DOKUZ EYLUL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ANALYSIS OF OCHRATOXIN A IN TURKISH GRAPE VINEGARS

by

H. Mine KURTBAY

June, 2007

İZMİR

ANALYSIS OF OCHRATOXIN A IN TURKISH GRAPE VINEGARS

A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of
Dokuz Eylül University
In Partial Fulfillment of
the Requirements for the Degree of Master of Science
in Chemistry

by
H. Mine KURTBAY

June, 2007
İZMİR

M. Sc. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"ANALYSIS OF OCHRATOXIN A IN TURKISH GRAPE VINEGARS"** completed by **H. Mine KURTBAY** under supervision of **Prof. Dr. Melek MERDİVAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

.....

Prof. Dr. Melek MERDİVAN

Supervisor

....

(Jury Member) (Jury Member)

…….

Prof. Dr Cahit HELVACI

Director

Graduate School of Natural and Applied Science

ACKNOWLEDGMENTS

I wish to thank my supervisor, Prof. Dr. Melek Merdivan, for her encouragement, guidance and support. In particular, for teaching me how to write scientific proposals, reports and, of course, this thesis, I am deeply grateful to her.

I want to thank Serap Seyhan for helping me during laboratory studies, discussions and for her valuable contributions.

I am also grateful to Research Foundation of Dokuz Eylul University (Project 06-KB-FEN-29) for the financial support.

I would like to thank to my family being with me all my life and in this situation for their unique patience and supports.

Finally, I would prefer to say to all my teachers and friends.

H. Mine KURTBAY

ANALYSIS OF OCHRATOXIN A IN TURKISH GRAPE VINEGARS

ABSTRACT

Determination of Ochratoxin A (OTA) is important because of its toxicity and also mycotoxicoses causes economic losses in the world seriously. For these purposes, the development of analytical methodologies for determination of OTA becomes essential.

In this study, a reversed phase high performance liquid chromatography fluorescence detector (RP-HPLC-FLD) was used to determine the amount of OTA in Turkish grape vinegars in water/acetonitrile/acetic acid (48.5:50.5:1.0, v/v/v) mobile phase at 1.5 mL min^{-1} flow rate with 50 $^{\circ}C$ oven temperature. The whole analysis was completed less than five minutes at 458 nm emission wavelength.

The direct injection and solid phase extraction (SPE) method were applied to all samples. For accuracy, standard addition method was used at 0.05, 0.10, 0.15 mL spiking volumes to all vinegars. Accu Bond SPE cartridges with octadecyl-silica stationary phase (C18-500 mg) were used for preconcentration of OTA for vinegar samples with 5-fold concentration.

Detection and quantitation limits of the method were found as 0.26 ng mL^{-1} and 1.0 ng mL^{-1}, respectively. For robustness within and between-day repeatability of the method for OTA the results of ten repeated measurements was found as 3.44 % and 5.22 %, respectively.

The results for the amount of OTA in grape vinegars were determined as in the range of 1.24 and 2.25 ng mL^{-1}.

Keywords: Ochratoxin A, Vinegar, Direct injection, Solid phase extraction (SPE), High performance liquid chromatography (HPLC)

TÜRK ÜZÜM SİRKELERİNDE OKRATOKSİN A ANALİZİ

ÖZ

Okratoksin A' nın (OTA) toksik özellik göstermesi ve mikotoksine maruz kalmanın dünyada ciddi ekonomik kayıplara yol açmasından dolayı tayini önemlidir. Bu amaçla OTA' nın belirlenmesi için analitiksel metotlar geliştirilmesi gereklidir.

Bu çalışmada, bazı Türk üzüm sirkelerindeki OTA miktarı ters fazlı yüksek performans sıvı kromatografisi floresans dedektörü kullanılarak su/asetonitril/asetik asit (48,5:50,5:1, v/v/v) hareketli fazında, 1,5 mL dk^{-1} akış hızı ve 50 $^{\circ}C$ fırın sıcaklığında belirlenmiştir. Tüm analiz 458 nm emisyon dalga boyunda beş dakikadan kısa sürede tamamlanmıştır.

