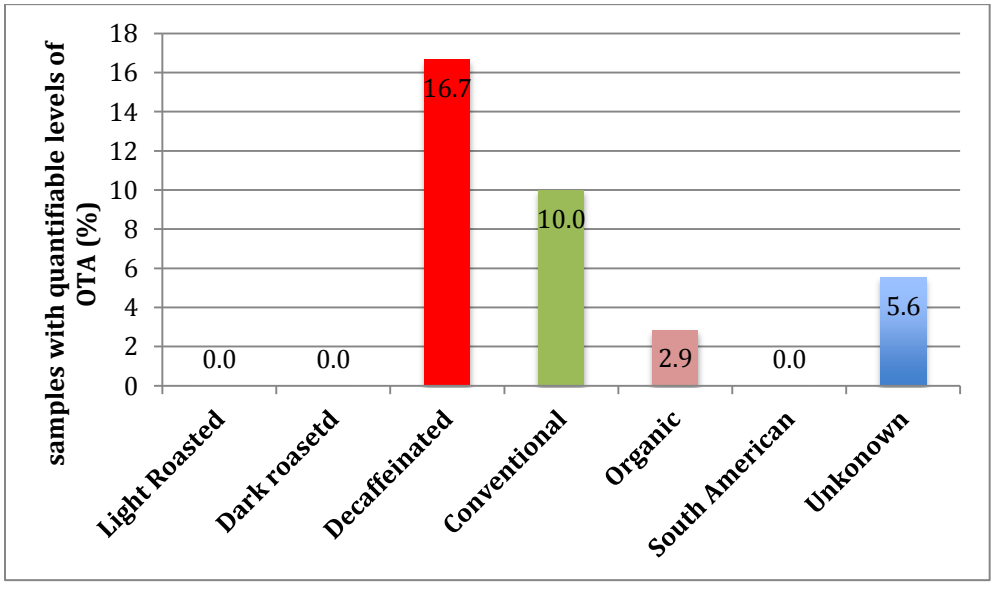

Figure 7. Incidence of contamination of coffee samples with OTA at quantifiable levels

Table 14. Summary of results for OTA analysis in coffee samples

Type of coffee	State	Nebraska				Minnesota			Illinois			Texas			California		
		(μg/kg)															
	Samples	S1	S2	S3	S4	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
Decaffeinated/ unknown origin/ conventional	Subsample1	NQ[1]	NQ	ND	NA[3]	ND	ND	ND	**1.8**	ND	ND	NQ	**2.0**	NQ	ND	**2.1**	ND
	Subsample2	ND[2]	NQ	ND	NA	ND	ND	ND	ND	ND	ND	NQ	**2.5**	NQ	ND	NQ	ND
Decaffeinated/ Unknown origin/ organic	Subsample1	ND	ND	ND	NA	ND	ND	ND	**1.7**	ND	ND	**3.1**	ND	ND	NQ	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	**6.5**	ND	ND	ND	ND	ND
Dark roasted/ unknown origin/ conventional	Subsample1	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dark roasted/ unknown origin/ organic	Subsample1	NQ	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dark roasted/ S. A origin/ conventional	Subsample1	ND	ND	ND	NA	ND	NA	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	NA	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dark roasted/ S. A origin/ organic	Subsample1	ND	ND	ND	ND	ND	ND	ND	NQ	ND	NA	ND	ND	NA	ND	ND	ND
	Subsample2	ND	ND	ND	ND	ND	ND	ND	ND	ND	NA	ND	ND	NA	ND	ND	ND
Light roasted/ unknown origin/ conventional	Subsample1	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Light roasted/ unknown origin/ organic	Subsample1	NQ	NQ	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Light roasted/ S. A origin/ conventional	Subsample1	ND	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND	ND
Light roasted/ S.A origin/ Organic	Subsample1	ND	ND	ND	NA	ND	ND	ND	ND	NA	NA	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	NA	ND	ND	ND	ND	NA	NA	ND	ND	ND	ND	ND	ND

NQ[1] = Non-quantifiable; ND[2] = Non-detectable; NA[3] = Not analyzed; LOD = 0.47 μg/kg and LOQ = 1.6 μg/kg; S.A[4]= South and Central America

3.2.2 Cocoa

The purchased cocoa samples were segregated into two groups, depending on the type of processing - Dutch or regular. In the Dutch process, alkali is included in the extraction process of cocoa from cocoa beans, but in the regular process no alkali was used. From each group, 10 samples were analyzed in duplicate, using 3% aqueous sodium bicarbonate: methanol (50:50) as the extraction solvent. Out of the total number of samples (20), 35% (7/20) were contaminated with OTA at detectable levels, with 30% (6/20) of the samples having levels above the quantifiable limit of the method. Among the samples evaluated, 20% (4/20) exceeded the Italian regulatory limit for OTA. The quantified levels of OTA ranged between 1.6 μg/kg - 18.0 μg/kg. Table 15 shows the results obtained for the analysis of OTA in cocoa samples. In different surveys done by Bonvehi (2004) and Amezqueta et al. (2004) in various cocoa samples, a contamination range of 0.1-23.1 μg/kg and 0.04-14.8 μg/kg were reported, respectively.

Dutch processed cocoa samples showed a higher incidence of OTA than regular processed cocoa. Among the analyzed Dutch processed samples, 50% (5/10) were contaminated with OTA; while only 10% (1/10) of the regular processed samples were contaminated. For the samples analyzed in this study an overall incidence of 35% contamination was observed.

The levels of OTA in cocoa samples were similar to the ones reported by others in the literature. Interestingly, even though Bonvehi (2004) and Amezqueta et al. (2008) reported similar levels of OTA in cocoa to the ones shown in this study, their studies

reported a higher incidence of OTA contamination, 76% and 63%, respectively. Figure 8 shows the incidence of OTA contamination in cocoa samples.

Table 15. Summary of results for OTA analysis in cocoa sample

Type of cocoa	State	Nebraska		Minnesota		Illinois		Texas		California	
		(µg/kg)									
	Samples	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
Dutch Process	Subsample1	ND[2]	**3.8**	**4.5**	ND	**1.9**	**1.6**	ND	ND	ND	ND
	Subsample2	**18.0**	NQ[1]	ND	ND	ND	ND	ND	ND	ND	ND
Regular Process	Subsample1	**3.3**	ND	NQ	ND	ND	ND	ND	ND	ND	ND
	Subsample2	**2.6**	ND	ND	ND	ND	ND	ND	ND	ND	ND

NQ[1]=Non-quantifiable; ND[2]=Non-detectable; LOD= 0.47 µg/kg and LOQ= 1.6 µg/kg

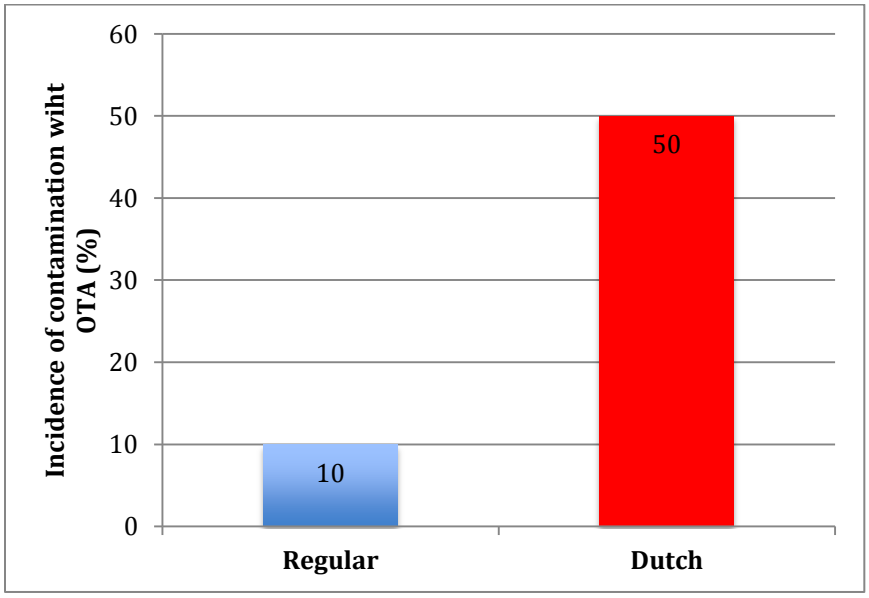

Figure 8. Incidence of contamination of cocoa samples with OTA at quantifiable level

3.2.3 Meat

The purchased meat samples were segregated into two groups, pork and ham. The method suggested by Jorgensen and Petersen (2002) was followed for analysis, with dichloromethane: ethyl acetate (1:3) used as the extraction solvent. A total of 30 samples (15 pork and 15 ham) were analyzed in duplicate. Out of the total samples (30), 10% (3) were contaminated with OTA at detectable levels, where 6.7% (2) were ham samples and 3.4% (1) were pork samples. Only one sample showed an OTA level above the quantifiable limit (0.75 μg/kg). The OTA levels found in the meat samples ranged between 0.6-0.9 μg/kg. Table 16 shows the results for OTA analysis in meat samples.

The levels of OTA found in meat, along with the incidence of contamination reported here are comparatively lower than previously reported in the literature. According to a survey done by Monaci et al. (2003), the levels of OTA in pig tissues ranged from 0.26-3.05 μg/kg, with 96.3% of the total samples testing positive for OTA. In other studies, the detected levels of OTA ranged from 0-15 μg/kg in kidneys from pigs, 0-2.9 μg/kg in meat samples (Jorgenson and Petersen, 2001), 0.6-5.6 μg/kg in dry cured hams (Dall'Asta et al., 2010), and a range of 0.28-7.28 μg/kg in dry cured ham samples (Toscani et al., 2007).

Table 16. Summary of results for OTA analysis in meat samples

Type of meat	State	Nebraska				Minnesota				Texas				California		
		(μg/kg)														
	Samples	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3
Ham	Subsample1	ND[1]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	ND	ND	ND	ND	ND	NQ[2]	NQ	ND	ND	ND	ND	ND
Pork	Subsample1	ND	ND	ND	ND	ND	ND	ND	ND	NQ	ND	ND	ND	ND	ND	ND
	Subsample2	ND	ND	ND	ND	ND	ND	ND	ND	**0.9**	ND	ND	ND	NQ	ND	ND

ND[1] = Non-detectable; NQ[2] = Non-quantifiable; LOD = 0.22 μg/kg and LOQ = 0.75 μg/kg

3.3 Validation of ELISA test kit for quantification of OTA in meat samples

The same method used for OTA extraction from meat samples for analysis by HPLC was used for validating the Veratox™ (Neogen, MI, USA) ELISA test kit with few changes, because the extraction procedure suggested by the kit's manufacturer proved not to be suitable for OTA extraction from meat in preliminary experiments. Therefore, the extraction solvent dichloromethane: ethyl acetate (1:3) was used. As part of the procedure, a 10 ml aliquot of the filtrate was evaporated to dryness and the residue was re-suspended into a solution for subsequent analysis with the ELISA test kit.

3.3.1 Recovery experiments

Different solutions were tested to dissolve the residue after the evaporation step. The performance of the different solutions was evaluated based on the recovery rate obtained after extracting OTA from meat samples spiked with 20 ppb of toxin and analyzing the

extract using the Veratox™ ELISA test kit. Recovery rates were calculated as previously described in Equation 1. For each resuspension solvent tested, 5 replicates were prepared. The different percent recoveries were calculated. The different resuspension solvents used were 70% methanol, 50% methanol and methanol+PBS (2:30).

