Method Validation for the Quantitative analysis of Ochratoxin A in wheat by HPLC with Fluorescence Detector.

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Biotechnology

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CERTIFICATE

This is to certify the dissertation entitled "**Method Validation for the Quantitative analysis of Ochratoxin A in wheat by HPLC with Fluorescence Detector***"* Submitted by *'**Ms. Shreedipti Sahoo**'* in partial fulfilment of the requirement for the degree of Master of Science in Biotechnology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No. *'**1661037**'*& Registration No. *'**16529850263**'* is a *bonafide* research work carried out by her under my guidance and supervision from '***18-12-2017***'to'***11-5-2018***'.

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CERTIFICATE

This is to certify that the dissertation entitled "**Method Validation for the Quantitative analysis of Ochratoxin A in wheat by HPLC with Fluorescence Detector**"submitted by '***Ms.Shreedipti Sahoo, Roll No-1661037, Registration No.'16529850263***' to the School of Biotechnology, KIIT University, Bhubaneswar-751024, for the degree of Master of Science in Biotechnology is her original work, based on the results of the experiments and investigations carried out independently by her during the period from '***18-12-2017***'to '***11-52018***' of study under my guidance.

Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University.

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DECLARATION

I hereby declare that the dissertation entitled "**Method Validation for the Quantitative analysis of Ochratoxin A in wheat by HPLC with Fluorescence Detector**"submitted by me, for the degree of Master of Science to KIIT University is a record of *bona fide* work carried by me under the supervision and guidance of '**Mrs.Vanajakshi V, Senior Tecnical officer(1),Food Safety and Analytical Quality Control Laboratory, Department in CSIR - Central Food Technological Research Institute, Mysuru, Karnataka**. I further declare that, the results of the work have not formed the basis for the award of any other degree to any candidate of any university.

Date:

Place: (***Shreedipti Sahoo***)

ABSTRACT

Mycotoxins are natural contaminants in raw materials like food and feeds. Ochratoxin is one of the most prevalent toxin among all mycotoxins mainly they found in 3 types in nature as Ochratoxin A, B, and C. OchratoxinA is a proven carcinogen in animals and is classified as a class 2B, possible human carcinogen by the International Agency for Research on Cancer. *Aspergillus ochraceus* and *Peni-cillium verrucosum*, and species of *Penicillium*, *Petromyces*, and *Neopetromyces* arecommon microbes which are able to produce Ochratoxin A. Ochratoxin A has generally recognized on rice, soya, wheat, corn, coffee, cocoa, ground almond and dried fruits. Also found on corn derivatives like :flour, bread, beer, grape juice and wine.

Several methods have been described for detection of Ochratoxin A in food matrix such as ELISA, TLC, LC-MS/MS. The purpose of this study was to develop and validate a modern, practical, fast and efficient method for Ochratoxin A in wheat using matrix-matched calibration by Liquid Chromatography with Fluorescence Detector (LC-FLD). Standardization of HPLC was carried out by using different concentration of acetic acid in mobile phase. Optimization of extraction Ochratoxin A procedure to be done for the quantification of Ochratoxin A in wheat sample which purchase from local market of Mysuru. Finally for method validation such as linearity, specificity and accuracy total recovery% was calculated with LOD and LOQ. The observed LOQ is 6.25 and LOD is 1.8. Recovery range is 76.13 - 91.76 % and relative standard deviation (% RSD) is 1.54 - 6.0 % for single extraction. In case of triple extraction recovery ranged as 71.46 - 90.76 % and %RSD is 0.45 - 0.46 %.

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Date:

Place: Shreedipti Sahoo

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Abbreviations/Acronyms

BEN - Balkan Endemic Nephropathy

CIN - Chronic Interstitial Nephropathy

DON - Deoxynivalenol

ELISA - Enzyme Linked ImmunoSorbent Assay

FAO - Food and Agricultural Organisation of the United Nation

FLD - Fluorescence Detector

HPLC - High Performance Liquid Chromatography

IAC - Immunoaffinity Column Chromatography

IARC - International Agency for Research on Cancer

LC/MS/MS – Liquid Chromatography-Mass Spectrometry

LOD - Limit Of Detection

LOQ - Limit Of Quantification

MMS - Matrix Match Standard

NTP - National Toxicollogy Program

OTA - OchratoxinA

RSD - Relative Standard Deviation

SPE - -Solid Phase Extraction

TLC – Thin Layer Chromatography

WHO - World Health Organization

ZEA - Zearalenone

1. Introduction

Background and Context

Food safety refers to limiting the presence of those risk wheather cronic or acute, that may make food harmful to the health of the consumer. Food safety is about handling, storing, producing and preparing food in such a way as to supress infection and contamination in the food production chain and to help ensure that food quality also enhance the quality to maintain good health. Mainly contaminats in food can be grouped according their origin and nature. Essentially, these are natural toxins (seafood toxin, mycotoxins), microbiological (bacteria, viruses, parasites), extraneous matter (biological, chemical, physical), other chemical compounds (pesticides, toxic metals, lubricants), packing materials, and poisons introduced through tampering. Additives were at one time a major concern, but today, microbilogical issues are the greatest, followed by pesticide and animal drug residues and antimicrobial drug resistance.(E Todd, 2003)

Mycotoxins are natural contaminants which are produced as secondary metabolites by various fugal species in foodstuffs and feed during production, storage, transportation, often under warm and humid conditions. Mycotoxins are classified as nephrotoxins, neurotoxins, hepatotoxins, and immunotoxins. Common mycotoxins include aflatoxins, ochratoxins, patulin, fumonisins, trichothecenes. Aflatoxins have the most acute toxic effects in human and carcinogenic effects in animal among all mycotoxins. They can grow on a variety of different crops and foodstuffs including nuts, cereals, spices and dried fruits.(Kieu Thi Ngoc Nguyen, 2014)

Ochratoxins produced by several fungal species mainly produced by *Aspergillus ochraceus* and *Penicillum verrucosum,* has the most toxic effect among all mycotoxins. Depending on their chemical structure they are three types Ochratoxin A, Ochratoxin B and Ochratoxin C. Ochratoxin A has been commonly found in cereal and starch rich foods with spices, coffee, wheat, dried fruits, wine, beer, grapes and meat. Scientific investigation observed that food may be mainly contaminated with Ochratoxin A during storage, and it is stable during most food processing stages. Ochratoxin A was classified by the International Agency for Research on Cancer(IARC) as possible carcinogen for

humans (Group 2B;12). Ochratoxin A can be nephrotoxic, mutagenic, hepatotoxic, teratogenic and carcinogenic.The long half-life of Ochratoxin A, together with frequent exposure of humans by ingestion of Ochratoxin A contaminated food, results in a high frequency Ochratoxin A in human blood samples collected around the world. The most frequently found mycotoxin in the blood of people exposed to mycotoxins in their food is Ochratoxin A.(Risk Assessment Studies,May 2006)

Scientific methods for the identification and quantification of Ochratoxin A need to be valid and accurate for routine monitoring. Many methods have been developed for extraction, clean up and detection of Ochratoxin A in different food samples such as immunoaffinity column chromatography (IAC), solid-phase microextraction, ELISA, TLC, LC/MS/MS. Among these, IAC is the most commonly used due to it's high specificity and efficiency based on a specific antigen-antibody reaction. TLC and ELISA are most frequent methods but in some cases separation may not be satisfactory and matrix interferences takes place respectively. Currently LC/MS/MS plays a significant role in analysis of Ochratoxin A in foods and feeds which is hazardous to human and animal health, monitoring low level of Ochratoxin A is especially important. Because most of the sample matrixes are very complicated, especially the biological samples, a pretreatment procedure based on SPE or liquid-liquid extraction is often required to remove the interfering matrix components and concentrate the targeted Ochratoxin A.(Ping Ding, 2013)

Scope and Objectives

1. There is a need to develop a simple method for extraction and determination of Ochratoxin A in food sample. Hence the present studies focused on the following objectives.
2. Standardization of HPLC condition for detection and quantification of Ochratoxin A.
3. Optimization of extraction procedure of Ochratoxin A spiked in wheat sample.
4. Preparation of MMS calibration curve for estimation of Ochratoxin A in wheat sample.
5. Validation of extraction and estimation efficiency of Ochratoxin A in spike wheat sample using MMS calibration curve.