Tüm örneklere direkt (doğrudan) enjeksiyon ve katı faz ekstraksiyon metodu uygulanmıştır. Yöntemin doğruluğu için sirke örneklerine 0,05, 0,10, 0,15 mL hacimlerde OTA standardından eklenmiştir. Sirke örneklerindeki OTA' nın ön deriştirilmesi Accu Bond marka oktadesil-silika durgun fazlı (C18-500 mg) katı faz ekstraksiyon kolonları kullanılarak 5 kat deriştirme faktörü ile gerçekleştirilmiştir.

Doğruluk için gözlenebilme ve tayin sınırı değerleri sırasıyla 0,26 ng mL^{-1} ve 1,0 ng mL^{-1} olarak belirlenmiştir. Okratoksin A' nın tayininde cihazın tekrarlanabilirliği için gün içi ve günler arası tekrarlanabilirlikler ardarda yapılan on ölçüm ile sırasıyla % 3,44ve % 5,22 olarak belirlenmiştir.

Üzüm sirkelerdeki OTA miktarı 1,24 ile 2,25 ng mL^{-1} arasında değişen değerlerde hesaplanmıştır.

Anahtar sözcükler: Okratoksin A, Sirke, Direkt, Katı Faz Ekstraksiyonu (SPE), Yüksek Performanslı Sıvı Kromatografisi (HPLC)

CONTENTS

Page

THESIS EXAMINATION RESULT FORM ii
ACKNOWLEDGEMENTS iii
ABSTRACT iv
ÖZ v

CHAPTER ONE–INTRODUCTION 1

1.1 The Mycotoxins 1
1.1.1 Kinds of Mycotoxins 1
1.1.1.1 Ochratoxin A 2

1.2 Contaminated Food and Beverages 3

1.3 Effects of Mycotoxicoses 4

1.4 Maximum Tolerable Limits 5

1.5 Determination of Mycotoxins 10
1.5.1 Chromatographic Determination 10
1.5.1.1 Gas Chromatography (GC) 10
1.5.1.2 Thin Layer Chromatography (TLC) 11
1.5.1.3 High Performance Liquid Chromatography (HPLC) 12
1.5.2 Screening Methods 14

1.6 Extraction and Clean-up Procedures 14
1.6.1 Solid phase extraction (SPE) 15
1.6.1.1 SPE columns 16
1.6.2 Immunoaffinity Clean-up (IAC) 17
1.6.2.1 IAC Columns 18
1.6.3 Liquid phase extraction (LPE) 19

1.7 Related Studies 20

1.8 Objective of This Work 23

CHAPTER TWO–EXPERIMENTAL METHODS AND MATERIALS. 25

2.1 Preparing the OTA Standard Solutions 25

2.2 Optimization Procedure in HPLC for Separation 26
2.2.1 Optimization of Oven Temperature 27
2.2.2 Optimization of Mobile Phase Ratio 27
2.2.3 Optimization of Flow Rate 27

2.3 Sampling and Storage 27

2.4 Sample Preparation 28
2.4.1 Direct Procedure 28
2.4.2 SPE Procedure 28

2.5 Statistical Analyze for Validation of the Method 29

CHAPTER THREE – RESULTS AND DISCUSSIONS 31

3.1 Selection of wavelength 31

3.2 Optimization Procedure for OTA in HPLC 31
3.2.1 Optimization of Oven Temperature 32
3.2.2 Optimization of Mobile Phase Ratio 33
3.2.3 Optimization of Flow Rate 34

3.3 Calibration curve of OTA 35

3.4 Determination of OTA in Vinegars 37
3.4.1 Direct Procedure 38
3.4.2 SPE procedure 40

3.5 Analytical Figures of Merit 45

3.6 Analysis of Vinegar Samples 46

CHAPTER FOUR – CONCLUSION 48

REFERENCES 50

CHAPTER ONE

INTRODUCTION

1.1 The Mycotoxins

Mycotoxins are toxic secondary metabolites produced by about 200 identified filamentous fungi, as e.g. *Fusarium, Aspergillus* and *Penicillium* species, growing under a wide range of climatic conditions on agricultural commodities in the field and during storage. Their growth is especially based on the moisture on air and food. Their occurrence in food, beverages and feed has been recognized as potential threat to human and animal health, either caused by direct contamination of plant materials or products thereof, or by "carry over" of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed (Zóllner & Mayer-Helm, 2006).