Table 17 shows the recovery rates obtained for each solvent used. Among the solvents tested, methanol:PBS (2:30) showed the highest recovery rate at 91.8% with a recovery range between 83-97%. ANOVA results are presented in Appendix C. The recovery results described here were similar to the results reported by other studies, where recovery rates varied between 67-90% (Toscani et al, 2007) and 87±13% (Bozzo et al., 2008). However, those studies used HPLC methods; while here an immunoaffinity method was under evaluation. Prior to this data no reports in the literature were found on the use of ELISA test kits for quantification of OTA in meat, which does not allow for direct comparison of method performance.

Table 17. Recovery rates obtained for each resuspension solvents tested when OTA was quantified by ELISA

Resuspension solvent	**Recovery rate (%)**					
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Average±Std dev
Methanol:PBS (2:30)	92.8	83.0	93.3	92.3	97.5	91.8±5.3[a]
70% Methanol	31.5	24.5	36.8	93.3	31.8	33.3±28.2[b]
50% Methanol	29.8	25.8	21.8	94.3	30.0	26.7±30.4[c]

Average recoveries with different superscript showed statistical difference by ANOVA ($p<0.05$).

3.3.2 Linearity

A series of standards (0, 5, 10, 15, 20 and 25 ppb) was prepared from the standards provided with the ELISA test kit. All the standards were evaluated using the procedure provided by the kit's manufacturer and the absorbance of each standard was measured in triplicate at 650 nm for plotting the standard curve. Table 18 shows the absorbance results for each standards and the characteristics of the standard curve.

Table 18. Absorbance obtained for each OTA standard including average values, standard deviation and standard curve characteristics.

OTA level (ppb)	**Absorbance**			
	Rep 1	**Rep 2**	**Rep 3**	**Average±Std dev**
0	1.52	1.56	1.63	1.57±0.06
5	0.95	0.90	1.03	0.96±0.07
10	0.79	0.73	0.78	0.77±0.04
15	0.63	0.63	0.62	0.63±0.01
20	0.61	0.64	0.61	0.62±0.02
25	0.56	0.57	0.59	0.57±0.02
R^2				**0.97**
Slope				**-1.56**
Y-INT				**1.52**

3.3.3 Cross-reactivity

Ochratoxin A and ochratoxin B (Sigma, Ltd) were used to evaluate the specificity of the kit's antibodies to OTA. The cross reactivity of each form of the toxin was calculated based on Equation 2. For these experiments different solvents used for resuspension were spiked with 10 ppb of OTA. Two different solvents were used as resuspension solvents, the one recommended by the manufacturer as the extraction solvent (50% methanol) and the one tested here as resuspension solvent (methanol:PBS (2:30)). For each solvent 5

replicates were prepared for each toxin. Table 19 shows the results for the cross reactivity experiments. Based on the results shown in Table 19, different levels of reactivity were observed for each solvent tested for OTA, with 50% methanol showing much less reactivity than methanol:PBS. Here, a 100% reactivity with OTA was desired since it is the analyte of interest. To verify that the solvent under evaluation that was giving the lowest level of reactivity (50% methanol) had been prepared correctly for the experiment, a 100 µl aliquot of this solvent was injected into the HPLC. Based on the results shown in Table 19, the solvent had been prepared correctly since 100% reactivity was observed and 50% methanol was really not the best solvent to be used with the kit under the tested conditions. Interestingly, as mentioned before, this is the suggested solvent given by the kit protocol. Higher reactivity for OTA was observed with the extraction solvent proposed by this validation study (83.4%); while 50% methanol showed only 67% of reactivity. ANOVA results are presented in Appendix D.

$$\text{Cross-Reactivity (\%)} = \frac{\text{[Toxin] as determined by the kit X 100}}{\text{10 ppb}}$$

Equation 2. Calculation of cross reactivity for different forms of ochratoxin

Also, according to results in Table 19, ochratoxin B did not show any cross-reactivity with the antibodies of the kit. Since, neither of the solvents under evaluation detected it, this suggests that the kit has antibodies that are specific for OTA.

Table 19. Cross reactivity rates (%) obtained for different forms of OTA in different solvents when toxin was quantified by ELISA

Solvent (10 ppb)	Average cross reactivity (%)±SD[3]		
	ELISA Test kit		HPLC
	Methanol:PBS[1] (2:30)	50% Methanol	50% Methanol
Ochratoxin A	83.5±6.9[a]	67.7±3.9[b]	101.2±5.5[c]
Ochratoxin B	<LOD[2]	<LOD	NA[4]

Average recoveries with different superscript showed statistical difference by ANOVA ($p<0.05$). (Appendix D)
PBS[1] = Phosphate buffer solution (pH 7.3)
LOD[2] = Limit of detection (1 ppb)
SD[3] = Standard deviation
NA[4] = Not analyzed

3.3.4 Robustness

To test the robustness of the ELISA test kit, changes in the protocol, more specifically changes in incubation times were made. Four different combinations of incubation time intervals were used. The standard combination (10 min/10 min) and three other combinations (10 min/12 min, 12 min/10 min and 12 min/12 min) were tested. For these tests, the resuspension solvent, methanol:PBS (2:30) was spiked with 15 ppb of OTA. The protocol suggested by the manufacturer was followed; except for changes in the incubation times as previously mentioned. Six replicates for each combination were prepared and the recovery rates were calculated as previously described. Table 20 shows the recovery rates (%) for the different incubation periods tested.

An analysis of variance (ANOVA) was done to evaluate the robustness of the ELISA test kit, as related to the incubation periods. The results from the ANOVA showed that there was a significant difference between the standard incubation time and the tested ones based on the recovery rates obtained for the different trials. However trial 10/12 was similar to trail 12/12. The results from ANOVA are presented in Appendix E. All the

tested incubation times showed lower recovery rates than the standard one. These results highlight the importance of following the manufacturer's protocol to avoid underestimating the levels of OTA in the samples.

Table 20. Recovery rates (%) obtained for different incubation times when OTA was quantified by ELISA

Trial	1st Incubation time	2nd Incubation time	Recovery rate (%)± SD[1]
10/10	10 min	10 min	111.4±5.1[a]
10/12	10 min	12 min	83.9±6.1[b]
12/10	12 min	10 min	97.1±5.2[c]
12/12	12 min.	12 min	87.7±5.8[b]

Average recoveries with different superscript showed statistical difference by ANOVA ($p<0.05$)
SD[1] = Standard deviation

3.3.5 Repeatability

To evaluate the repeatability of the ELISA test kit for analysis of OTA in meat, the recovery rates obtained by different individuals, using different samples, analyzed on different days were compared. For this experiment, meat samples were spiked with 0, 5, and 10 ppb of ochratoxin A. Two individuals performed the experiment independently for each of the mentioned spiking levels, in duplicate, on 5 different days. The recovery rates were calculated as previously described and Table 21 shows the values obtained for each sample, by each individual, on each day.

An ANOVA analysis was done for each operator for each spiking level. Considering the results, no significant difference was observed between the recovery rates obtained by the operators for each spiking level. This indicates that the method is very repeatable in the

course of several days with samples spiked at different levels by a given operator. Also, no significant difference was observed between the two operators for any of the spiking levels tested, denoting that the method is very repeatable among different operators as well. The ANOVA results are presented in the Appendix F.

Table 21. Average recovery rates for duplicate samples obtained by each operator, for each spiking level, on each different day.

Spiking level	0 ppb	5 ppb	10 ppb
	Recovery rate (%)		
Operator 1			
Day 1	<LOD	103.8	100.7
Day 2	<LOD	75.6	90.2
Day 3	<LOD	62.5	70.6
Day 4	<LOD	96.8	121.4
Day 5	<LOD	73.9	79.5
Average ± SD[1]		85.5±17.2[a]	92.5±19.7[b]
Operator 2			
Day 1	3.7	78.4	83.8
Day 2	<LOD	102.4	105.0
Day 3	<LOD	87.5	102.5
Day 4	<LOD	77.4	54.2
Day 5	<LOD	76.6	79.2
Average ± SD		84.5±10.9[a]	84.9±20.5[b]

Average recoveries with different superscript showed statistical difference by ANOVA ($p<0.05$)
SD[1] = Standard deviation

3.4 Detection of ochratoxin A in dried fruits using ELISA test kits

The contamination levels of four different dried fruits - dates, figs, apricots and raisins with OTA were evaluated using an ELISA based method. Veratox™ (Neogen, MI, USA) ELISA test kits were used and the protocol suggested by the manufacturer was followed.

Samples were segregated as mentioned in the sampling plan (Section 2.4.1) and analyzed for OTA in duplicate. Table 22 shows the average levels of OTA quantified in the samples, along with the minimum and maximum levels of contamination. The incidence of OTA contamination in the samples is also shown in Table 22.

A total of 49 samples of dried fruits were analyzed for OTA and 65% (32/49) of them showed detectable levels of OTA. Among the raisin samples, 100% (18/18) showed OTA levels above detectable levels, 95% (17/18) above quantifiable limits. Among the dates samples, 70% (7/10) showed OTA levels above detectable levels, 30% (3/10) above quantifiable limits. Among the figs samples, 18% (2/11) showed OTA levels above detectable levels, 9% (1/11) above quantifiable limits. Among the apricot samples, 36% (4/11) showed OTA levels above detectable levels, 9% (1/11) above quantifiable limits. The detected levels of OTA in the samples ranged between 1.0-21.2 μg/kg. When the European limit for OTA in dried vine fruits (10 μg/kg) was considered, 27% of the raisin samples exceeded the regulatory limit. Regarding the type of farming, there was no significant difference between conventional and organic based samples. ANOVA results are presented in Appendix G.

In a survey of dry vine fruits for OTA, low levels (0.11-0.39 μg/kg) of the toxin were detected (Romero et al., 2005); but high incidence of ochratoxigenic fungi was reported. MacDonald et al. (1999) reported contamination levels of dried vine fruits with OTA ranging from 0.2-53.6 μg/kg; while Magnoli et al. (2003) reported levels ranging from

1.0 to 7.5 μg/kg with 74% of the samples contaminated with OTA. The results obtained in this study were similar to ones reported by MacDonald et al. (1999).

Table 22. Incidence of contamination of dried fruits with OTA, along with average, maximum and minimum quantifiable levels of contamination

Type of fruit	Type of farming	Incidence of samples positive for OTA (%)	Incidence of contamination with OTA at quantifiable levels (%)	Minimum OTA (ppb)	Maximum OTA (ppb)	Average (ppb) ±Std dev
Dates	Conventional (N=5)	60 (n=3)	40.0 (n=2)	2.0	2.7	2.4±0.5
	Organic (N=5)	80.0 (n=4)	20.0 (n=1)	2.1		NA
Figs	Conventional (N=5)	0.0(n=0)	0 (n=0)	NA[2]	NA	NA
	Organic (N=5)	33 (n=2)	20(n=1)	2.7		NA
Apricots	Conventional (N=6)	80(n=4)	20 (n=1)	2.2		NA
	Organic (N=5)	0(n=0)	0 (n=1)	NA	NA	NA
Raisins	Conventional (N=7)	100 (n=7)	100 (n=7)	2.3	16.0	7.0±1.6
	Organic (N=11)	100 (n=11)	90.0 (n=10)	3.6	21.2	8.3±5.4

LOD = Limit of detection (1 ppb)
NA[1] = Not applicable

3.5 Isolation and identification of fungi from dried fruits

3.5.1 Fungal incidence

Samples of all dried fruits (both positive and negative for OTA) were directly plated on DRBC agar to allow for the growth of the fungal microbiota present in them. Different groups of samples, conventional or organic farming, seem to show varied prevalence of contamination as listed in Table 23. Based on the results showed in Table 23, figs seem to present the lowest incidence (8.3%) of contamination with fungi; while dates showed the

highest incidence (92%). Figures 9, 11 and 12 show some of the samples with fungal growth; while Figure 10 shows a sample without any fungal contamination.