1.3Achievements

Results of the study show following achievements

1. Linearity of Standard graph
2. Optimization of HPLC condition with different concentration of acetic acid in mobile phase
3. Matrix match standard with different concentration
4. Quantification of extraction procedure
5. LOD, LOQ and RSDr (%)

1.4 Overview of Dissertation

The main goal of this dissertation was to develop a validated method for identification and quantification of Ochratoxin A in wheat. Linearity and Linear range of Ochratoxin A in methanol was achieved as describe in 4.2.4. Optimization of Ochratoxin A from spiked wheat sample was done which was mentioned in 4.2.6. Linearity, linear range, LOD and LOQ of matrix matched standard observed. LOD and LOQ was 6.25ppb and 5ppb respectively. Recovery was achieved in range of 71.94-91.76% for single extraction where % RSD is 1.54-6.0 % and 71.46-90.76% for triple extraction where % RSD is 0.45-0.46 %. The validation parameters measured are within acceptable limits and were considerable which is reported in (EU, 519, 2014)

2.Review of Literature

Food Safety

Food safety involves handling, storing, and preparing food to prevent infection and help to make sure that our food keeps enough nutrients for us to have a healthy diet. Food safety is everybody's concern, and it is difficult to find anyone who has not encountered an unpleasant moment of foodborne illness at least once in the past year. It is threatened by numerous contaminants, which can originate from environmental pollution, such as toxic metals and organic halogenated compounds; chemicals used in the production of food, such as pesticides and veterinary drugs; contaminants formed during food production and cooking; contaminants arising from food packing, or natural toxins in food. The highest concern was reported for pesticides in fruit, vegetables and cereals, with 72% of the respondents being very or fairly worried. (Agneta Oskarsson, 2011)

According to the World Health Organization (WHO), foodborne diseases kill an estimated 2.2 million people annually, 1.9 million of them children. Food safety also has potential impact on at least 4 of the 8 milennium development goals set by the United Nations for 2015.(Ana Marusic, Food safety and Security, 2011) The "WHO Five keys to safer food" serve as the basis for educational programmes to train food handlers and educate the consumers. They are especially important in preventing foodborne illness. The five keys are as follows.

- Keep food surface clean. Wash all utensils, plates, platters and cutlery as soon as used.
- Separate raw food from cooked food.
- Cook food thoroughly, to the appropriate temperature.
- Keep food at safe temperatures, both for serving and storage.
- Use safe water and raw materials.

There are different ways to maintain food safety such as good agricultural practices should be applied to reduce microbial and chemical hazards. Organic farming has been promoted in many countries of the South-East Asia Region, as there is a significant segment of health-conscious people. Food safety requires due attention during harvest, transport, processing, storage and finally during food preparation and storage by consumers. (World Health Organization)

Food Contaminants

It has been revealed that food contamination, either from microbiological or chemical origin, is the highest concern for consumers. Food contamination is one of the major drawbacks in food distributions. Food contamination has been recorded in history for as early as 8,000 years ago. The US Centre for Disease Control and Prevention confirmed more than 11,000 foodborne infections in the year 2013, with several agents like viruses, bacteria, toxins, metals and other chemicals causing food contamination. Further more, food contaminants has become more serious in recent years due to the development of industry and the consequent environmental pollution. Besides that, the ingestion of contaminated food with pesticides and heavy metals could cause gastrointestinal infections. (Irfan A. Rather, 2017)

Food contaminations can also takes place during transportation. It can cause by from vehicle exhausts of petrol and diesel or because a cross contamination in the vehicle used for food transportation. Cleaning and disinfecting during food processing eliminate the presence of possible microorganisms to a significant level. Chemicals used as cleaners or disinfectants must be good for food contact surfaces and need to be accepted by the legislation. The addition of sanitizers could be in permitted levels with minimum concentration in processed fruits and vegetables, to quantify the residual chemicals present in the food is important in order to certify that they have been completely removed. (Cristina Nerin, 2015)

Heating treatment in the production process in another source of contaminants. High cooking temperature at home and industries is the common method for food process. The use of high temperature for cooking paired with external factors leads to the formation of toxic compounds that leave an impact on the food safety and quality. Food packing also leads to food contaminants due to the use of several additives like antioxidants, stabilizers, plasticizers, and slipping agents to improve the packing material properties. Food storage in another reason that can lead to toxins in food. Some of the contaminating factors include direct sunlight that speeds deterioration of food and packing and adsorption of unwanted off-odors.

Types of Food Contaminants

Generally food contaminants include environmental contaminants, food processing contaminants, unapproved adulterants and food additives, and migrants

from packaging materials. Food processing contaminants include those undesirable compounds, which are formed in the food during baking, heating, canning, roasting, fermentation, or hydrolysis. Environmental contaminants are impurities that are either introduced by human or occurring naturally in water, air or soil. The direct food contact with packing materials can lead to chemical contamination due to the migration of some harmful substances into foods. (Irfan A. Rather, 2017)

Health Impacts by Food Contaminants

Food contaminants are the main reason behind cancer it can also adversely affect children's neurological development and the immune response. Pesticides in the food as contaminants also show severe health implications. High level of these chemicals in the food cause neural and kidney damage, congenital disabilities, reproductive problems, and can prove to be carcinogenic. The accumulation of pesticides in the tissue of the body can also result in metabolic degradation. Exposure to such chemicals in the fetal stages of development can cause brain injury and such lifelong disabilities at much lower doses than those which can affect adult brain function. Heavy metals consumption is also associated with malnutration and increases the rates of gastrointestinal diseases. Metals such as cadmium and lead can easily enter into the food chain.

Foodstuffs and feeds can be contaminated in a variety of ways, mainly by the presence of microorganisms. In addition to the risk of the presence of microorganisms themselves, a great danger in their metabolites which can be highly toxic and harmful to the consumers health. Among them, mycotoxins are the most important hazard since they represent the increasing threat from the aspects of health and economy. (Bojana Danilovic, 2017)

Mycotoxin

One of the major drawbacks in food safety is the naturally occuring food contaminants known as Mycotoxins. They are secondary metabolites produced by certain types of moulds. They are chemically stable and can't be destroyed either during food processing or by heat treatment, thus for controling these compounds in food is significant for health,agricultural production and food processing. The chemical structures of mycotoxin vary noticeably, but they are all low molecular weight organic compound.

History of Mycotoxin

For the 1st time Mycotoxins arised in 1960 due to the cause of a feed related mycotoxicosis called turkey X disease, which was later proved to be caused by aflatoxins, appeared in farm animals in England. The genera responsible for production of Mycotoxin include: *Aspergillus*, *Pencilium and Fusarium*. Due to the contamination in agricultural field during the time of transportation, storage and processing when the environmental conditions of temperature and humidity allow to develop fungal spores naturally present in the environment, it result in the production of Mycotoxin. Temperature, moisture, water activity, pH and oxygen concentration are remarkable parameter in production of Mycotoxins.

Types of Mycotoxin

Various types of mycotoxins are found which are mainly responsible for the mycotoxicosIs such as Aflatoxins(AF B1,AF B2,AF G1 and AF G2), Patulin, Deoxynivalenol(DON), Ochratoxin(OTA), Zearalenone(ZEA), Fumonisins(FB1,FB2), T2 and HT-2 toxins. The occurance of these multiple mycotoxins give toxic effects due to the consumption of contaminated food. Aflatoxins are produced by *Aspergillus* specis of fungi such as *A. flavus* and *A. parasiticus* but AF B1 is the most toxic they can cause liver cancer in many animal species.

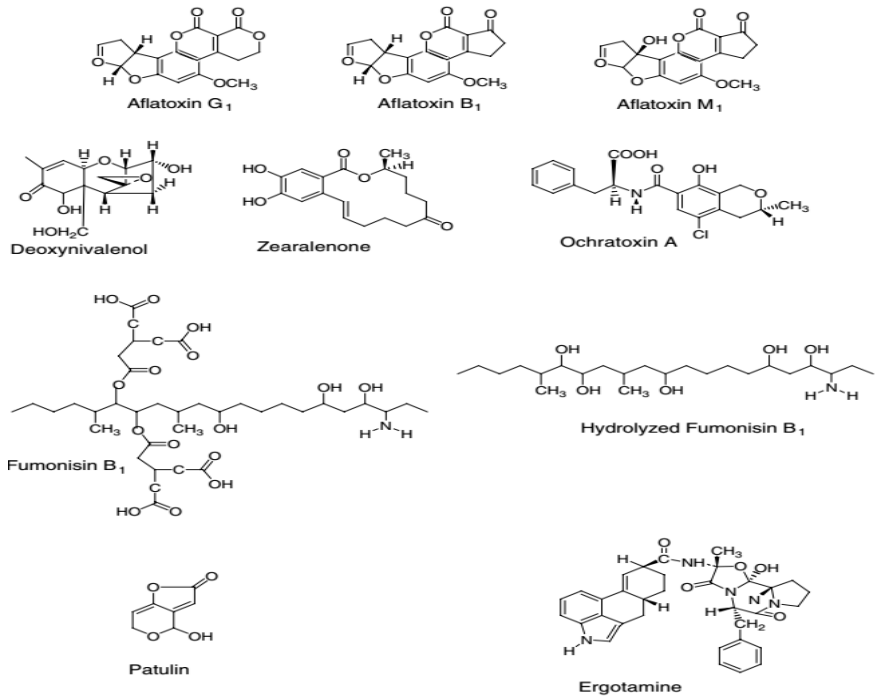


Fig 2.1: Chemical structures of mycotoxins found in foods (Food Mycotoxins, 2006)

Source of Mycotoxin

Mycotoxins can occur in foods such as groundnuts, tree nuts, maize, rice, figs and other dried foods, spices, crude vegetable oils and cocoa beans, due to the result of fungal contamination. Patulin is a type of mycotoxin produced by *Aspergillus*, *Penicilium* and *Paecilomyces*fungal species. *P.expansum* is especially found in fruits and vegetable molds.