1.1.1 Kinds of Mycotoxins

Some hundred different mycotoxin species have been discovered so far exhibiting a great structural diversity (Figure 1.1). Most of them offer considerable thermal and chemical stability. They cannot or can only partly be removed by food processing or by other suitable decontamination procedures. Today, aflatoxins, trichothecenes, ochratoxin A (OTA), zearalenone (ZON), fumonisins, moniliformin, patulin and citrinin receive by far the greatest attention due to their frequent occurrence and their severe effects on animal and human health. Factors contributing to their presence or production in food and feed include storage, environmental and ecological conditions, as temperature, relative humidity, the substrate and the use of fungicides, though the interrelations between all these factors are not yet well understood and toxin production cannot reasonably be predicted (Zóllner & Mayer-Helm, 2006)

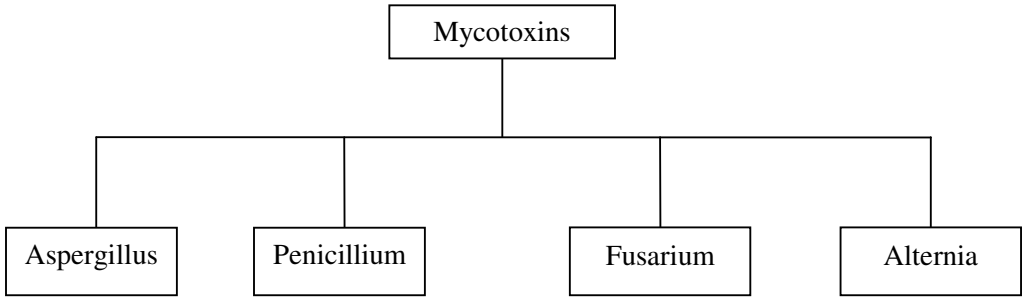


Figure 1.1 Some mycotoxins and their producer fungus

1.1.1.1 Ochratoxin A

Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* families and are weak organic acids consisting of a derivative of an isocoumarin as indicated in Figure 1.2. The family of ochratoxins consists of three members, A, B, and C which differ slightly from each other in chemical structures. On the other hand, OTA, 7-(L-β-3-phenylalanylcarbonyl)carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methyl isocumarin, is the most abundant and hence the most commonly detected member but is also the most toxic of the three (The Government of the Hong Kong Special Administrative Region, 2006).

As in other mycotoxins, OTA can contaminate a wide variety of foods as a result of fungal infection in crops, in the field during growth, at harvest, in storage and in shipment under favorable environmental conditions especially when they are not properly dried. OTA may be present in a foodstuff even when the visible mould is not seen. OTA is especially found mainly in cereal and cereal products**.** Besides cereals and cereal products, OTA is also found in a range of other food commodities, including coffee, cocoa, wine, beer, pulses, spices, dried fruits, grape juice, pig

kidney and other meat and meat products of animals exposed to feedstuffs contaminated with this mycotoxin (The Government of the Hong Kong Special Administrative Region, 2006).