Table 23. Prevalence of fungal contamination in conventional and organic farming samples of dried fruits

Type of fruit	Type of farming	Incidence of contamination with molds	Total Isolates
Dates	Conventional (N=5)	88.0±13.0	44
	Organic (N=5)	96.0±5.5	59
Figs	Conventional (N=5)	8.4±20.4	11
	Organic (N=5)	8.3±20.4	14
Apricots	Conventional (N=6)	0.0	0
	Organic (N=5)	74.0±26.1	35
Raisins	Conventional (N=7)	55.7±43.9	25
	Organic (N=11)	80.0±33.1	53

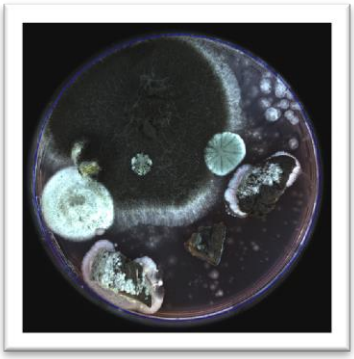

Figure 9. Fig sample showing fungal contamination

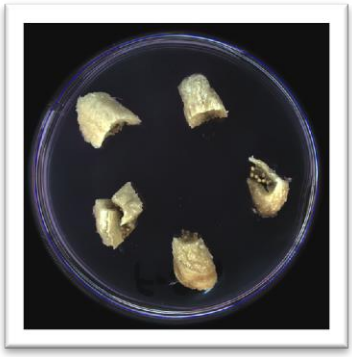

Figure 10. Fig sample showing absence of fungal contamination

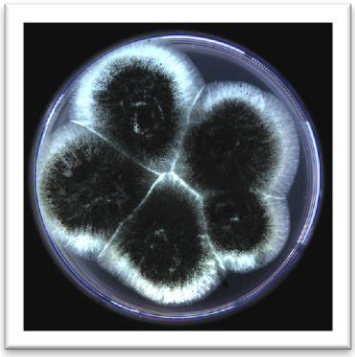

Figure 11. Raisin sample showing black molds

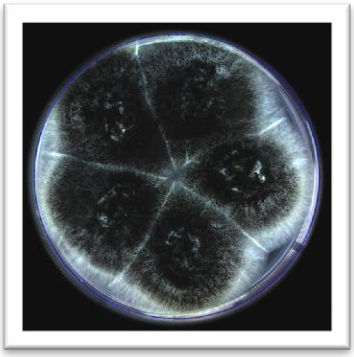

Figure 12. Raisin sample showing black molds

3.5.2 Isolation of fungal microbiota from dried fruits

From the plates used to evaluate the fungal incidence in dried fruits, isolates were obtained to represent the fungal microbiota of the sample. Isolates were purified and temporarily stored on PDA slants until further evaluation. Molds were allowed to grow for 7 days at 25^{o}C before being stored at 4^{o}C

3.5.3 Characterization of fungal isolates using PCR and genomic sequencing

Isolates stored under refrigeration were reactivated by growing on PDA slants for 7 days at 25^{o}C. Once the cultures were actively growing, they were inoculated in Sabauraud (Sigma Aldrich, Ltd) broth for mycelial production. After 2-3 days mycelia were harvested and the DNA was extracted and amplified in a PCR reaction. The PCR products were sent for DNA sequencing; however, before sending the PCR products to sequencing, the PCR reaction was verified by running the amplified DNA products on

1% agarose gel for 1 h at 40V. Figure 13 represents some of the amplified DNA products on agarose gel.

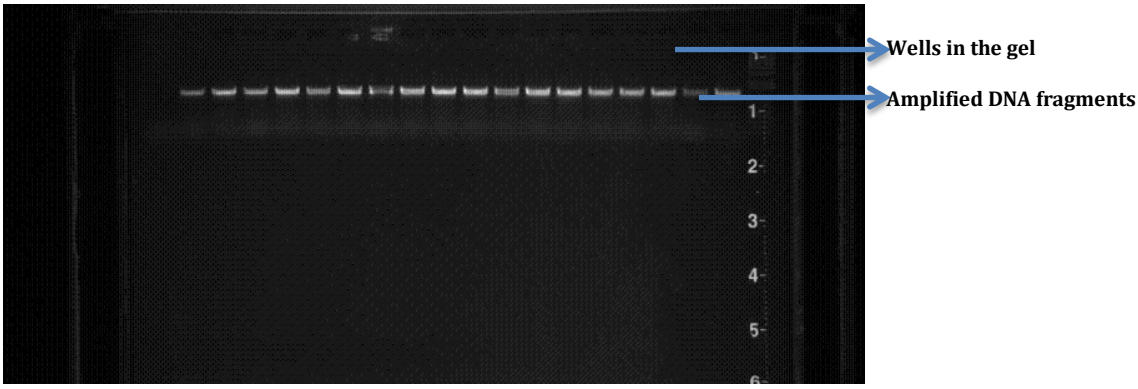

Figure 13. Amplified DNA in 1% agarose gel

For the purpose of identifying the fungal isolates, the definition of species given by Drancourt et al., (2000) was followed. According to them, a species match is defined as a strain when there is ≥ 99% 16S rDNA gene sequencing similarity to strains previously deposited in GenBank. The sequences obtained were used to identify the isolates by comparing their similarity to other sequences that had been previously deposited in the GenBank DNA database, by using the BLAST algorithm.

Using forward and reverse sequences, with the help of the software, Bioedit a sequence was obtained. While trying to identify isolates using the consensus sequence, no results were obtained since they did not show similarity with any of the sequences in the GenBank. Because the forward sequence was generated by a primer related to a portion of the DNA that is more conserved, only the forward sequences were used for identification of the fungal isolates. The fungal microbiota obtained in this study from all dry fruits consisted of 93% (107/116) of *Aspergillus* species. A total of 11 different

strains were identified from the total fungal isolates. Table 24 shows the different species and strains isolated from each dried fruit according to the best match or highest similarity to sequences previously deposited in the GenBank.

Table 24. Different fungal species and strains isolated from dried fruits

Dried Fruit	Fungal species	Strain	Incidence (%)
Apricots	*Aspergillus tubingensis*	CBS 122.49	8.4
	Aspergillus tubingensis	SZX-6	41.7
	Aspergillus tubingensis	A4S5_21	8.4
	Aspergillus niger	KAML02	8.4
	Aspergillus niger	Uf221	33.4
	Eurotiomycetes	DC482	8.4
Figs	*Aspergillus niger*	KAML02	25.0
	Aspergillus niger	Uf221	25.0
	Aspergillus tubingensis	SZX-6	50.0
Dates	*Aspergillus niger*	KAML02	11.7
	Aspergillus niger	Uf221	18.4
	Aspergillus niger	AL-26	1.6
	Aspergillus tubingensis	SZX-6	56.7
	Aspergillus tubingensis	Uf125	3.4
	Aspergillus tubingensis	A4S5_21	1.6
	Eurotiomycetes	DC482	5.0
	Eurotium	sp	1.6
Raisins	*Aspergillus niger*	KAML02	69.3
	Aspergillus niger	Uf221	7.7
	Aspergillus niger	MUM05.13	2.5
	Aspergillus tubingensis	SZX-6	10.3
	Eurotiomycetes	DC482	7.7
	Pencillium citrinum	ESF19M	2.6

Black Aspergilli are the most common fungi present in dried vine fruits and some species within this group are capable of producing OTA. *Aspergillus niger* var *niger* and *Aspergillus niger* var *awamori* were isolated in higher frequency from dried vine fruits (Magnoli et al., 2004). Chulze et al. (2006) reported that *Aspergillus niger* was isolated

from 60% of the dried vine fruit samples. Romero et al. (2005) reported that 95% of the total isolates from dried vine fruits were black Aspergilli.

Because *A. carbonarius* has been reported as a major *Aspergillus* species isolated from dried vine fruits (Accensi et al., 1999), comparison was done between the sequences obtained in this study and *A. carbonarius* sequences from GenBank using the BLAST algorithm. The percent of similarity between them was only around 95-97%, while higher similarity was observed with *A. niger* and *A. tubingensis*. Because the percent identity of the sequences to *A. niger* or *A. tubingensis* was 99% or higher, one could argue with confidence that the strains obtained in this study are mostly *A. niger*. Appendix H shows a table where each of the 116 isolates is presented along with the top hit for that isolate, the percent identity, and the E value according to GenBank.

3.5.4 Phylogenetic analysis of fungal isolates

Based on the DNA sequences obtained after PCR reaction with the primer ITS1F, phylogenetic trees were created for the fungal microbiota isolated from each type of dried fruits. The software Mega 5 was used for this procedure and only forward sequences were used for developing the phylogenetic trees. Phylogenetic trees for aprictos, figs, raisins and dates are shown in Figures 14, 15, 16 and 17, respectively.