Fig 2.2:Food contaminated with Fungi(http://mentalfloss.com)

It observes that they can damage immune system in animals. Fruits such as apricots, grapes, apples, pears, olives, cereals are contaminated with Patulin. Historically it discover that apple juice are highly contaminated by patulin. Ochratoxin is also one of the family of mycotoxin that found in three secondary metabolite forms A, B and C. All are produced by *Penicillium* and *Aspergillus* species. They are carcinogen, nephrotoxin and also can cause tumor in the human urinary tract. They are mainly found in wheat, corn, oats, cheese and meat products of animals consuming ochratoxin-contaminated grains.

Microbes responsible for Mycotoxins

Table: 2.1 Major classes of mycotoxin producing fungi and mycotoxins (www.mycotoxins.info)

Major genera of mycotoxin producing fungi	Species	Mycotoxins
	*A. flavus* *A. parasiticus* *A. nomius*	Aflatoxin (B1, B2, G1,G2)
	A. ochraceus	Ochratoxin (OchratoxinA)
	A. clavatus	Patulin
	*F. verticillioides* *F. proliferatum*	Fumonisin (B1, B2, B3) Fusaric acid
	F. graminearum *F. culmorum*	Zearalenone
	*P. verrucosum* *P. viridicatum*	Ochratoxin (OchratoxinA)
	P. cirtinum *P. verrucosum*	Citrinin
	P. expansum *P. claviforme*	Patulin

Health Hazards

Sometimes mycotoxins are also found in different organs and tissue, due to which they can also enter the human food chain through the animal products such as meat, milk or egg. The toxic reaction of mycotoxin on animal and human health is known as mycotoxicosis. They also have the potential for both acute and chronic health effects by ingestion,skin contact and inhalation. These highly toxic materials can enter into the blood stream and lymphatic system also prevent protein synthesis and damage macrophage systems. Also found in dried fruits such as beans and nuts. To date more than 300 mycotoxins having varying degree of toxicity only few of them are widely accepted as remarkable food or animal feed safety risk. According to the survey of the Food and Agricultural Organisation of the United Nation(FAO) it is found that 25% of the World's food crops are contaminated with mycotoxins.

OchratoxinA

Ochratoxins are toxic secondary metabolites produced by some fungi. It is the 1st major group of mycotoxins defined after the discovery of aflatoxins. There are 3 structural members of ochratoxins :A, B and C which slightly differ from one another. Ochratoxin A is the most significant and extensive fungal toxin of this group than Ochratoxin B and C are of lesser importance. These type of toxins produced by mould species of *Aspergillus ochraceus*, *Aspergillus niger, Aspergillus carbenarius, Penicillium verrucosum* and species of *Penicillum*, *Petromyces* and *Neopetromyces* which grow on a variety of agricultural products. The source of Ochratoxin found in food like grapes, dried vine fruits, fruit juice, wine, beer, coffee, dairy products, spices, pork, poultry and chocolate. They are also found easily in the human food chain directly via plant products such as oil seeds, nuts, cereal grains.

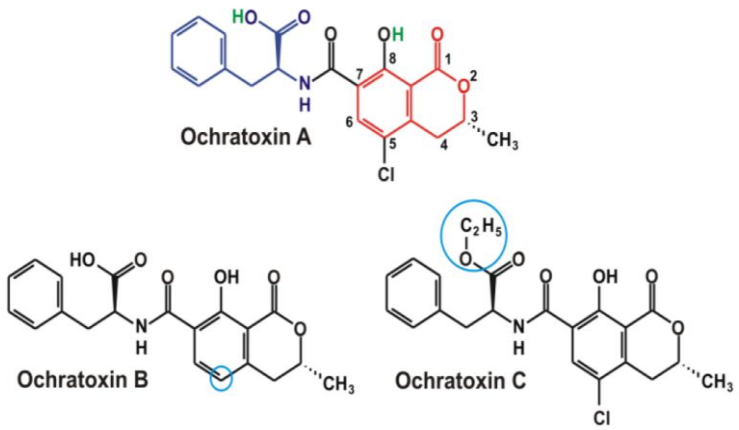


Fig 2.4:Chemical structures of Ochratoxins (http://www.mdpi.com)

History

It was 1st reported in South Africa as a Secondary metabolites produced by a strain inadvertenly referred to as *Aspergillus ochraceus*. (M.Peraica,[1]Bulletinof the World Health Organization, 1999) *Aspergillus ochraceu*s is the well known species of ochratoxin producing Aspergillus. The colonies of *Aspergillus ochraceus* grow rapidly(45 to 55mm in days). The optimum temperature for their growth is 25^0c. They are highly resistant to sunlight and survives sun-drying because of it's black spores and therefore grows at high temperature. (Risk Assessment Studies, 2006)

The reason behind the growth of the mould and subsequent production of Ochratoxin A is dependent upon various factors including temperature, humidity and water activity during the harvesting , drying and storage of the crops. Specially they grow where the crops are not properly dried. (S.O Fapohunda,November 2014)

Health Hazards

Ochratoxin A is proven carcinogen in animals and is classified as a class 2B, possible human carcinogen by the International Agency of Research on Cancer. The National Toxicollogy Program(NTP) has designated Ochratoxin A as "reasonably anticipated to be human carcinogen" based on sufficient evidence of carcinogenicity in experimental animals. (Janette H.Hope[1] and Bradley E.Hope[2], 2011)

Acute renal failure in one person, possibly caused by inhalation of Ochartoxin A in a granary which had been closed for 2years, was reported in Italy. The symptoms developed after 24 hour of transitory epigastric tension, respiratory distress and retrosternal burning. In Tunisia, Ochratoxin A has been observed in high concentrations in the blood and food of patients with kidney impairment of unknown etology. It has also been found in many countries, both in food, feed and in humans.(M. Peraica[1],1999)

In vitro and invivo research has indicated cerebellar, hippocampal and other adverse neurological effects due to Ochratoxin A. A single dose of Ochratoxin A to swiss mice was corresponded with remarkable oxidative damage in six brain regions - the cerebellum, hippocampus, caudate putamen, pens medulla, substantia nigra and cerebral cortex. (Janette H. Hope[1] and Bradley E. Hope[2]2011) Level of Ochratoxin A in human can be measured by detection of Ochratoxin A in human blood and breast milk, has been detected in various countries in Europe.

Ochratoxin A can also cause inhibition of macromolecular synthesis, inhibition of mitochondrial respiration and increased lipid peroxidation.(M.Peraica[1],1999) Ochratoxin A has been considered as a reason of various human nephropathies since the 1970s including Balkan Endemic Nephropathy (BEN) and Chronic Interstitial Nephropathy (CIN) (Travis R Bui-Klimke and Felicia Wu, 2016)

The tenacity of Ochratoxin A in the human body prolonged as it has a blood half-life of 35 days after a single oral dosage, due to unfavorable elimination toxicokinetics. The long half-life of Ochratoxin A, together with frequent exposure of humans by ingestion of Ochratoxin A contaminated food, results in a high frequency Ochratoxin A in human blood samples collected around the world. The most frequent found mycotoxin in the blood of people exposed to mycotoxins in their food is Ochratoxin A.(Risk Assessment Studies,May 2006)

It is found that baking and roasting have been reported to reduce the toxin content by more than 20%. However, physical treatment of grain, such as scouring while cleaning the grain prior to milling, can result in a >50% reduction of Ochratoxin A contamination in the resultant wheat flour.

OchratoxinA Detection Methods

Detection and quantification of Ochratoxin A are more significant due to it's adverse effect. Quality control of food and feed requires extraction and analysis, including TLC, HPLC, MS, and immunochemical methods. Each of these methods has it's advantages and disadvantages. However, with regard to costs and rapidity, immunochemical methods have gained much interest in the last decade.There are several methods for determination of Ochratoxin A. ELISA and LC-fluorescence detection (FLD) are the most frequently used methods. Currently, LC/MS/MS plays an important role in the analysis of Ochratoxin A because of it's high sensitivity and selectivity. Monitoring low level of Ochratoxin A is especially important because Ochratoxin A in food and feeds is potentially hazardous to human and animal health. Because most of the sample matrixes are very complicated, especially the biological samples, a pretreatment procedure based on SPE or liquid-liquid extraction is often required to remove the interfering matrix components and concentrate the targeted Ochratoxin A. (Ping Ding, 2013)

Many recent methods have been reported for extraction and cleanup of Ocharatoxin A in various food matrixes such as immunoaffinity chromatography, solid-phase microextraction, hollow fiber liquid-phase micoextraction, depressive liquid-liquid micoextraction the quick,easy, cheap, effective, rugged and safe method. Among all these, IAC is the most commonly used due to it's high specificity and efficiency. However, IAC columns have some shortcomings, like rather high cost, no ability to be reused, and limited shelf life. (Kieu Thi Ngoc Nguyen, 2014)