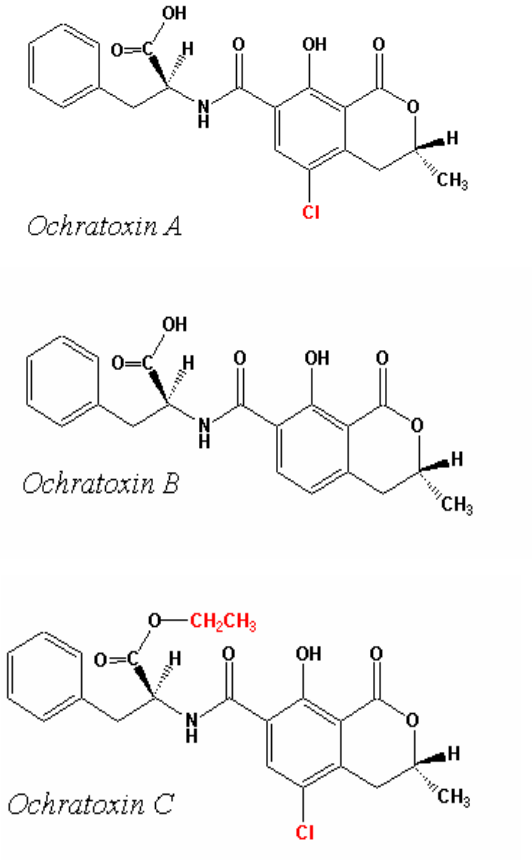


Figure 1.2 Structures of ochratoxins

1.2 Contaminated Foods and Beverages

Today several food and feeds are known to contaminate with mycotoxins such as; aflatoxins, ochratoxin A, patulin, citrinin, cyclopiazonic acid, fumonisins, moniliformin, zearalenone, etc. Table 1.1 shows some of the mycotoxins and their contaminated foods and beverages.

Table 1.1 Some mycotoxins and their contaminated foods and beverages.

Mycotoxin	Contaminated Foods and Beverages
Aflatoxins (B1, B2, G1, G2, M1, M2)	Cereals, maize, nuts and peanuts, rice, cocoa, soya, animal feed, spices, dried figs, meat products, milk, cheese
Ochratoxin A	Wheat, barley, corn, peanut, nuts, soya, cocoa, coffee, wine and dried wine fruits, beer and cheese
Zearalenone	Corn, barley, oats, wheat
Fumonisins	Maize, cereal
Moniliformin	Corn
Patulin	Apple and apple products, apple pure, apple juice, apple vinegar
Citrinin	Wheat, cereal, barley, rice, maize, cheese, dried fruits
Cyclopiazonic Acid	Cheese, tomato and products, maize, rice
Tenuazonic Acid	Tomato, sunflower seeds, olive, sorghum

1.3 Effects of Mycotoxicoses

Although evidence of mycotoxicoses can be traced back to ancient times and the middle ages (ergotism), it was, however, not before 1960, that mycotoxins were identified as potential health hazard, when 100.000 turkeys died from an acute necrosis of the liver after consuming groundnuts infected by *Aspergillus flavus* and contaminated by aflatoxins (X-disease) (Zóllner & Mayer-Helm, 2006).

The toxic properties of mycotoxins in humans and animals include severe nephrotoxic, neurotoxic, carcinogenic, immunosuppressive and estrogenic effects. Less critical compounds and chronic intake of small amounts of mycotoxins may reduce feed intake and weight gain in animals and cause diarrhoea in humans (Zóllner & Mayer-Helm, 2006).

Surveillance studies showed that mycotoxin contamination also is a worldwide problem. Economic losses deriving from that are tremendous including reduction of livestock production and agricultural production, health care, veterinary and regulatory costs (Zóllner & Mayer-Helm, 2006).

1.4 Maximum Tolerable Limits

As mycotoxins are the potential health risks to humans and animals, national and regional food authorities have addressed the mycotoxin problem by adopting regulatory limits and are continuously amending existing regulations (Krska & Molinelli, 2007). Measures have been set up by authorities in many countries to monitor and control mycotoxin levels, especially for agricultural import products from third world countries. Maximum tolerable levels and guideline levels have been established for aflatoxins, OTA, ZON and deoxynivalenol (DON), etc down to the ppb to ppt level in different food and feed products (Table 1.2).

Table 1.2 Mycotoxin regulatory and consultative limits (RHM Technology, 2003).