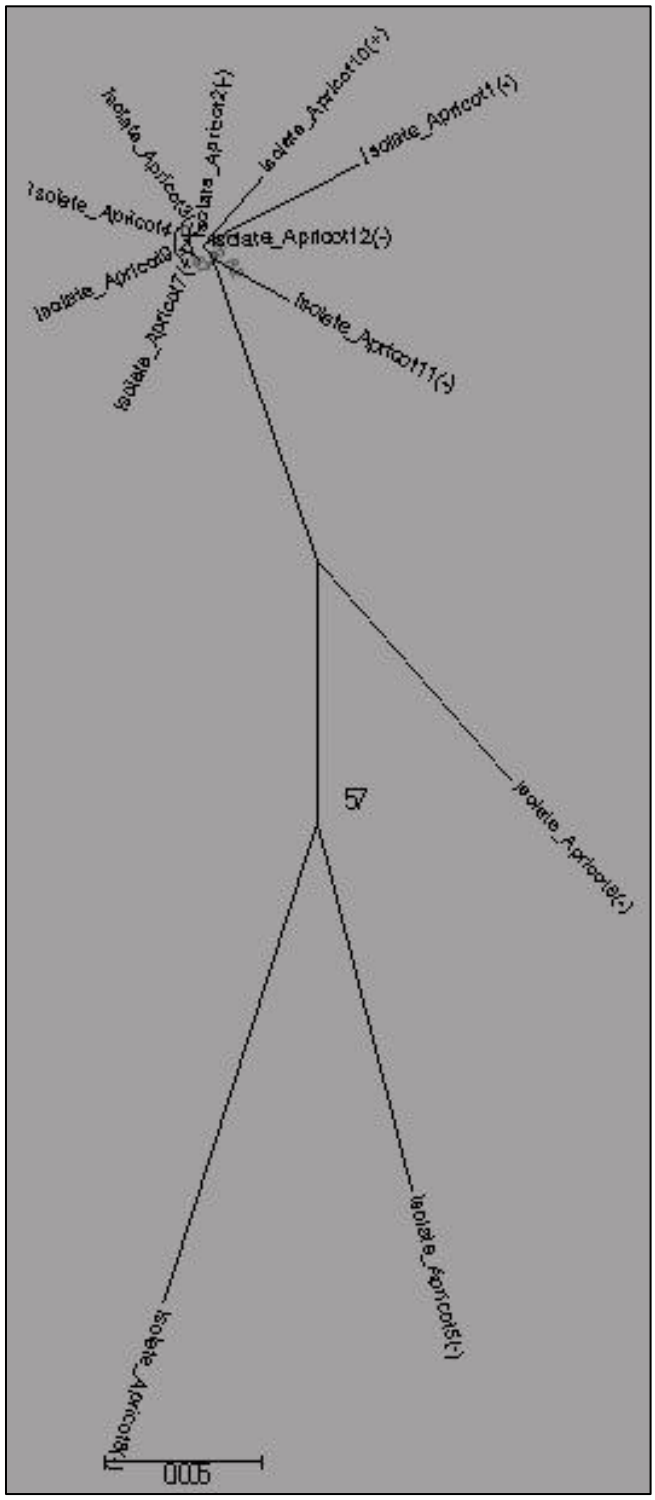

Figure 14. Phylogenetic tree for fungal microbiota isolated from apricots

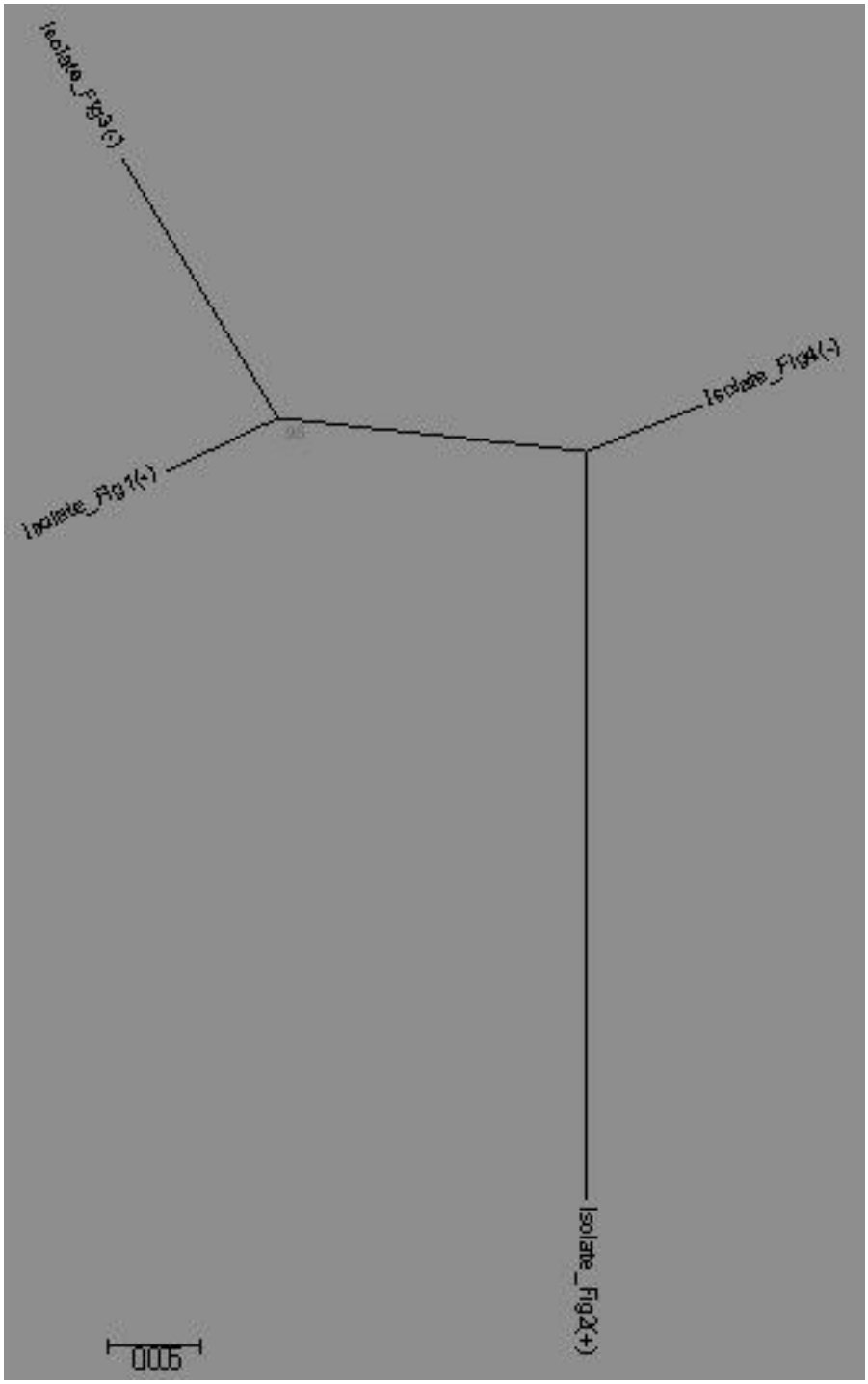

Figure 15. Phylogenetic tree for fungal microbiota isolated from figs

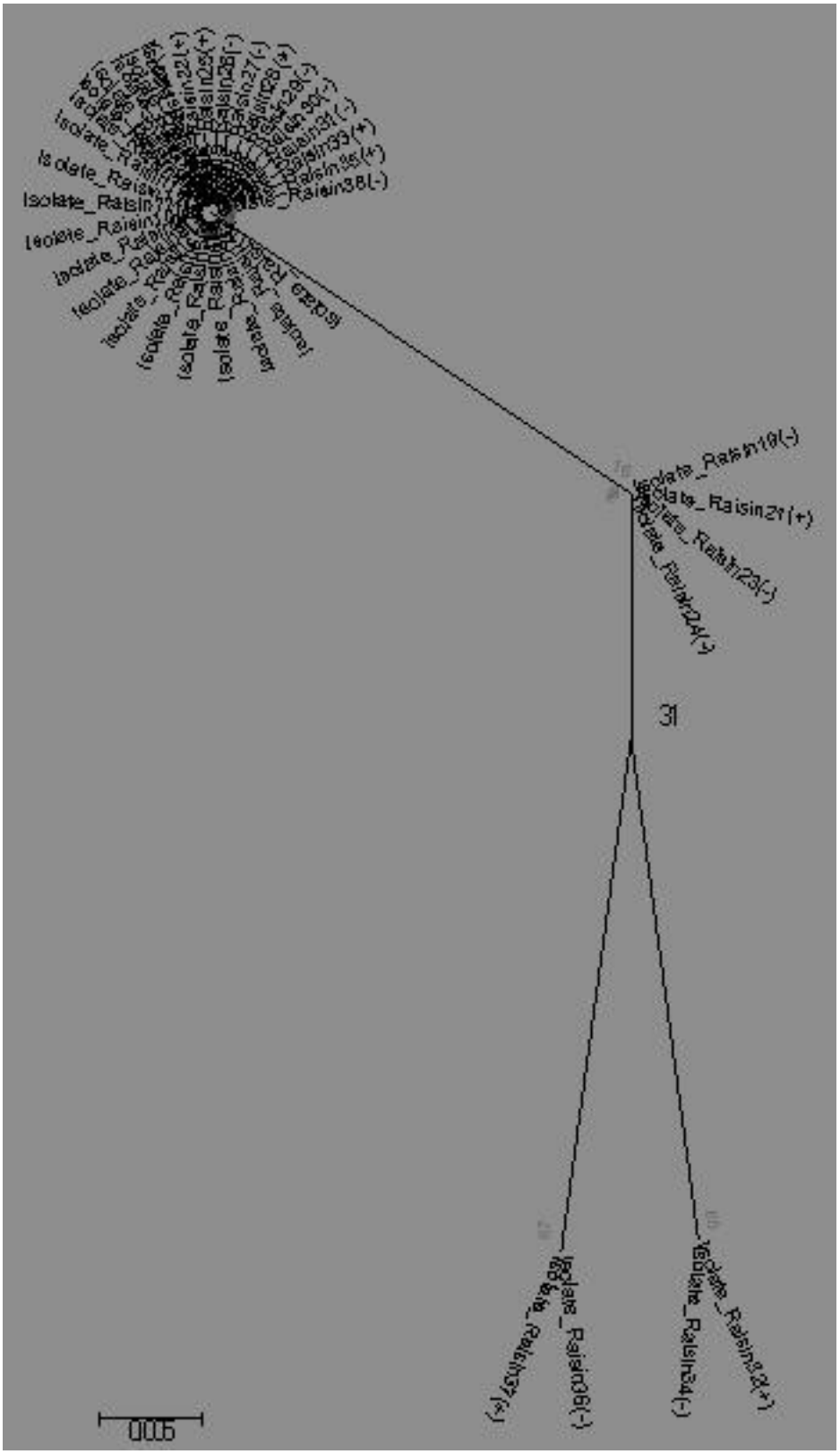

Figure 16. Phylogenetic tree for fungal microbiota from raisins

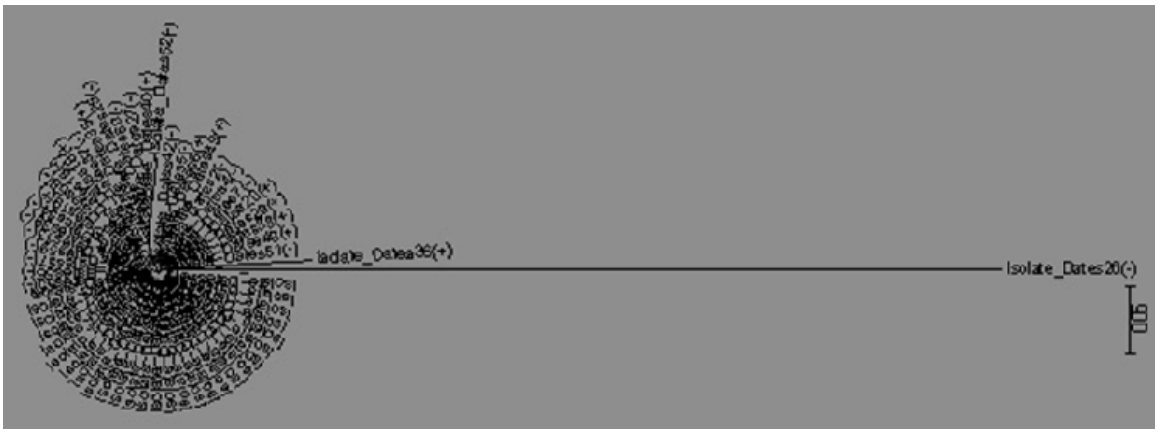

Figure 17. Phylogenetic tree for fungal mictobiota isolated from dates

Based on the phylogenetic trees, there seems to be very little genetic diversity among the isolates obtained from each dried fruit. Most of them are clustered together with a few isolates separated by a few nucleotides, given scale shown in the phylogenetic trees.

When the information in the phylogenetic trees is compared to the information provided in Appendix H, basically all isolates obtained from the dried fruits could be identified as either *Aspergillus niger* or *Aspergillus tubingensis*, with not much genetic diversity among the isolates. The only isolate that seems to be most apart from the others is isolate 26 obtained from dates. When the GenBank hits for this isolates were considered it was, however identified as *A. niger* as well.

The species *Aspergillus niger* and *Aspergillus tubingensis* are closely related and both belong to the *Aspergillus niger* aggregate. According to Pitt and Hocking (2009), the two species are morphologically undistinguishable and for practical purposes they can both be called as *A. niger*.