Recently it examined that Ultrasonic extraction is a procedure for extracting nanovolatile and semivolatile organic compounds from solids. The ultrasonic process ensures intimate contact of the sample matrix with the extraction solvent. Because ultrasonic extraction is rapid and economical, it may be considered an ideal method for extracting a large number of samples and suitable for routine monitoring. Recent studies have reported the use of ultrasonication to extract fumonisins B^1 and B^2 in corn and 220 undesirable chemical residues in infant formula. (Kieu Thi Ngoc Nguyen, 2014)

Detection of Ochratoxin A by AOAC method

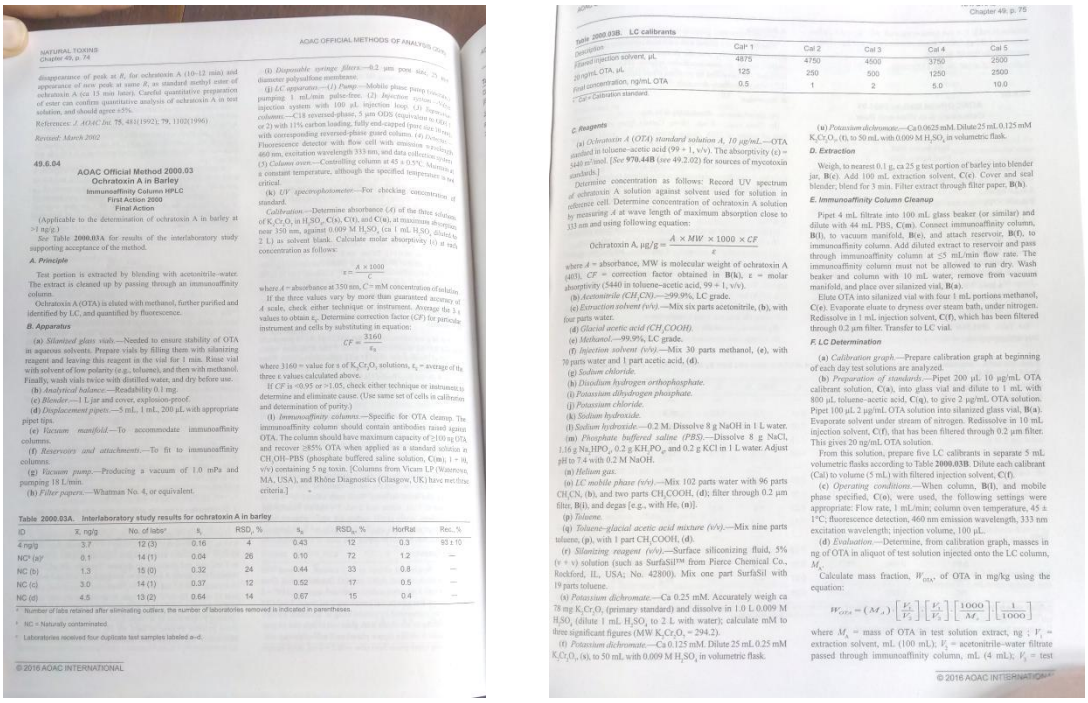


Fig 2.5 : AOAC detection method in barley 2000.03

Regulation of Ochratoxin A in EU

Table: 2.2 Regulation of Ochratoxin A in diiferent food and feed

(https://www.romerlabs.com)

OCHRATOXIN A

Ochratoxin in Food

Commodity	Maximum Level
Unprocessed cereals	5 ppb
All products derived from unprocessed cereals (including cereal products and cereal grains intended for direct human consumption)	3 ppb
Dried wine fruit (currants, raisins and sultanas)	10 ppb
Roasted coffee beans and ground roasted coffee	5 ppb
Soluble coffee (instant coffee)	10 ppb
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % volume) and fruit wine	2 ppb
Flavoured wine, flavoured wine based drinks and flavoured wine product cocktails	2 ppb
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated must as reconstituted, intended for direct human consumption	2 ppb
Species of spices (including white and black pepper, nutmeg, ginger and turmeric, excluding *Capsicum* spp.)	15 ppb
Capsicum spp. spices (including chillies, chilli powder, cayenne and paprika)	20 ppb
Mixtures of spices	15 ppb
Liquorice root	20 ppb
Liquorice extract	80 ppb
Baby foods and processed cereal based foods for infants and young children	0.5 ppb
Dietary foods for special medical purposes intended specifically for infants	0.5 ppb
Wheat gluten not sold directly to the consumer	8 ppb

Ochratoxin in Feed

Commodity	Guidance Level
Cereals and cereal products	0.25 ppm
Complementary and complete feeding stuffs for pigs	0.05 ppm
Complementary and complete feeding stuffs for poultry	0.1 ppm
Complementary and complete feeding stuffs for cats and dogs	0.01 ppm

Table 2.3 : Regulation of Ochratoxin A by FSSAI in India

Ochratoxin A in Food	Maximum Level
Wheat/ Barley	20 µg/Kg
Grape wine white	20 µg/l
Grape wine red	20 µg/l
Carbonated wine	20 µg/l
Fruit wine other than grapes	20 µg/l
Fortified wine	20 µg/

3 Aims and Objectives

There is a need to develop a simple method for extraction and determination of Ochratoxin A in food sample. Hence the present studies focused on the following objectives.

Standardization of HPLC condition for detection and quantification of Ochratoxin A.

Optimization of extraction procedure of Ochratoxin A spiked in wheat sample.

Preparation of MMS calibration curve for estimation of Ochratoxin A in wheat sample.

Validation of extraction and estimation efficiency of Ochratoxin A in spike wheat sample using MMS calibration curve.

4 Materials and Methods

4.1 Materials

Glassware

- Volumetric flasks
- Measuring cylinders
- Erlenmeyer flask
- Separating Funnel
- Sample vials and inserts

Plasticware

- Centrifuge tubes
- Microtips
- Centrifuge tubes

Apparatus

- Mixer grinder
- Orbital shaker
- Rotary evaporator
- Weighing balance (Sartorius ME 5, and Mettler Toledo AG204)
- Centrifuge (Eppendorf 5427 R)
- Sonicator (P Selecta ultrasons)
- Micropipettes – 20 µl, 200 µl & 1000 µl (Brand)
- HPLC 1525 with FLD detector (Waters, Milford, MA, USA)
- Symmetry C_{18} column (4.6 x 250 mm, 5.0 µ, Waters)
- Water bath
- Whatman no.1 filter papers

Reagents

- Acetonitrile(CH_3CN)≥99.9%(LC) grade
- Acetic acid-(LC) grade
- Extraction solvent(v/v)-Mix six parts acetonitrile with four parts water
- Methanol-99.9%(LC) grade
- Ethyl actate
- Ochratoxin A (Sigma)

4.2 Methods

4.2.1 Preparation of Ochratoxin A Standard Solution

Ochratoxin A (OTA) standard solutions-Stock solution contains-10µg/ml. 15.3µl Ochratoxin A(from standard stock solution) was aspirated into 10 mL glass- stoppered volumetric flask,volume was made up to 10ml with acetic acid-acetonitrile (1:99), vigorously shaken to dissolve. The actual concentration was determined by calculation using formula:

$$\text{Ochratoxin A}(\mu g/ml) = \frac{(\text{Absorbance} \times \text{Mol. Wt} \times 1000)}{\text{molar absorptivity}}$$

The molar absorptivity of Ochratoxin A in acetic acid and benzene (1:99)=5550

The molecular weight Ochratoxin A =403.8.

Finally the working stock solution was made by taking 1ml from acetic acid, acetonitrile standard solution and made up the volume to 10ml with methanol in standard volumetric flask. The working stock Ochratoxin A standard solution contains 10µg/10ml.

4.2.2 LC-MS/MS of Standard Ochratoxin A

The chromatographic separations were carried out using a quaternary pump Alliance 2695 HPLC system coupled with tandem mass spectrometry consisting of triple quadropole mass analyser and is also equipped with electron spray ionization source (Quattro Premier Micromass Mass spectrophotometer (MS/MS).

4.2.3 Optimization of HPLC Method using Different Concentration of acetic acid in mobile phase

HPLC conditions:

Column :Symmetry C_{18} column (4.6 x 250mm, 5 µ particle size, Waters)

Flow rate : 1 mL/min

Column temperature : 40 ^{0}C

Injection volume : 20 µL

Mobile phase : Water: methanol: acetic acid (31:68.5:0.5)

Optimization of acetic acid concentration in mobile phase was standardized HPLC condition with different concentration of acetic acid i.e, 2.5%, 0.5%, 0.1%. In the above mentioned mobile phase the different concentration of acetic acid i.e 2.5%, 0.5%, 0.1% and water concentration 29%, 31%, 31.4% respectively was studied keeping methanol constant to 68%. Chromatographic analyses were performed using HPLC 1525 system(Waters, Milford, MA, USA) with FLD detector and separation were achieved using an Symmetry C_{18} column (4.6 x 250 mm, 5µl particle size, Waters) with Fluorescence detector with isocratic mobile phase. Where excitation wave length is 333nm and emission wave length is 460nm.