EXISTING REGULATORY LIMITS IN THE EUROPEAN COMMISION, (EC)	
MYCOTOXIN, COMMODITY	LIMIT ($\mu g\ kg^{-1}$)
AFLATOXINS (TOTAL = B1+B2+G1+G2)	TOTAL
Nuts and dried fruit and processed products for direct human consumption	4
Groundnuts for sorting or other physical treatment prior to human consumption or use as an ingredient in foodstuffs	15
Other nuts and dried fruits for sorting or other physical treatment prior to human consumption or use as an ingredient in foodstuffs	10
Cereals and processed products, Cereals for sorting or other physical treatment prior to human consumption or use as an ingredient in foodstuffs	4
Maize for sorting or other physical treatment prior to human consumption or use as an ingredient in foodstuffs	10
Spices	10
AFLATOXIN M1	**M1**
Milk and milk products	$0.05\mu g\ L^{-1}$
Infant formulae	0.025
Dietary foods for special medical purposes	0.025
PATULIN	LIMIT ($\mu g\ kg^{-1}$)
Fruit juices, fruit nectar, in particular apple juice and fruit juice ingredients in other beverages, Concentrated fruit juice after reconstitution, Spirit drinks, cider and other fermented drinks from apples or containing apple juice	50

Table 1.2 (Continued)

EXISTING REGULATORY LIMITS IN THE EUROPEAN COMMISION, (EC)	
MYCOTOXIN, COMMODITY	LIMIT (µg kg^{-1})
Solid apple products including apple compote and puree	25
Apple juice and solid apple products including apple compote and apple puree for infants and young children, other baby food	10
OCHRATOXIN A	
Raw cereal grains including rice and buck wheat / cereal products	5 / 3
Dried wine fruits (currants, raisins, sultanas)	10
Table wine (red, white and rose) and other wine and/or grape must based beverages, aromatised wines, aromatised wine based drinks, aromatised wine-product cocktails and fruit wines	2
Grape juice, grape juice ingredients in other beverages, including grape nectar and conc. grape juice as reconstituted Grape must and conc. grape must as reconstituted	2
Roasted coffee beans and ground roasted coffee (except soluble coffee)	5
Soluble coffee (instant coffee)	10
Baby foods and processed cereal based foods for infants and young children (dry matter)	0.5
Dietary foods for special medical purposes intended for infants (dry matter apart from milk and products as reconstituted)	0.5

Table 1.2 (Continued)

EXISTING REGULATORY LIMITS IN THE EUROPEAN COMMISION, (EC)	
MYCOTOXIN, COMMODITY	LIMIT ($\mu g\ kg^{-1}$)
DEOXYNIVALENOL	
Unprocessed cereals other than durum wheat, oats and maize	1250
Unprocessed durum wheat and oats	1750
Unprocessed maize (1 July 2007, if no specific level set before this date)	1750
Cereal flour, including maize flour, semolina, maize grits and maize meal, pasta (dry)	750
Bread, pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based food for infants and young children and baby food (dry matter basis)	200
ZEARALENONE (also referred to as ZEA, ZON, F2)	
Unprocessed cereals other than maize	100
Unprocessed maize (if no specific levels set before July 2007)	200
Cereal flour except maize flour	75
Maize flour, maize meal, maize grits and refined maize oil	200
- Bread, pastries, biscuits, maize snacks and maize based breakfast cereals (if no specific levels set before 1 July 2007), other cereal snacks and breakfast cereals	50
- Processed maize-based foods for infants and young children, other processed cereal-based food for infants and young children (dry matter basis)	20

In contrast to EC, in Turkey only a few mycotoxins and their limits have been determined in some foods and beverages by Tarım ve Köyişleri Bakanlığı.. Table 1.3 shows the maximum tolerable limits of some mycotoxins in some foods and feed.