3.5.5 Evaluation of potential for ochratoxin A production by fungi isolated from dried fruits

All the fungal isolates were streaked on YES medium and incubated for 7 days at 25^{o}C. Detection of OTA was done according to the method suggested by Gonzalez-Salgado (2009). Figure 18 shows the incidence of OTA and non-OTA producers among total fungal isolates, with 36.8% of all the fungal isolates being able to produce OTA. Figure 19 and Figure 20 show the incidence of OTA and non-OTA producers isolated from dried fruits that were negative and positive for contamination with OTA, respectively. Among

the isolates obtained from dried fruit samples positive for OTA, 44.4% of the fungal isolates were OTA producers. In contrast, only 29.3% of the fungal isolates obtained from dried fruit samples negative for OTA were able to produce OTA.

High incidence of fungal contamination in dried fruits and high incidence of toxin producers among them emphasize the importance of controlled storage of these products. In light of the findings in this study, dried fruits should be stored in a very dry and cool environment. A package that allows for any moisture migration can lead to a hazardous situation where mold spores are allowed to germinate and produce toxin during storage.

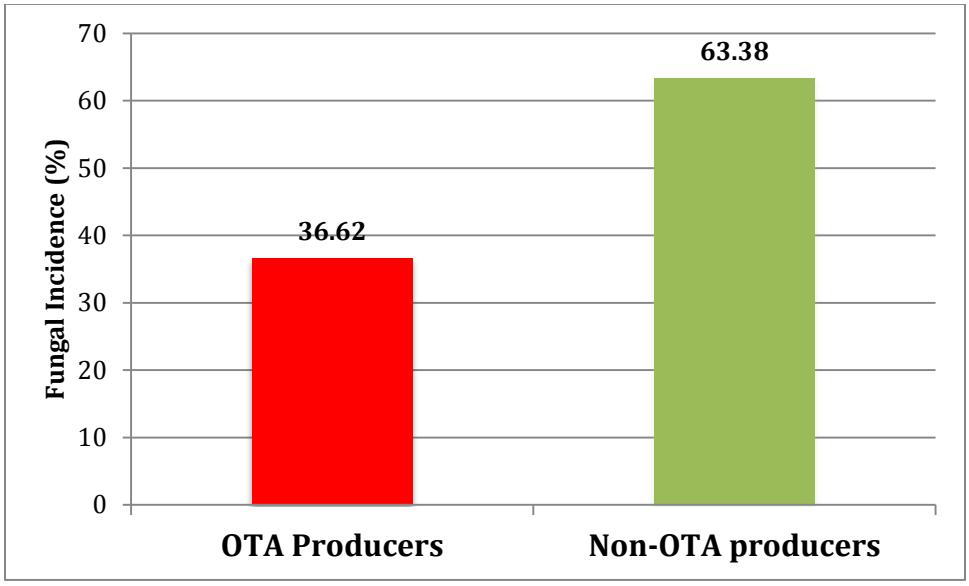

Figure 18. Incidence of OTA and non-OTA producers among total fungal isolates

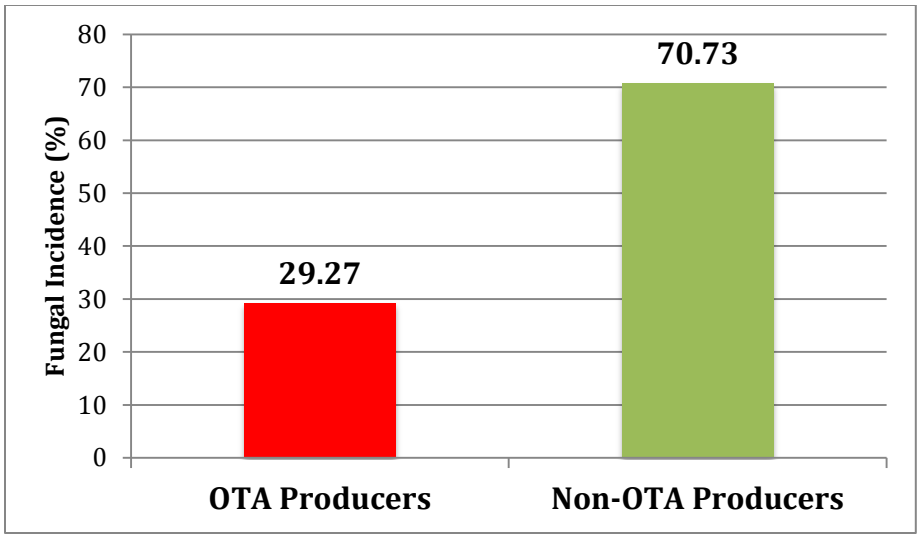

Figure 19. Incidence of OTA and non-OTA producers among fungal isolates from dried fruit samples negative for OTA

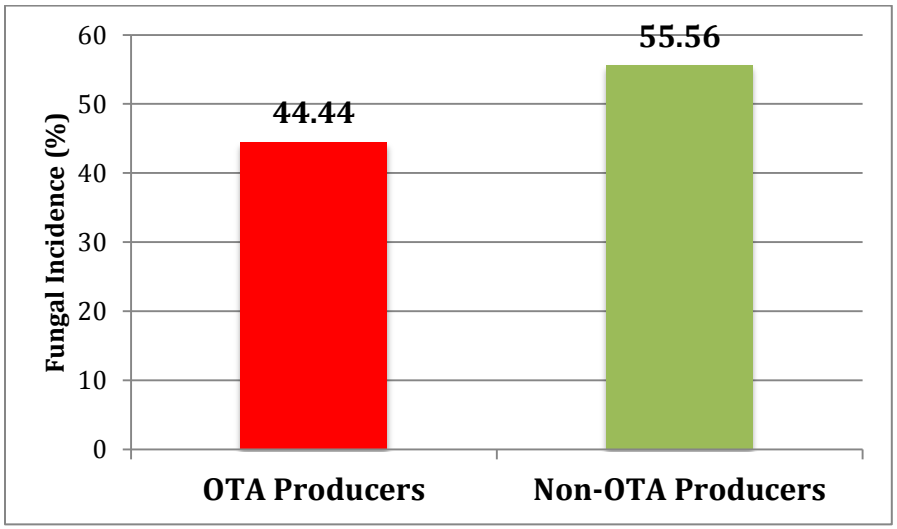

Figure 20. Incidence of isolated OTA and non-OTA producers isolated from dried fruit samples positive for OTA

Based on their genetic sequences, all isolates obtained from dried fruits were compared to the sequences in the GenBank and the best match for them along with their ability to produce OTA is shown in Table 25. According to the data provided in Table 25, most of the OTA producers were either identified as *A. niger* or *A. tubingensis*

Table 25. Isolates best match to sequences deposited in the GenBank along with their ability to produce OTA

Strains	Total isolates	% OTA producers in individual species
Eurotium sp.	1	100
Eurotiomycetes sp. DC482	2	50.0
Aspergillus niger strain MUM05.13	1	100
Aspergillus niger strain AL-26	1	0.0
Aspergillus niger isolate Uf221	20	15.0
Aspergillus niger strain KAML2	37	27.0
Penicillium citrinum strain ESF19M	1	0.0
Aspergillus tubingensis isolate A4S5_21	1	100
Aspergillus tubingensis isolate Uf125	2	50.0
Aspergillus tubingensis strain SZX-6	45	11.1
Aspergillus tubingensis strain CBS 122.49	1	0.0

Romero et al. (2005) reported that 15% of the fungal isolates obtained from dried vine fruit samples, were ochratoxigenic. They reported that *A. carbonarius* was the main OTA producer. Magnoli et al. (2004) isolated molds from dried vine fruits and among the isolated *Aspergillus* strains 28% were ochratoxigenic and again *A. carbonarius* was the main OTA producer. Chulze et al. (2006) reported that *Aspergillus niger* was the predominant species in dried fruit but not a potential OTA producer; while *Aspergillus carbonarius* was the important OTA producer in dried vine fruits.

Based on the literature reports, *A. carbonarius* is the major OTA producer in dried fruits; however the results in this study showed that *Aspergillus niger* and *Aspergillus tubingensis* were the major producers of OTA in dried fruit samples tested. The possible reason for the fact is that *A. niger* and *A. tubingensis* were shown to be the major OTA producers in contrast with the data in the literature for dried fruits could be explained by the geographical source of the samples. Medina et al. (2005), identified isolates of *A.*

tubingensis capable of producing OTA. Accensi et al. (2001) and Abarca et al. (1994) reported the occurrence of OTA producing *A. niger* isolates. Even though these two species have not been reported before as OTA producers- just a small fraction of them was shown to produce the toxin-10% of *A. niger* (Abraca et al., 1994) and 14% of *A. tubingensis* (Medina et al., 2005). In this study, 24% of the *A. niger* strains were capable of producing OTA; while 14% of *A. tubingensis* was able to produce the toxin. The dried fruits analyzed, especially raisins in this project were from different United States, while the other reports were for fruits from Turkey, Argentina and Australia.

Chapter 4. CONCLUSIONS AND FUTURE RESEARCH

4.1 Conclusions

- In this study, the extraction solvent, 3% aqueous sodium bicarbonate: methanol (50:50) showed the best recovery results (83.2% and 82.7%) compared to other solvents for analysis of OTA in coffee and cocoa, respectively. In meat, dichloromethane: ethyl acetate (1:3) was the extraction solvent of choice with a 97.0 % average recovery rate.

- Decaffeinated coffee samples showed the highest incidence of contamination (16.7%) with OTA among all the coffee samples analyzed. Coffee samples from conventional farming seemed to show higher incidence of contamination (10.0%) than their organic counterparts (2.7%). Coffee samples with unknown origin seemed to show higher incidence of OTA (5.5%) than samples from South America (0%).

- Among the cocoa samples analyzed, 1 in 4 samples were contaminated with OTA. Dutch processed samples seemed to show higher incidence (50.0%) of OTA than regular processed cocoa products (10.0%).

- Among the meat samples analyzed, only one of the ham samples showed OTA levels above the limit of quantification.

- In the validation study of an ELISA test kit for quantification of OTA in meat, the average recovery (91.8%), repeatability (no difference between two operators)

and linearity of the standard curve indicated that the kit would be suitable for such analysis, if the proposed extraction procedure was followed.

- The cross-reactivity experiment showed that the method is very specific for OTA, especially when the proposed resuspension solvent is used (83.5%). The results of the robustness showed that the incubation times proposed by the manufacturer must be closely followed to avoid under estimation of OTA levels.

- Among the dried fruits surveyed, raisins showed the highest incidence of samples positive fro OTA (100%), with 27.8% of the samples showing OTA levels above the European regulatory limit (10 ppb) and OTA levels ranging between 3.0-21.2 ppb. No samples from apricots, dates and figs exceeded the regulatory limit but detectable OTA levels ranged between 1.0-2.7 ppb.

- When the fungal incidence in dried fruits was evaluated, 100% of raisins were contaminated, while apricots, figs and dates showed fungal incidence of 37%, 8.3% and 92%, respectively.

- According to the phylogenetic trees obtained based on the sequences of isolates from dried fruits, there is not much diversity in the population of apricots, raisins, figs and dates as most of the fungal isolates had identical sequences with a few differing by a few nucleotides.