4.2.4 Linearity and Linear range of Ochartoxin A in Methanol

Linearity and linear range of quantification of Ochratoxin A was done by HPLC using 1, 0.5, 0.25, 0.1, 0.5 and 0.025ppm solution of Ochratoxin A in methanol. The HPLC condition followed was given in **4.2.3**. The concentration of acetic acid in mobile phase was 0.5%, water was 31%, methanol was 68%.

4.2.5 Preparation of Standard and Matrix matched Calibration curve

Standard sample (control) was used for the preparation of matrix matched standard. Different concentration of standard solution of Ochartoxin A in 1ml methanol as given in table **4.1** was prepared. 50 µL of these standards solution were reconstituted of 200 µL using purified blank wheat sample extract in methanol and injected into HPLC. HPLC condition was as described in **4.2.3**.

Table: 4.1 Concentrations of Ochratoxin A used to prepare MMS calibration curve

Standard OchratoxinA solution	**OchratoxinA in 20µL of MMS injected**
2ppm/2000ppb	10ng
1ppm/1000ppb	5ng
0.5ppm/500ppb	2.5ng
0.25ppm/250ppb	1.25ng
0.1ppm/100ppb	0.5ng
0.05ppm/50ppb	0.25ng
0.025ppm/25ppb	0.125ng

4.2.6 Optimization of Ochratoxin A Extraction from spiked wheat Sample

Single Extraction

100g wheat sample was spiked with Ochratoxin A in the concentration level of 80, 40, 20, 10ppb. The sample was thoroughly mixed to ensure uniform distribution of Ochratoxin A in the sample. 25gm of each of the Ochratoxin A spiked wheat sample was ground to coarse grits using mixer grinder. The ground wheat was suspended in 100ml of extraction solvents acetonitrile-water (60:40) in a conical flask and agitated on orbital shaker at 120ppm for 20 min. The solvents was filtered through Whatman no.1 filter paper. The Ochratoxin A in the filterate was extracted into 50ml of ethyl acetate with constant agitation for 5min using a separating funnel. The organic phase of acetonitrile and ethyl acetate containing Ochratoxin A was collected in a round bottom flask. The Ochratoxin A extract was evaporated to dryness at 45℃ in rotary evaporator. The dry Ochratoxin A was taken in 1ml of methanol. Which was further analyzed for Ochratoxin A by HPLC method as given in **4.2.3**.

Triple Extraction

100g wheat sample was spiked with Ochratoxin A in the concentration level of 320, 160, 80, 40, 20, 10ppb. The sample was thoroughly mixed to ensure uniform distribution of Ochratoxin A in the sample. 25gm of Ochratoxin A spiked wheat sample was ground to cost grits using mixer grinder. The ground wheat was suspended in 100ml of extraction solvents acetonitrile-water (60:40) in a conical flask and agitated on orbital shaker at 120ppm for 20 min. The solvents was filtered through Whatman no.1 filter paper. The whole filtrate was concentrate up to 25ml by using rotary evaporator. The concentrated Ochratoxin A agitated with 25ml of ethyl acetate for 5min using a separating funnel for three times. The organic phase of acetonitrile and ethyl acetate containing Ochratoxin A was pooled together in a round bottom flask. The filtrate extract was finally passed with anhydrous sodium sulphate bed to eliminate water content. The Ochratoxin A extract in ethyl acetate was evaporated to dryness at 45^0c in rotary evaporator. The dry Ochratoxin A was taken in 1ml of methanol. Which was further analyzed for Ochratoxin A by HPLC method as given in **4.2.3**.

4.2.7 Analytical method validation:

Limits of detection (LODs) and quantification (LOQs)

Limits of detection (LODs) and quantification (LOQs) were evaluated by spiking blank pulse samples with Ochratoxin A, and subjecting to HPLC analysis. LODs and LOQs were determined as the lowest amount of Ochratoxin A for which the signal-to-noise ratios (S/N) were higher than 3 and 10, respectively.

Recovery and Repeatability

Recovery was evaluated by analyzing blank samples that were spiked with Ochratoxin A before extraction. The wheat sample was spike with Ochratoxin A at the concentration of 80ppb, 40ppb, 20ppb, 10ppb in case of single extraction and 320ppb, 160ppb, 80ppb, 40ppb, 20ppb and 10ppb for triple extraction which is mentioned in **4.2.6**. The % recovery of Ochratoxin A evaluated from spiked wheat sample was estimated by HPLC method as described in **4.2.3.** The results obtained were compared with the expected result to assess the recovery and accuracy of the method. The recovery was calculated based on the matrix matched standards.

Repeatability (intraday precision) was evaluated by spiking wheat samples at different concentration levels of (10, 20, 40, 80, 160, and 1000 ppb), in duplicates/triplicates. The recovery was calculated based on the matrix matched standards. The results obtained were compared with the expected result to assess the recovery and precision of the method. The precision of the method was determined by calculating the relative standard deviation (RSD, %), for the repeated measurements.

5 Results and Discussion

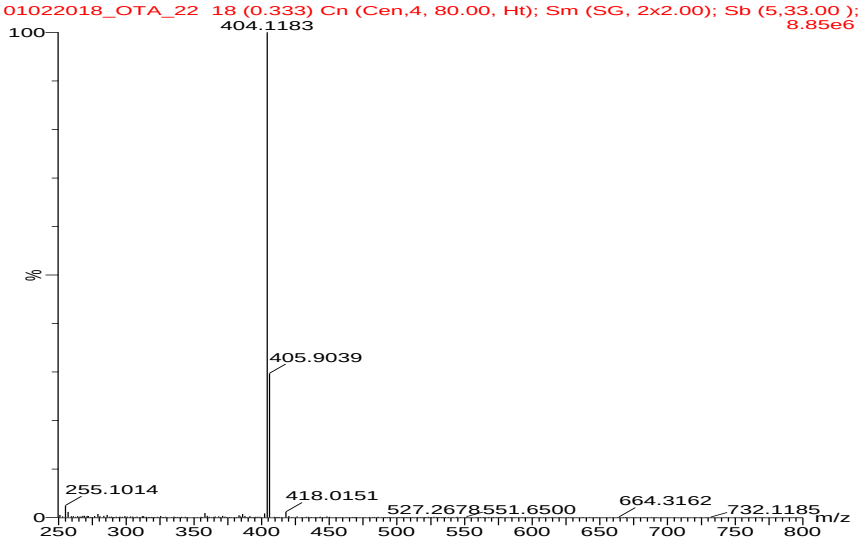
One of the major global concern world wide in food safety is the detection of mycotoxin in food. Because of there food regulators are continually lowering the maximum residue limits (MRL) of many mycotoxins especially aflatoxins, Ochratoxin etc. This demand further improvement in the analytical methods with lower detection limits, which warrants development of sensitive and selective methods. AOAC Official methods for mycotoxins, especially Ochratoxin A analysis involve the use of IAC column (AOAC 2000.03). Though these methods are still in use for the analysis of Ochratoxin A in wheat samples, other methods involving HPLC and LC-MS/M have been developed. European Union maximum limits for Fusarium toxins (deoxynivalenol, zearalenone and fumonisins B1 and B2) in cereals and cereal-based products have been established by Commission Regulation (EC) No 1881/2006.

LC-MS/MS Analysis

Based on the literature survey, Ochratoxin A was analyzed using ESI positive mode. MS tuning parameters were optimized by direct infusion of 1 ppm solution of Ochratoxin A in methanol at a flow rate of 20 µL/min. The MS fragmentation data was obtained using ESI positive mode. Ochratoxin A fragmentation patterns were obtained by high energy collision induced dissociation using LC-MS/MS. The MS was operated in multiple reaction monitoring (MRM) mode, by monitoring one parent ion and two daughter ions of Ochratoxin A.