Table 1.3 Max limits of some mycotoxins in Turkey*

MYCOTOXIN	COMMODITY	LIMIT (ng g^{-1})
AFLATOKSİN B1/ (B1+B2+G1+G2)	Nut, groundnut, and other oily dry fruits, oily seeds, fig, raisin and dried fruits and processed foods	5/10
	Cereals and derivates	2/4
	Baby foods and feed	1/2
	Spices	5/10
	Other foods	5/10
AFLATOKSİN M1	Milk	0.05
	Milk powder	0.5
	Cheese	0.25
	Baby foods	0.05
OCHRATOXIN A	Dried wine fruit	10
	Unprocessed cereal grain	5
	Processed cereal and cereal products	3
PATULIN	Apple juice and derivates and vinegar	50

Tarım ve Köyişleri Bakanlığı- Koruma ve Kontrol Genel Müdürlüğü, 2002

1.5 Determination of Mycotoxins

1.5.1 Chromatographic Determination

Driven by regulatory authorities, like the European Commission and the Food and Drug Administration (FDA), extensive analytical efforts have been made to enable fast and reliable analysis of a large number of samples for surveillance and monitoring purposes on the ppb to ppt level in a wide variety of complex food environments. Especially for the highly toxic and carcinogenic aflatoxins, fast and highly sensitive (ppt level) screening assays based on immunochemical methods are commercially available. Similar test kits which are easily applicable to automation are also available for OTA and ZON as well as for other relevant mycotoxins, enabling the screening in complex food and feed matrices (Zóllner & Mayer-Helm, 2006).

In this context chromatography coupled to different detection principles is used to confirm unambiguously positive findings and to enable exact quantification when a maximum tolerable level or a relevant guideline level has been exceeded.

1.5.1.1 Gas Chromatography (GC)

The routine use of GC is restricted to a limited number of mycotoxins, especially A- and B-trichothecenes. This is because of their non-fluorescent and weak UV-VIS absorption properties, which require derivatisation in order to facilitate detection, and makes the determination of trace levels unreliable. A more widespread use of GC methodology for other mycotoxins is mainly limited by insufficient volatility of analytes, the lack of suitable derivatisation agents, or the availability of alternative easy-to-use methods. GC methods have been reported for patulin, sterigmatocystin and zearalenone, but these are not widely used (European Mycotoxin Awareness Network, 2000).

1.5.1.2 Thin Layer Chromatography (TLC)

Despite the fact that many other techniques have been increasingly applied in the determination of certain varieties of mycotoxins, TLC is still popular due to its coherent features such as line principle, higher sample throughput, lower operating costs, convenience in identification by comparison of spot color, R_F values and UV–Vis spectrum, and is the method of choice for both semi-quantitative and quantitative purposes (Lin, Zhang, Wang, Wang & Chen, 1998).

Sample preparation is needed for most analytical methods and so it is for TLC analysis of mycotoxins because the sample matrix is usually very complicated. The procedures for extraction and clean-up of given matrices depends on the physicochemical properties of the mycotoxins. For example, Brand-burn et al. reported the use of a pH-bonded cartridge for the clean-up of extracts from cottonseed (Brandburn, Jewers, Jones & Tomlins, 1989). Silica gel columns were also used for the purification of mycotoxins.

Of the layers used, a silica gel layer on a glass plate or on a plastic sheet (Matysik & Giryn, 1996) is the most widely employed, with normal-phase development. However, phenyl non-polar bonded silica gel, silanized silica gel, octadecyl-bonded silica gel, silica gel impregnated with a proper organic acid and polyamide has also been used as alternative methods to analyze different mycotoxins.

Among a number of solvent systems used in literature, it is difficult to evaluate the relative advantages between them. However, a few solvents like chloroform, ethyl acetate, acetone, methanol, toluene, acetic acid, water and ether have often been employed in the initial systems with various percentages. In the separation of mycotoxins, tank development has been the common configuration. Comparison was made between tank and continuous linear development configurations. Of course, instrumental TLC has been increasingly performed in the mycotoxin analysis.

1.5.1.3 High Performance Liquid Chromatography (HPLC)

HPLC is the most widely used of all of the analytical separation techniques (Skoog & James, 1992). The reasons behind this popularity are its sensitivity, its ready adaptability to accurate quantitative determinations, its suitability for separating nonvolatile species or thermally fragile ones, and above all, its widespread applicability to substances that are of prime interest to industry, to many fields of science, and to the public. In analytical HPLC, the focus is to obtain information about the sample compounds. The information that can be obtained includes identification, quantification and resolution of a compound.