- A total of 116 (black molds) isolates were obtained from the different dried fruits. Among them 93% (107/116) were identified based on their genome sequence as *Aspergillus niger* and/or *Aspergillus tubingensis.*

- The high fungal incidence observed in dried fruits associated with the high incidence of ochratoxigenic isolates; highlight the importance of good storage practices for this kind of product. Dried fruits are highly hygroscopic and if the storage conditions (packaging, temperature, relative humidity) are not adequate the spores present in the product may germinate and toxin can be produced.

- *A. niger* (24.0%, 14/59) and *A. tubingensis* (14.0%, 7/49) were the main OTA producers in the dried fruit samples analyzed.

4.2 Future Research

Considering those results, the following future research could be done to allow for a better understanding of the OTA contamination and toxin production patterns in food commodities:

1. Additional survey (i.e. year 2) of OTA levels in coffee, cocoa and meat would better support a future risk assessment for OTA in these food commodities.

2. A comparison of ochratoxin A levels in green coffee and roasted coffee would provide an understanding of the role of processing conditions on the toxin levels of the final product.

3. Expand the validation of Veratox™ (Neogen, MI) kit for meat analysis by directly comparing values obtained with the test kit and by HPLC.

4. Compare the sequence of the ITSI region of the isolates obtained in this study with the sequence of the reference strains of *A. niger* and *A. carbonarius* to verify the findings reported here.

5. Survey vineyards in North America to evaluate if the fungal microbiota and toxigenic species associated with grapes and raisins in this portion of the world are different than what has been reported in the literature, especially regarding the fact that *A. niger* would be the major OTA producer in these products.

6. Define storage conditions that would favor mold growth and toxin production in dried fruits to help manufactures prevent the spoilage of the product or production of toxin during storage.

APPENDIX

Appendix A: Statistical analysis for the recovery experiments in coffee

```
data Coffee;
input Coffee $ @@;
do obs = 1 to 3;
     input Recovery @@;
     output;
end;
datalines;
1 74.7 74.1 83.0
2 13.1 12.5 14.1
3 64.1 45.6 43.7
;
proc print; run;
proc glimmix;
class Coffee;
model Recovery=Coffee / solution;
lsmeans Coffee / diff cl ;
run;
```

The SAS System

The GLIMMIX Procedure

Model Information

Data Set	WORK.COFFEE
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
Coffee	3	1 2 3

Number of Observations Read	9
Number of Observations Used	9

Dimensions	
Covariance Parameters	1
Columns in X	4
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	9

Optimization Information	
Optimization Technique	None
Parameters	4
Lower Boundaries	1
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	43.89
AIC (smaller is better)	51.89
AICC (smaller is better)	91.89
BIC (smaller is better)	51.06
CAIC (smaller is better)	55.06
HQIC (smaller is better)	48.56
Pearson Chi-Square	304.80
Pearson Chi-Square / DF	50.80

Parameter Estimates

Effect	Coffee	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		51.1333	4.1150	6	12.43	<.0001
Coffee	**1**	26.1333	5.8195	6	4.49	0.0041
Coffee	**2**	-37.9000	5.8195	6	-6.51	0.0006

Parameter Estimates

Effect	Coffee	Estimate	Standard Error	DF	t Value	Pr > |t|
Coffee	**3**	0	.	.	.	.
Scale		50.8000	29.3294	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Coffee	2	6	61.22	0.0001

Coffee Least Squares Means

Coffee	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	77.2667	4.1150	6	18.78	<.0001	0.05	67.1976	87.3357
2	13.2333	4.1150	6	3.22	0.0182	0.05	3.1643	23.3024
3	51.1333	4.1150	6	12.43	<.0001	0.05	41.0643	61.2024

Differences of Coffee Least Squares Means

Coffee	_Coffee	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	**2**	64.0333	5.8195	6	11.00	<.0001	0.05	49.7935	78.2732
1	**3**	26.1333	5.8195	6	4.49	0.0041	0.05	11.8935	40.3732
2	**3**	-37.9000	5.8195	6	-6.51	0.0006	0.05	-52.1398	-23.6602

Appendix B: Statistical analysis for the recovery experiments in cocoa

```
data Cocoa;
input Cocoa $ @@;
do obs = 1 to 3;
     input Recovery @@;
     output;
end;
datalines;
1 107.5 99.9 62.5
2 36.9 43.4 38
;
proc print; run;
proc glimmix;
class Cocoa;
model Recovery=Cocoa / solution;
lsmeans Cocoa / diff cl ;
run;
```

The SAS System

The GLIMMIX Procedure

Model Information	
Data Set	WORK.COCOA
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
Cocoa	2	1 2

Number of Observations Read	6

Number of Observations Used	6

Dimensions	
Covariance Parameters	1
Columns in X	3
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	6

Optimization Information	
Optimization Technique	None
Parameters	3
Lower Boundaries	1
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	36.31
AIC (smaller is better)	42.31
AICC (smaller is better)	66.31
BIC (smaller is better)	40.47
CAIC (smaller is better)	43.47
HQIC (smaller is better)	38.27
Pearson Chi-Square	1184.71
Pearson Chi-Square / DF	296.18

Parameter Estimates

Effect	Cocoa	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		39.4333	9.9361	4	3.97	0.0166
Cocoa	**1**	50.5333	14.0518	4	3.60	0.0228

Parameter Estimates

Effect	Cocoa	Estimate	Standard Error	DF	t Value	Pr > |t|
Cocoa	**2**	0	.	.	.	.
Scale		296.18	209.43	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Cocoa	1	4	12.93	0.0228

Cocoa Least Squares Means

Cocoa	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	89.9667	9.9361	4	9.05	0.0008	0.05	62.3796	117.55
2	39.4333	9.9361	4	3.97	0.0166	0.05	11.8463	67.0204

Differences of Cocoa Least Squares Means

Cocoa	_Cocoa	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	**2**	50.5333	14.0518	4	3.60	0.0228	0.05	11.5194	89.5473

Appendix C: Statistical analysis for the recovery experiments of OTA in meat using ELISA test kit

```
data MeatR;
input MeatR $ @@;
do obs = 1 to 5;
      input Recovery @@;
      output;
end;
datalines;
1 92.8 83.0 93.3 92.3 97.5
2 31.5 24.5 36.8 42.0 31.7
3 29.8 25.8 21.8 26.3 30.0
;
proc print; run;
proc glimmix;
class MeatR;
model Recovery=MeatR / solution;
lsmeans MeatR / diff cl ;
run;
```

The SAS System

Obs	MeatR	obs	Recovery
1	1	1	92.8
2	1	2	83.0
3	1	3	93.3
4	1	4	92.3
5	1	5	97.5
6	2	1	31.5
7	2	2	24.5
8	2	3	36.8
9	2	4	42.0
10	2	5	31.7
11	3	1	29.8
12	3	2	25.8
13	3	3	21.8
14	3	4	26.3

Obs	MeatR	obs	Recovery
15	3	5	30.0

The SAS System

The GLIMMIX Procedure

Model Information	
Data Set	WORK.MEATR
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
MeatR	3	1 2 3

Number of Observations Read	15
Number of Observations Used	15

Dimensions	
Covariance Parameters	1
Columns in X	4
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	15

Optimization Information

Optimization Information	
Optimization Technique	None
Parameters	4
Lower Boundaries	1
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	78.66
AIC (smaller is better)	86.66
AICC (smaller is better)	92.37
BIC (smaller is better)	88.60
CAIC (smaller is better)	92.60
HQIC (smaller is better)	85.94
Pearson Chi-Square	330.08
Pearson Chi-Square / DF	27.51

Parameter Estimates

Effect	MeatR	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		26.7400	2.3455	12	11.40	<.0001
MeatR	**1**	65.0400	3.3170	12	19.61	<.0001
MeatR	**2**	6.5600	3.3170	12	1.98	0.0714
MeatR	**3**	0	.	.	.	.
Scale		27.5067	11.2295	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
MeatR	2	12	233.07	<.0001

MeatR Least Squares Means

MeatR	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper

MeatR Least Squares Means

MeatR	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	91.7800	2.3455	12	39.13	<.0001	0.05	86.6696	96.8904
2	33.3000	2.3455	12	14.20	<.0001	0.05	28.1896	38.4104
3	26.7400	2.3455	12	11.40	<.0001	0.05	21.6296	31.8504

Differences of MeatR Least Squares Means

MeatR	_MeatR	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	**2**	58.4800	3.3170	12	17.63	<.0001	0.05	51.2528	65.7072
1	**3**	65.0400	3.3170	12	19.61	<.0001	0.05	57.8128	72.2672
2	**3**	6.5600	3.3170	12	1.98	0.0714	0.05	-0.6672	13.7872

Appendix D: Statistical analysis for the cross-reactivity experiments using ELISA test kit

```
data CrossreactivityA;
input CrossreactivityA $ @@;
do obs = 1 to 6;
      input Recovery @@;
      output;
end;
datalines;
1 76.3 89.4 82.1 77.9 91.2 91.2
2 64.7 64.3 75.1 67.9 65.9 67.7
;
proc print; run;
proc glimmix;
class CrossreactivityA;
model Recovery=CrossreactivityA / solution;
lsmeans CrossreactivityA / diff cl ;
run;
```

The SAS System

Obs	CrossreactivityA	obs	Recovery
1	1	1	76.3
2	1	2	89.4
3	1	3	82.1
4	1	4	77.9
5	1	5	91.2
6	1	6	91.2
7	2	1	64.7
8	2	2	64.3
9	2	3	75.1
10	2	4	67.9
11	2	5	65.9
12	2	6	67.7

The SAS System

The GLIMMIX Procedure

Model Information	
Data Set	WORK.CROSSREACTIVITYA
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
CrossreactivityA	2	1 2

Number of Observations Read	12
Number of Observations Used	12

Dimensions	
Covariance Parameters	1
Columns in X	3
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	12

Optimization Information	
Optimization Technique	None
Parameters	3
Lower Boundaries	1

Optimization Information	
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	66.26
AIC (smaller is better)	72.26
AICC (smaller is better)	76.26
BIC (smaller is better)	73.17
CAIC (smaller is better)	76.17
HQIC (smaller is better)	71.26
Pearson Chi-Square	308.69
Pearson Chi-Square / DF	30.87

Parameter Estimates

Effect	CrossreactivityA	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		67.6000	2.2682	10	29.80	<.0001
CrossreactivityA	**1**	17.0833	3.2077	10	5.33	0.0003
CrossreactivityA	**2**	0	.	.	.	.
Scale		30.8688	13.8050	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
CrossreactivityA	1	10	28.36	0.0003

CrossreactivityA Least Squares Means

CrossreactivityA	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	84.6833	2.2682	10	37.33	<.0001	0.05	79.6294	89.7372
2	67.6000	2.2682	10	29.80	<.0001	0.05	62.5461	72.6539

Differences of CrossreactivityA Least Squares Means									
CrossreactivityA	**_CrossreactivityA**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
1	**2**	17.0833	3.2077	10	5.33	0.0003	0.05	9.9360	24.2306

Appendix E: Statistical analysis for the robustness experiments using ELISA test kit

```
data robutness;
input robutness $ @@;
do obs = 1 to 6;
     input Recovery @@;
     output;
end;
datalines;
1 110.34 113.05 115.33 112.55 115.33 101.72
2 95.07 80.77 87.02 79.21 82.05 79.52
3 88.19 95.41 97.72 97.18 103.65 100.27
4 76.75 88.53 94.58 88.13 89.76 88.53
;
proc print; run;
proc glimmix;
class robutness;
model Recovery=robutness / solution;
lsmeans robutness / diff cl ;
run;
```