The Full-scan MS analyses of Ochratoxin A was carried out to check the purity as well as to confirm the presence of protonated molecules. (Fig.5.1) depicts the full scan MS/MS spectra of Ochratoxin A,with their corresponding daughter ions. Based on the experience and available literature, a capillary voltage of 3.5 kV was selected and tuned the MS to obtain the cone voltage and collision energy for Ochratoxin A. Under LC-MS conditions, very good resolution was observed for Ochratoxin A. The cone voltage is 22V. The molecular ion 404.1183 $[M+H]^+$ was produced in positive–electron spray ionization (ESI) mode along with m/z 239.001 and 358.0640, which has been reported as a fingerprint ion from Ochratoxin A.

a)



b)

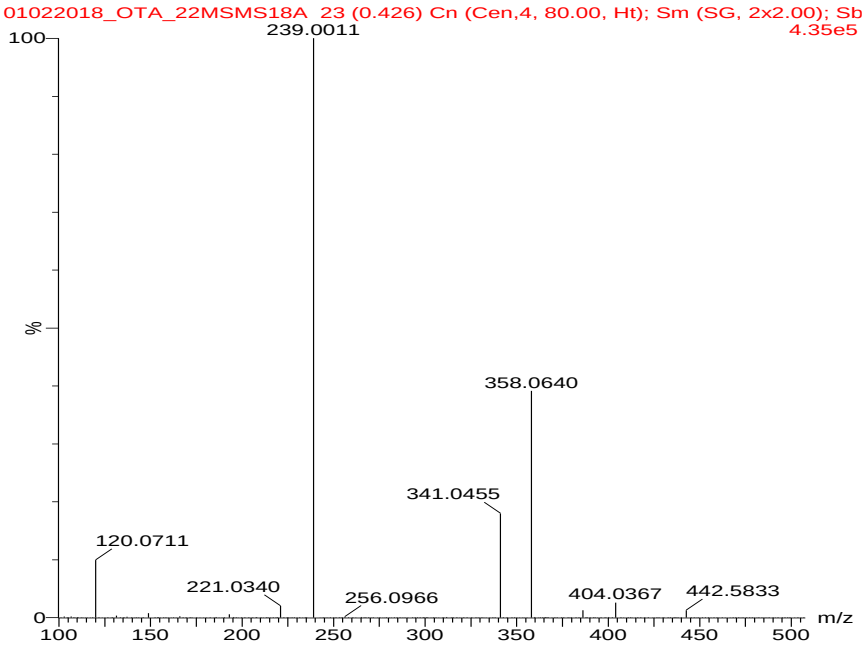


Fig. 5.1:(a) Full scan of Parent ion by MS, (b) Full scan of Daughter ion by MS/MS spectra of Ochratoxin A

HPLC-FLD Analysis

Optimization of HPLC method

An HPLC analytical method for the analysis of Ochratoxin A was explored in this present investigation. Since Ochratoxin A is a polar molecule, and the more polar nature of this molecule coupled with high solubility in organic solvent (methanol) and water, makes HPLC as a suitable technique for Ochratoxin A analysis. The most

commonly used detector for Ochratoxin A analysis is Fluorescence detector, which detects Ochratoxin A at 333 nm and emission at 460nm.

The separation procedure forOchratoxin A utilized the Symmetry C_{18} column (4.6 x 250 mm, 5µ, Waters) at a column temperature of 40ºC. The isocratic chromatographic separations were performed with 68.5% methanol, 0.5% acetic acid and 31% water, at a flow rate of 1 mL/min. The detector used is FLD detector at 333nm and emission at 460nm. The HPLC run was performed for 15-minutes, where a single prominent peak was obtained. The HPLC chromatograms based on the HPLC conditions are depicted in Fig.5.2. A good resolved peak of Ochratoxin A, with good peak shape was evident (Fig.5.2). Based on the peak shape/symmetry and resolution, a 15min HPLC isocratic elution described in the methods section (Fig.4.2.3), was selected. The 15-min run gave good retention/separation for Ochratoxin A . The retention times (t_R) in min. were 10.6 ± 0.5 for Ochratoxin A.

HPLC is having advantage of high sensitivity, good selectivity and reliable repeatability. However, HPLC system is expensive, and samples need to be purified before analysis. Otherwise, HPLC is a widely used analytical technique for quantification of mycotoxins, like aflatoxins, ochratoxins, etc. thus the developed and optimized HPLC method will be routinely used for Ochratoxin A analysis.

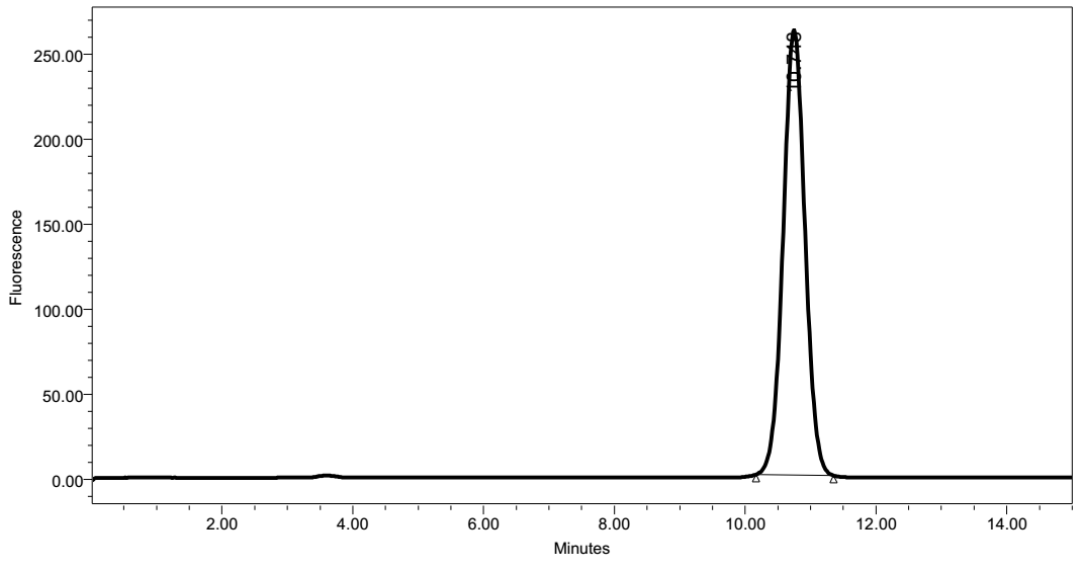


Fig.5.2: HPLC chromatograms of Ochratoxin A (0.25 ppm), showing the Ochratoxin A peak

Linearity and linear range of Ochratoxin A in methanol

To examine the linearity and the linear range of quantification of Ochratoxin A, without any interference, different amount of Ochratoxin A dissolved in clean methanol were subjected to HPLC analysis, as described in the methods section.The linear range in methanol was found to be 0.025 to 1 ppm (Table 5.2; Fig. 5.3). The standard calibration graph of Ochratoxin A showed good regression equation with the correlation coefficients (r^2) values greater than 0.99. The r^2 values were 0.991 for Ochratoxin A, which indicated good linearity (Fig. 5.3).

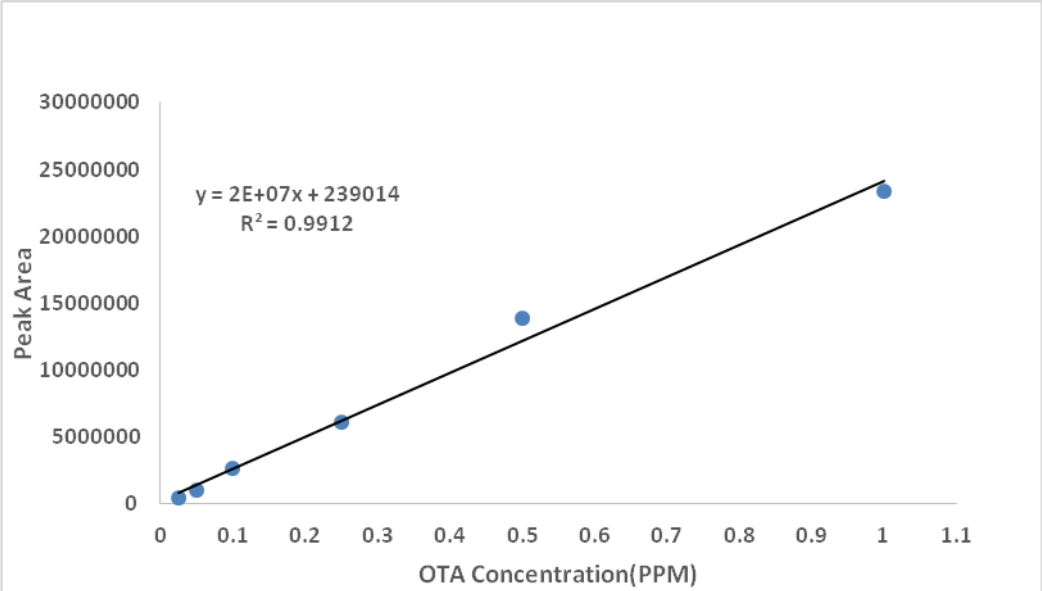


Fig. 5.3: Standard calibration graph of Ochratoxin A in methanol by HPLC

Table 5.1 : Linearity and Linear range of Ochratoxin A in Methanol and wheat matrix

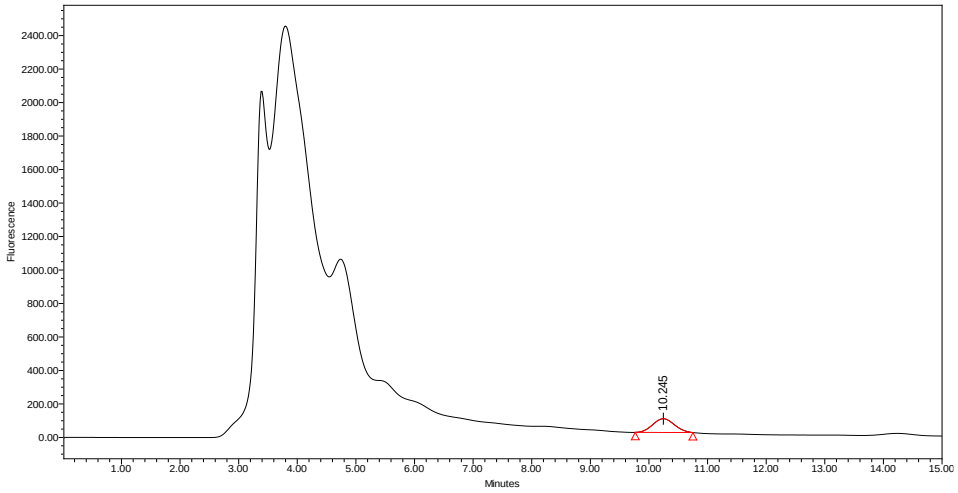
Ochratoxin A	Regression equation	r^2	Linear range (ppb)
Methanol	y = 2E+07x + 239014	0.9912	25 – 1000
Matrix-matched	y= 1E+06x - 238344	0.9971	6.25-1000

Optimization of Sample Extraction

Extraction solvent was selected form AOAC method (2000.03) but the use of IAC column for purification of sample is tedious job for clean up. From different literature survey it's found that TLC plate is also used in FSSAI method from which Ochratoxin A can be quantified.

Extraction of Ochratoxin A from spiked at different concentration was performed wheat sample taken place with single and triple extraction using ethyl acetate. 10, 20, 40, 80ppb concentration of spiking was done with single extraction and 10, 20, 40, 80, 160 and 320ppb concentration was done with triple extraction. Finally it was observed that both single and triple extraction is good enough but triple extraction is good enough for higher concentration.

a)



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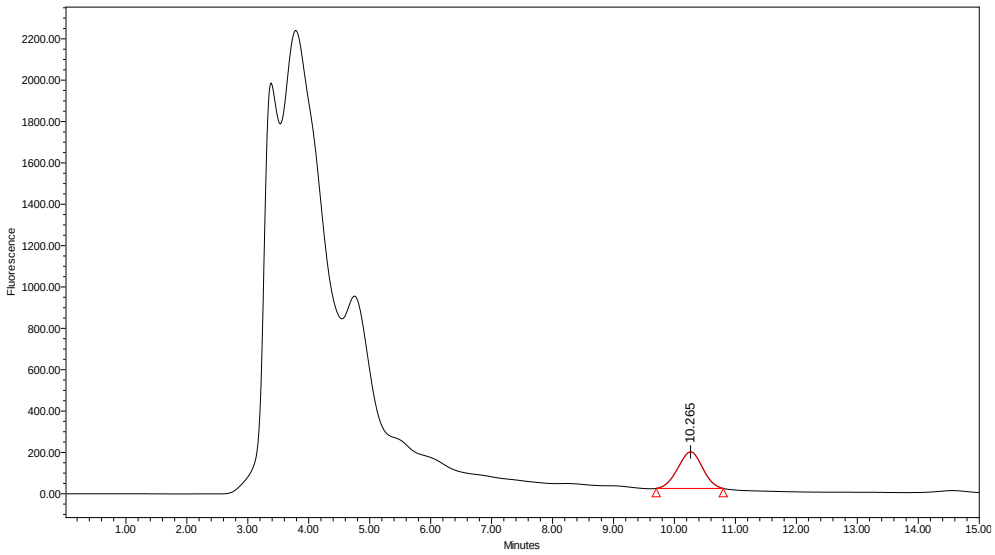
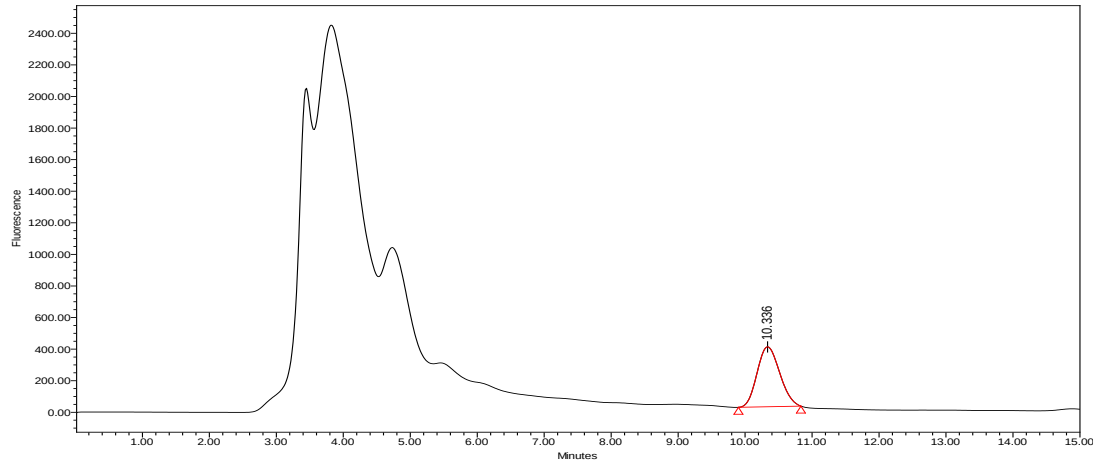


C)



d)

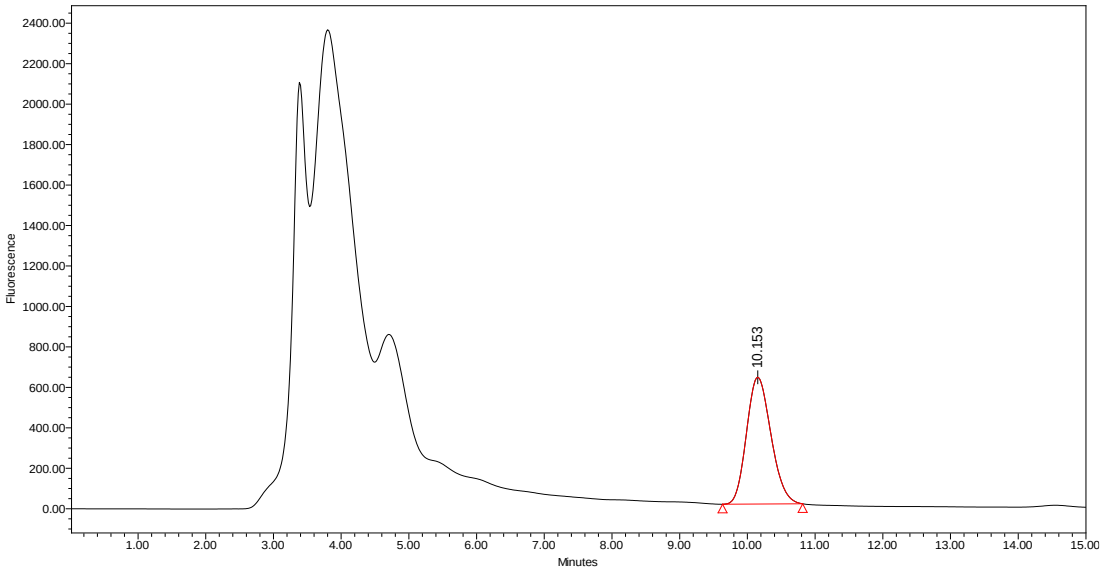


Fig:5.4 Different concentration of spike sample(a)10ppb, (b)20ppb, (c)40ppb, (d)80ppb

Validation of developed HPLC method

Method validation is the process of scientifically proving the acceptability of the analytical method for its intended purpose. Method validation was performed in terms of linear range, linearity, LODs, LOQs, recovery (accuracy) and repeatability (%Relative Standard Deviation [RSD], precision). Validation of the method is conducted in order to check the performance of the methodology. The method was validated for wheat samples essentially in compliance with the requirements set by Commission Regulation (EC) No 401/2006 of 23 Feb 2006 & 519/2014 of 16 May 2014.

Linearity, linear range, LOD and LOQ of matrix-matched standards with wheat

The blank wheat samples were extracted using the developed method, as described earlier. The extract (150µL) was mixed 50 µL of Ochratoxin A standards at a final concentration level of 6.25, 12.5, 25, 62.5, 125, 250, 500 &1000 ppb, and the linearity was evaluated by subjecting to HPLC analysis. A matrix matched standard graph yielded good regression equation with a correlation coefficients (r^2) values more than 0.9971 for wheat, indicating good linearity (Table 5.1;fig 5.5, 5.6).No significant interference from the wheat samples was observed in the RTs corresponding to Ochratoxin A. The linearity for Ochratoxin A was r^2 = 0.9971, using LC-FLD (Fig.5.6)It is obvious that no interference observed corresponding to the RT of Ochratoxin A.