The SAS System

The GLIMMIX Procedure

Model Information

Data Set	WORK.ROBUTNESS
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
robutness	4	1 2 3 4

Number of Observations Read	24
Number of Observations Used	24

Dimensions	
Covariance Parameters	1
Columns in X	5
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	24

Optimization Information	
Optimization Technique	None
Parameters	5
Lower Boundaries	1
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	132.84
AIC (smaller is better)	142.84
AICC (smaller is better)	147.12
BIC (smaller is better)	147.82
CAIC (smaller is better)	152.82
HQIC (smaller is better)	143.81
Pearson Chi-Square	627.28
Pearson Chi-Square / DF	31.36

Parameter Estimates

Effect	robutness	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		87.7133	2.2863	20	38.36	<.0001
robutness	**1**	23.6733	3.2334	20	7.32	<.0001
robutness	**2**	-3.7733	3.2334	20	-1.17	0.2569
robutness	**3**	9.3567	3.2334	20	2.89	0.0090
robutness	**4**	0	.	.	.	.
Scale		31.3638	9.9181	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
robutness	3	20	28.58	<.0001

robutness Least Squares Means

robutness	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	111.39	2.2863	20	48.72	<.0001	0.05	106.62	116.16
2	83.9400	2.2863	20	36.71	<.0001	0.05	79.1708	88.7092
3	97.0700	2.2863	20	42.46	<.0001	0.05	92.3008	101.84
4	87.7133	2.2863	20	38.36	<.0001	0.05	82.9441	92.4825

Differences of robutness Least Squares Means

robutness	_robutness	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	**2**	27.4467	3.2334	20	8.49	<.0001	0.05	20.7020	34.1913
1	**3**	14.3167	3.2334	20	4.43	0.0003	0.05	7.5720	21.0613
1	**4**	23.6733	3.2334	20	7.32	<.0001	0.05	16.9287	30.4180
2	**3**	-13.1300	3.2334	20	-4.06	0.0006	0.05	-19.8747	-6.3853

Differences of robutness Least Squares Means									
robutness	**_robutness**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
2	**4**	-3.7733	3.2334	20	-1.17	0.2569	0.05	-10.5180	2.9713
3	**4**	9.3567	3.2334	20	2.89	0.0090	0.05	2.6120	16.1013

Appendix F: Statistical analysis for the repeatability experiments using ELISA test kit

1) Low spiking level

```
data ReapeatabilityLS;
input ReapeatabilityLS $ @@;
do obs = 1 to 5;
      input Recovery @@;
      output;
end;
datalines;
1 103.8 75.6 62.5 96.8 73.9
2 78.4 102.4 87.5 77.4 76.6
;
proc print; run;
proc glimmix;
class ReapeatabilityLS;
model Recovery=ReapeatabilityLS / solution;
lsmeans ReapeatabilityLS / diff cl ;
run;
```

The SAS System

The GLIMMIX Procedure

Model Information	
Data Set	WORK.REAPEATABILITYLS
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**

Class Level Information		
Class	**Levels**	**Values**
ReapeatabilityLS	2	1 2

Number of Observations Read	10
Number of Observations Used	10

Dimensions	
Covariance Parameters	1
Columns in X	3
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	10

Optimization Information	
Optimization Technique	None
Parameters	3
Lower Boundaries	1
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	68.60
AIC (smaller is better)	74.60
AICC (smaller is better)	80.60
BIC (smaller is better)	74.84
CAIC (smaller is better)	77.84
HQIC (smaller is better)	72.99
Pearson Chi-Square	1659.18
Pearson Chi-Square / DF	207.40

Parameter Estimates

Effect	ReapeatabilityLS	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		84.4600	6.4405	8	13.11	<.0001
ReapeatabilityLS	**1**	-1.9400	9.1082	8	-0.21	0.8367
ReapeatabilityLS	**2**	0	.	.	.	.
Scale		207.40	103.70	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
ReapeatabilityLS	1	8	0.05	0.8367

ReapeatabilityLS Least Squares Means

ReapeatabilityLS	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	82.5200	6.4405	8	12.81	<.0001	0.05	67.6683	97.3717
2	84.4600	6.4405	8	13.11	<.0001	0.05	69.6083	99.3117

Differences of ReapeatabilityLS Least Squares Means

ReapeatabilityLS	_ReapeatabilityLS	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	**2**	-1.9400	9.1082	8	-0.21	0.8367	0.05	-22.9435	19.0635

2) High spiking level

```
data ReapeatabilityHS;
input ReapeatabilityHS $ @@;
do obs = 1 to 5;
     input Recovery @@;
     output;
end;
datalines;
1 100.7 90.2 70.6 121.4 79.5
```

```
2 83.8 105.0 102.5 54.2 79.2
;
proc print; run;
proc glimmix;
class ReapeatabilityHS;
model Recovery=ReapeatabilityHS / solution;
lsmeans ReapeatabilityHS / diff cl ;
run;
```

The SAS System

The GLIMMIX Procedure

Model Information	
Data Set	WORK.REAPEATABILITYHS
Response Variable	Recovery
Response Distribution	Gaussian
Link Function	Identity
Variance Function	Default
Variance Matrix	Diagonal
Estimation Technique	Restricted Maximum Likelihood
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
ReapeatabilityHS	2	1 2

Number of Observations Read	10
Number of Observations Used	10

Dimensions	
Covariance Parameters	1
Columns in X	3
Columns in Z	0
Subjects (Blocks in V)	1
Max Obs per Subject	10

Optimization Information	
Optimization Technique	None
Parameters	3
Lower Boundaries	1
Upper Boundaries	0
Fixed Effects	Not Profiled

Fit Statistics	
-2 Res Log Likelihood	73.97
AIC (smaller is better)	79.97
AICC (smaller is better)	85.97
BIC (smaller is better)	80.21
CAIC (smaller is better)	83.21
HQIC (smaller is better)	78.36
Pearson Chi-Square	3246.30
Pearson Chi-Square / DF	405.79

Parameter Estimates

Effect	ReapeatabilityHS	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		84.9400	9.0087	8	9.43	<.0001
ReapeatabilityHS	**1**	7.5400	12.7403	8	0.59	0.5703
ReapeatabilityHS	**2**	0	.	.	.	.
Scale		405.79	202.89	.	.	.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
ReapeatabilityHS	1	8	0.35	0.5703

ReapeatabilityHS Least Squares Means

ReapeatabilityHS	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper

ReapeatabilityHS Least Squares Means

ReapeatabilityHS	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	92.4800	9.0087	8	10.27	<.0001	0.05	71.7058	113.25
2	84.9400	9.0087	8	9.43	<.0001	0.05	64.1658	105.71

Differences of ReapeatabilityHS Least Squares Means

ReapeatabilityHS	_ReapeatabilityHS	Estimate	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
1	**2**	7.5400	12.7403	8	0.59	0.5703	0.05	-21.8392	36.9192

Appendix G: Statistical analysis for comparison between OTA levels in conventional and organic dried fruits

```
data Rep Location Variety Aw;
input Rep@@ Farming$ Fruit$ OTA@@; cards;
1 Orgc Fig 0
2 Orgc Fig 0
3 Orgc Fig 0
4 Orgc Fig 0
5 Orgc Fig 0
1 Conv Fig 1.2
2 Conv Fig 2.7
3 Conv Fig 0
4 Conv Fig 0
5 Conv Fig 0
1 Orgc Apricot 0
2 Orgc Apricot 0
3 Orgc Apricot 0
4 Orgc Apricot 0
5 Orgc Apricot 0
1 Conv Apricot 1
2 Conv Apricot 2.2
3 Conv Apricot 1
4 Conv Apricot 1.1
5 Conv Apricot 0
6 Conv Apricot 0
1 Orgc Raisin 4.1
2 Orgc Raisin 1.6
3 Orgc Raisin 3.7
4 Orgc Raisin 3.6
5 Orgc Raisin 10
6 Orgc Raisin 16.6
7 Orgc Raisin 4.2
8 Orgc Raisin 5
9 Orgc Raisin 21.2
10 Orgc Raisin 4.7
11 Orgc Raisin 16.1
1 Conv Raisin 16
2 Conv Raisin 7.9
3 Conv Raisin 7.1
4 Conv Raisin 2.3
5 Conv Raisin 3
6 Conv Raisin 7.2
7 Conv Raisin 4.5
1 Orgc Dates 2.1
```

```
2 Orgc Dates 1.4
3 Orgc Dates 1.5
4 Orgc Dates 1.5
1 Conv Dates 1
2 Conv Dates 2.7
3 Conv Dates 1.9
4 Conv Dates 1.1
;
proc mixed;
class Farming Fruit;
model OTA=Farming Fruit Farming*Fruit;
lsmeans Farming Fruit Farming*Fruit/diff; run;
```

The Mixed Procedure

Model Information	
Data Set	WORK.AW
Dependent Variable	OTA
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	Levels	Values
Farming	2	Conv Orgc
Fruit	4	Apricot Dates Fig Raisin

Dimensions	
Covariance Parameters	1
Columns in X	15
Columns in Z	0
Subjects	1
Max Obs Per Subject	47

Number of Observations	
Number of Observations Read	47
Number of Observations Used	47
Number of Observations Not Used	0

Covariance Parameter Estimates	
Cov Parm	Estimate
Residual	14.9425

Fit Statistics	
-2 Res Log Likelihood	229.9
AIC (smaller is better)	231.9
AICC (smaller is better)	232.0
BIC (smaller is better)	233.5

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Farming	1	39	0.00	0.9471
Fruit	3	39	11.28	<.0001
Farming*Fruit	3	39	0.26	0.8513

Least Squares Means							
Effect	Farming	Fruit	Estimate	Standard Error	DF	t Value	Pr > |t|
Farming	Conv		2.5489	0.8422	39	3.03	0.0044
Farming	Orgc		2.4699	0.8318	39	2.97	0.0051
Fruit		Apricot	0.4417	1.1704	39	0.38	0.7079
Fruit		Dates	1.6500	1.3667	39	1.21	0.2346
Fruit		Fig	0.3900	1.2224	39	0.32	0.7514
Fruit		Raisin	7.5558	0.9345	39	8.09	<.0001

The Mixed Procedure

Least Squares Means

Effect	Farming	Fruit	Estimate	Standard Error	DF	t Value	Pr > |t|
Farming*Fruit	Conv	Apricot	0.8833	1.5781	39	0.56	0.5789
Farming*Fruit	Conv	Dates	1.6750	1.9328	39	0.87	0.3914
Farming*Fruit	Conv	Fig	0.7800	1.7287	39	0.45	0.6543
Farming*Fruit	Conv	Raisin	6.8571	1.4610	39	4.69	<.0001
Farming*Fruit	Orgc	Apricot	0	1.7287	39	0.00	1.0000
Farming*Fruit	Orgc	Dates	1.6250	1.9328	39	0.84	0.4056
Farming*Fruit	Orgc	Fig	-355E-17	1.7287	39	-0.00	1.0000
Farming*Fruit	Orgc	Raisin	8.2545	1.1655	39	7.08	<.0001

Differences of Least Squares Means

Effect	Farming	Fruit	_Farming	_Fruit	Estimate	Standard Error	DF	t Value	Pr > |t|
Farming	Conv		Orgc		0.07898	1.1837	39	0.07	0.9471
Fruit		Apricot		Dates	-1.2083	1.7993	39	-0.67	0.5058
Fruit		Apricot		Fig	0.05167	1.6923	39	0.03	0.9758
Fruit		Apricot		Raisin	-7.1142	1.4977	39	-4.75	<.0001
Fruit		Dates		Fig	1.2600	1.8336	39	0.69	0.4960
Fruit		Dates		Raisin	-5.9058	1.6556	39	-3.57	0.0010
Fruit		Fig		Raisin	-7.1658	1.5387	39	-4.66	<.0001
Farming*Fruit	Conv	Apricot	Conv	Dates	-0.7917	2.4952	39	-0.32	0.7527
Farming*Fruit	Conv	Apricot	Conv	Fig	0.1033	2.3407	39	0.04	0.9650
Farming*Fruit	Conv	Apricot	Conv	Raisin	-5.9738	2.1506	39	-2.78	0.0084
Farming*Fruit	Conv	Apricot	Orgc	Apricot	0.8833	2.3407	39	0.38	0.7079
Farming*Fruit	Conv	Apricot	Orgc	Dates	-0.7417	2.4952	39	-0.30	0.7679
Farming*Fruit	Conv	Apricot	Orgc	Fig	0.8833	2.3407	39	0.38	0.7079
Farming*Fruit	Conv	Apricot	Orgc	Raisin	-7.3712	1.9618	39	-3.76	0.0006
Farming*Fruit	Conv	Dates	Conv	Fig	0.8950	2.5931	39	0.35	0.7318
Farming*Fruit	Conv	Dates	Conv	Raisin	-5.1821	2.4229	39	-2.14	0.0388
Farming*Fruit	Conv	Dates	Orgc	Apricot	1.6750	2.5931	39	0.65	0.5221
Farming*Fruit	Conv	Dates	Orgc	Dates	0.05000	2.7334	39	0.02	0.9855
Farming*Fruit	Conv	Dates	Orgc	Fig	1.6750	2.5931	39	0.65	0.5221
Farming*Fruit	Conv	Dates	Orgc	Raisin	-6.5795	2.2570	39	-2.92	0.0059
Farming*Fruit	Conv	Fig	Conv	Raisin	-6.0771	2.2634	39	-2.68	0.0106
Farming*Fruit	Conv	Fig	Orgc	Apricot	0.7800	2.4448	39	0.32	0.7514
Farming*Fruit	Conv	Fig	Orgc	Dates	-0.8450	2.5931	39	-0.33	0.7463
Farming*Fruit	Conv	Fig	Orgc	Fig	0.7800	2.4448	39	0.32	0.7514
Farming*Fruit	Conv	Fig	Orgc	Raisin	-7.4745	2.0849	39	-3.59	0.0009
Farming*Fruit	Conv	Raisin	Orgc	Apricot	6.8571	2.2634	39	3.03	0.0043
Farming*Fruit	Conv	Raisin	Orgc	Dates	5.2321	2.4229	39	2.16	0.0370
Farming*Fruit	Conv	Raisin	Orgc	Fig	6.8571	2.2634	39	3.03	0.0043
Farming*Fruit	Conv	Raisin	Orgc	Raisin	-1.3974	1.8690	39	-0.75	0.4591
Farming*Fruit	Orgc	Apricot	Orgc	Dates	-1.6250	2.5931	39	-0.63	0.5345
Farming*Fruit	Orgc	Apricot	Orgc	Fig	3.55E-15	2.4448	39	0.00	1.0000
Farming*Fruit	Orgc	Apricot	Orgc	Raisin	-8.2545	2.0849	39	-3.96	0.0003
Farming*Fruit	Orgc	Dates	Orgc	Fig	1.6250	2.5931	39	0.63	0.5345
Farming*Fruit	Orgc	Dates	Orgc	Raisin	-6.6295	2.2570	39	-2.94	0.0055

The Mixed Procedure

Differences of Least Squares Means

Effect	Farming	Fruit	_Farming	_Fruit	Estimate	Standard Error	DF	t Value	Pr > |t|
Farming*Fruit	Orgc	Fig	Orgc	Raisin	-8.2545	2.0849	39	-3.96	0.0003

Appendix H:

Table 26. Identification of each isolate obtained from dried fruits according to information on GenBank

Isolated strain	Top hit by GenBank	% Identity	E-value
Isolatefig1	*Aspergillus tubingensis* strain SZX-6	97	6e-173
Isolatefig2	*Aspergillus niger* strain KAML02	96	5e-143
Isolatefig3	*Aspergillus tubingensis* strain SZX-6	95	2e-136
Isolatefig4	*Aspergillus niger* isolate Uf221	99	0.0
Isolateapricot1	*Eurotiomycetes* sp.DC482	100	0.0
Isolateapricot2	*Aspergillus niger* isolate Uf221	99	0.0
Isolateapricot3	*Aspergillus niger* isolate Uf221	99	0.0
Isolateapricot4	*Aspergillus niger* isolate Uf221	100	0.0
Isolateapricot5	*Aspergillus tubingensis* strain SZX-6	97	6e-173
Isolateapricot6	*Aspergillus tubingensis* strain SZX-6	98	4e-179
Isolateapricot7	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolateapricot8	*Aspergillus niger* strain KAML02	98	3e-176
Isolateapricot9	*Aspergillus niger* isolate Uf221	100	0.0
Isolateapricot10	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolateapricot11	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolateapricot12	*Aspergillus tubingensis* strain CBS 122.49	99	0.0
Isolatedates1	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates2	*Aspergillus niger* strain KAML02	99	0.0
Isolatedates3	*Aspergillus tubingensis* isolate Uf125	100	0.0
Isolatedates4	*Aspergillus niger* strain KAML02	99	0.0
Isolatedates5	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates6	*Aspergillus niger* strain KAML02	99	0.0
Isolatedates7	*Aspergillus niger* strain KAML02	99	0.0
Isolatedates8	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates9	*Aspergillus tubingensis* strain SZX-6	100	0.0
Isolatedates10	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates11	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates12	*Aspergillus niger* isolate Uf221	100	0.0
Isolatedates13	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates14	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates15	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates16	*Aspergillus tubingensis* strain SZX-6	98	6e-178
Isolatedates17	*Aspergillus tubingensis* strain SZX-6	98	4e-178
Isolatedates18	*Aspergillus tubingensis* strain SZX-6	97	9e-171
Isolatedates19	*Aspergillus tubingensis* strain SZX-6	98	1e-179
Isolatedates20	*Aspergillus niger* strain KAML02	99	0.0
Isolatedates21	*Aspergillus tubingensis* strain SZX-6	99	0.0

Isolatedates22	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates23	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates24	*Aspergillus tubingensis* strain SZX-6	100	0.0
Isolatedates25	*Aspergillus tubingensis* strain SZX-6	96	2e-146
Isolatedates26	*Aspergillus niger* strain AL-26	95	0.0
Isolatedates27	*Aspergillus tubingensis* strain SZX-6	97	8e-146
Isolatedates28	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates29	*Eurotiomycetes* sp. DC482	100	0.0
Isolatedates30	*Aspergillus niger* isolate Uf221	100	0.0
Isolatedates31	*Aspergillus tubingensis* isolate Uf125	99	0.0
Isolatedates32	*Aspergillus niger* isolate Uf221	100	0.0
Isolatedates33	*Eurotiomycetes* sp. DC482	99	0.0
Isolatedates34	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates35	*Aspergillus tubingensis* strain SZX-6	97	0.0
Isolatedates36	*Eurotium* sp	99	1e-163
Isolatedates37	*Aspergillus tubingensis* strain SZX-6	97	5e-148
Isolatedates38	*Aspergillus tubingensis* strain SZX-6	98	0.0
Isolatedates39	*Aspergillus tubingensis* strain SZX-6	97	0.0
Isolatedates40	*Aspergillus niger* isolate Uf221	100	0.0
Isolatedates41	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates42	*Aspergillus tubingensis* isolate A4S5	97	0.0
Isolatedates43	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates44	*Aspergillus tubingensis* strain SZX-6	100	0.0
Isolatedates45	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates46	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates47	*Aspergillus tubingensis* strain SZX-6	100	0.0
Isolatedates48	*Eurotiomycetes* sp. DC482	100	0.0
Isolatedates49	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates50	*Aspergillus niger* strain KAML02	100	0.0
Isolatedates51	*Aspergillus niger* strain KAML02	94	8e-162
Isolatedates52	*Aspergillus tubingensis* strain SZX-6	100	0.0
Isolatedates53	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates54	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates55	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates56	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolatedates57	*Aspergillus tubingensis* strain SZX-6	97	4e-174
Isolatedates58	*Aspergillus niger* isolate Uf221	99	0.0
Isolatedates59	*Aspergillus tubingensis* strain SZX-6	97	2e-172
Isolateraisin1	*Aspergillus niger* strain KAML02	100	0.0
Isolateraisin2	*Aspergillus niger* strain KAML02	100	0.0
Isolateraisin3	*Aspergillus niger* strain KAML02	99	2e-177
Isolateraisin3	*Aspergillus tubingensis* strain SZX-6	98	4e-179
Isolateraisin4	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin5	*Eurotiomycetes* sp. DC482	99	0.0
Isolateraisin6	*Aspergillus niger* strain KAML02	100	0.0

Isolateraisin7	*Aspergillus niger* isolate Uf221	100	0.0
Isolateraisin8	Aspergillus niger strain KAML02	99	0.0
Isolateraisin9	*Eurotiomycetes* sp. DC482	99	0.0
Isolateraisin10	*Aspergillus niger* strain KAML02	98	2e-177
Isolateraisin11	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin12	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin13	*Aspergillus niger* isolate Uf221	99	0.0
Isolateraisin14	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin15	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin16	*Aspergillus niger* strain KAML02	100	0.0
Isolateraisin17	*Aspergillus niger* strain KAML02	98	2e-177
Isolateraisin18	*Aspergillus niger* strain KAML02	97	4e-177
Isolateraisin19	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin20	*Aspergillus niger* strain KAML02	96	4e-129
Isolateraisin21	*Aspergillus niger* strain KAML02	97	8e-146
Isolateraisin22	*Aspergillus niger* strain KAML02	97	3e-150
Isolateraisin23	*Aspergillus niger* strain KAML02	98	1e-153
Isolateraisin24	*Aspergillus niger* strain MUMO5.13	99	0.0
Isolateraisin25	*Eurotiomycetes* sp.DC482	99	0.0
Isolateraisin26	*Aspergillus niger* strain KAML02	100	0.0
Isolateraisin27	*Aspergillus tubingensis* strain SZX-6	98	4e-174
Isolateraisin28	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin29	*Aspergillus niger* strain KAML02	99	0.0
Isolateraisin30	*Aspergillus tubingensis* strain SZX-6	99	0.0
Isolateraisin31	*Aspergillus niger* strain KAML02	96	4e-179
Isolateraisin32	*Aspergillus niger* isolate Uf221	99	0.0
Isolateraisin33	*Aspergillus nige*r strain KAML02	96	4e-139
Isolateraisin34	*Aspergillus niger* strain KAML02	98	4e-179
Isolateraisin35	*Aspergillus niger* strain KAML02	97	2e-146
Isolateraisin36	*Aspergillus niger* strain KAML02	96	2e-167
Isolateraisin37	*Aspergillus niger* isolate Uf221	99	0.0
Isolateraisin38	*Pencillium citrinum* strain ESF19M	95	4e-56

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