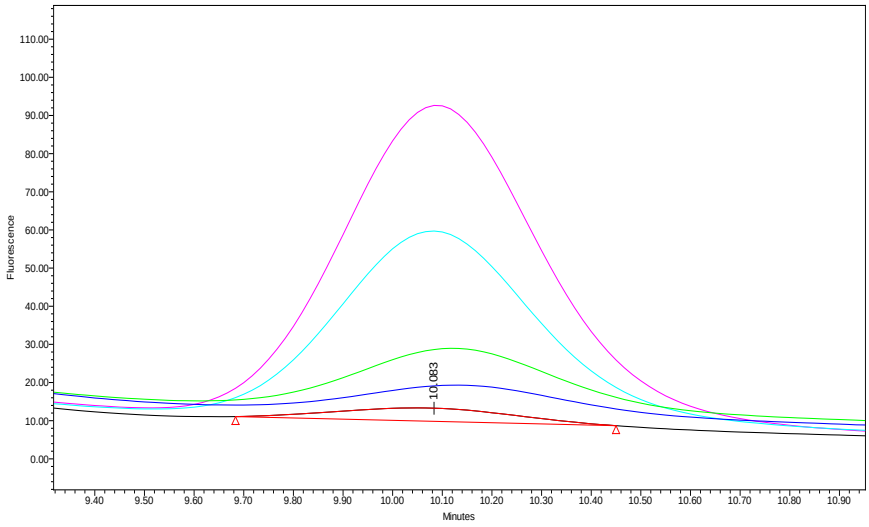


Fig:5.5 Overlay of chromatograms of matrix matched Ochratoxin A in wheat

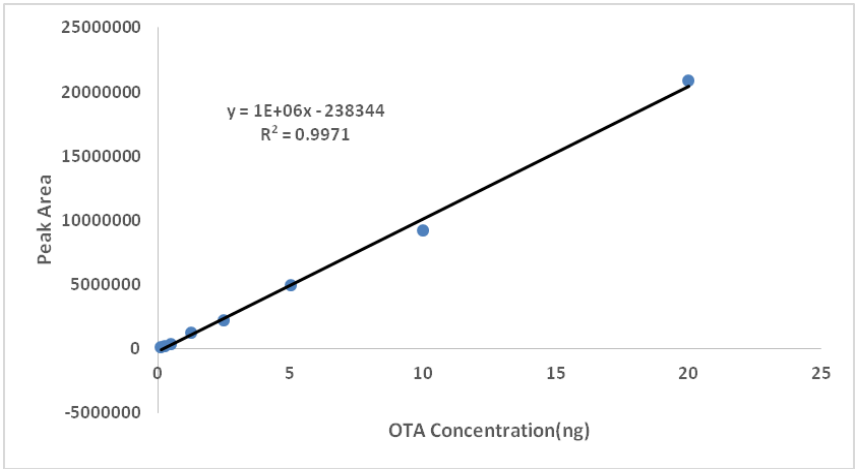


Fig. 5.6:Matrix matched calibration graph of Ochratoxin A

A signal-to-noise ratios of 3:1 and 10:1 were considered for LOD and LOQ, respectively. Table summarizes the LOD and LOQ of Ochratoxin A in matrix wheat sample extract. A comparatively low LOQ and LOD values (6.25 and 1.8 ppb [µg/kg, or ng/g or ng/ml]) were obtained for the Ochratoxin A by using this method. Earlier reported values were very high. LOD (s/n = 3) was 0.7µg/l and LOQ (s/n = 10) was 0.1µg/l for red wine (Shephard,et al., 2018).

Table 5.2: LOD and LOQ values of Ochratoxin A in wheat samples analyzed by HPLC

OchratoxinA	Wheat	
	LOQ (ppb)	LOD (ppb)
OchratoxinA	6.25	1.8

Recovery and Repeatability:

Method performance characteristics obtained from triplicate/quadruplicated analysis of wheat samples spiked with Ochratoxin A at 4 different levels, and quantified using matrix-matched calibration graphs, are presented in Table 5.6.

Each spiked wheat samples (25 g), corresponding final concentration of 10, 20, 40 and 80 ppb were extracted single time and 320, 160, 80, 40, 20 and 10ppb were extracted three times. These quality control samples were quantified using the matrix matched calibration curve, prepared on the same day, in two to three replicates. The recovery of single extracted spiked Ochratoxin A for wheat samples were found to be in the range of 76.13 - 91.76%. The precision of the method was determined by calculating the relative standard deviation (% RSD), for the repeated measurements. The relative standard deviations (% RSD) for the recovery of Ochratoxin A in wheat samples ranged from 1.54 – 6.0 % (Table5.3). The recovery of triple extracted spiked Ochratoxin A for wheat samples were found to be in the range of 71.46 – 90.76 %. The precision of the method was determined by calculating the relative standard deviation (% RSD), for the repeated measurements. The relative standard deviations (% RSD) for the recovery of Ochratoxin A in wheat samples ranged from 0.45–0.46% (Table5.4). The results are in consistent with the reported literature. The acceptable mean recovery between 70 and 110% for Ochratoxin A was reported (EU, 519,2014).

Table 5.3: Recovery (%) and repeatability of Ochratoxin A in wheat samples with single extraction

Mycotoxin	Spiked level (ppb)	% Recovery	SD	% RSD
OchratoxinA	3(10)	76.13	1.17	1.54
	3(20)	82.74	3.83	4.63
	3(40)	91.76	3.14	3.42
	3(80)	71.94	4.33	6.02

Table 5.4: Recovery (%) and repeatability of Ochratoxin A in wheat samples with triple extraction

Mycotoxin	Spiked level (ppb)	% Recovery	SD	% RSD
OchratoxinA	3(160)	90.76	0.42	0.46
	3(320)	71.46	0.32	0.45

These recoveries are in the range between 70 - 110%, they can be considered acceptable since they were repeatable. Furthermore, it is evident that relative standard deviations (%RSDr) were always lower than 20% for Ochratoxin A at the concentration levels assayed, indicating the stability of the developed method. This developed method meets requirements reported in the EU guidelines (Commission Regulation (EC) No 519/2014 of 16 May 2014), indicating that a method can be considered accurate and precise when accuracy data are comprised between 70 and 110 % with relative standard deviation (repeatability) not higher than 20%.

The new HPLC method enables quantification of Ochratoxin A with single analysis, which has simple sample preparation step and improved sensitivity. Additionally, HPLC is having advantage of high sensitivity, good selectivity and reliable repeatability. HPLC is a widely used analytical technique for quantification of mycotoxins, like aflatoxins, ochratoxins, etc. Thus the developed and optimized HPLC method will be routinely used for analysis of Ochratoxin A in food samples in our lab. However, further improvement and optimization along with validation of the method is essential for using this method for Ochratoxin A analysis in different food samples.

Conclusion

Mycotoxin is food contaminant which is a major health problem. Ochratoxin A produced by *A .ochraceus* is found to be nephropathy. The current study involved method validation for the quantitative analysis of Ochratoxin A in wheat by HPLC-FLD detector.

1. The standard Ohratoxin A LC-MS/MS revealed the the parent ion 404.1183 and daughter ion 239.001, 358.0640.
2. The HPLC condition for detection of Ochratoxin A with FLD was standardized with mobile phase Water, Methanol, Acetic acid (31.8:68.5:0.5).
3. The linearity and linear range of quantification of Ochratoxin A method was found to be 0.025 to 1 ppm where regression equation with correlation coefficient (r^2) value was >0.99. The regression equation was y =2E + 07x + 239014
4. Extraction of Ochratoxin A from spiked wheat sample was done by single extraction and triple extraction method.
5. MMS standard curve of Ochratoxin A in spiked wheat had regression equation y = 1E + 06x – 238344 with (r^2) >0.9971 with linear range 6.25 – 100ppb
6. The Limit Of Detection and Limit Of Quantification of Ochratoxin A in wheat sample by optimized HPLC condition was 1.8 and 6.25 respectively
7. In single extraction, the sample was spike at 10, 20, 40, and 80 ppb level and the % recovery range was 71.94 – 91.76 % with % RSD range 1.54 to 6.02
8. In single extraction, the sample was spike at 160, and 320 ppb level and the % recovery range was 71.46 – 90.76% with % RSD range 0.45 to 0.46. AOAC method involves use of IAC and TLC method recommended by FSSAI which can be overcome by the present method. This is a noble precise method and these findings gave way for simple, rapid extraction, detection and quantification of Ochratoxin A by HPLC method.

Summary

1. Conformation of Ochratoxin A was done by LC-MS/MS
2. Standardization of HPLC was carried out by using different concentration of acetic acid in mobile phase.
3. Optimization of extraction Ochratoxin A procedure to done for the quantification of Ochratoxin A in wheat sample.

4. Linearity was calculated by using standard curve
5. Optimization of sample extraction
6. LOD, LOQ and RSD % was calculated
7. Recovery and repeatability was achieved for both single and triple extraction of wheat sample

Evaluation

1. Presence of Ochratoxin A was conformed by LC-MS/MS
2. Standardization of HPLC-FLD detector
3. Linearity of Ochratoxin A in methanol
4. Optimization of sample extraction
5. LOD and LOQ was calculated
6. Recovery and repeatability was calculated

Future work

Reproducibility can done for more precise and significant result. This procedure can be optimized Ochratoxin A for other food sample such as apple juice, barley and wine.

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