Bonded-phase packings are classified as reversed-phase when the bonded coating is nonpolar in character and as normal-phase when the coating contains polar functional groups. Most commonly, the R group of the siloxane in these coatings of the reversed-phase is a C8 chain or a C18 chain. The mechanism by which these surfaces retain solute molecules is at present not entirely clear (Alshana, 2004).

Improving the resolution of a chromatographic column is based upon varying one of the three parameters (N, k', and α) (Table 1.4).

Table 1.4 Important relationships in HPLC

Name	**Equation**
Number of theoretical plates	$N = 16\ (t_R/W)^2$
Retention factor	$k' = (t_R - t_M) / t_M$
Selectivity factor	$\alpha = [(t_R)_B - t_M] / [(t_R)_A - t_M]$
Resolution	$R_s = 2\ [(t_R)_B - (t_R)_A] / [W_A + W_B]$

Where,

t_R : retention time, time between injection of a sample and appearance of a solute peak at the detector.

t_M : dead time, time required for an unretained species to pass through a column.

$(t_R)_A$: retention time of species A.

$(t_R)_B$: retention time of species B.

W_A : peak width at its base (in units of time) for species A.

W_B : peak width at its base (in units of time) for species B.

The retention factor (k') is experimentally the most easily manipulated of the three because of the strong dependence of this constant upon the composition of the mobile phase. For optimal performance, k' should be in the ideal range of 1 to 10; for complex mixtures, however, this range must often be expanded to perhaps 20 in order to provide time for peaks of all of the components to appear. Sometimes, adjustment of k' alone does not suffice to produce individual peaks with no overlap. If resolution is very poor (below 0.5) variation in selectivity factor (α) must be resorted to keep k' within a reasonable range. This can be achieved by choosing a different stationary phase or by changing the mobile phase identity.

Methods for mycotoxins are needed for compliance with tolerances and guidelines, for monitoring and survey work, and for research in areas such as epidemiology, mycology, metabolism, pharmacokinetics, food processing and decontamination. For more than 30 years, considerable research has been devoted to developing methods for detecting and determining mycotoxins in foods, feed, and biological fluids. In the case of OTA, the technique used in most studies is HPLC with fluorescence detection, mostly because a very low detection limit can be reached, due to the fact that OTA has natural fluorescence (Valenta, 1998).

1.5.2 Screening Methods

Enzyme-linked immunosorbent assays (ELISAs) have become one of the most useful tools for rapid monitoring of mycotoxins, especially for the screening of raw materials. Despite high matrix dependence and possible overestimation, the great advantages of microtitre plate ELISAs are speed, ease of operation, sensitivity, and high sample throughput. In recent years, test kits based mostly on direct competitive assays for field use have emerged. Interest is also increasing in rapid immunoassay-based methods, for example lateral flow devices enabling qualitative determination of mycotoxins on one-step strip tests within a few minutes, because of the need for rapid on-site (pre)-screening (Visconti, Lattanzio, Pascale & Haidukowski, 2005; Weingaertne, Krska, Praznik, Grasserbauer & Lew, 1997).

Several recently developed methods, for example fluorescence polarization immunoassays (FPI), surface plasmon resonance (SPR), and non-invasive methods based on infrared and acoustic techniques have much potential in mycotoxin analysis. Immunosensors are emerging as an alternative for screening and quantitative analysis of mycotoxins. Optical methods, for example fourier transform mid-infrared spectroscopy with attenuated total reflection (MIR-ATR) or near-infrared transmission spectroscopy are interesting techniques for detection of mycotoxins in grain, enabling reduction of sample preparation to an absolute minimum. Optical methods enable high sample throughput with no sample preparation other than grinding. Major restrictions so far are high matrix dependence and lack of appropriate calibration materials (Krska & Molinelli, 2007).

1.6 Extraction and Clean-up Procedure

Depending on the nature of the mycotoxin different analytical approaches and analytical tools target single classes and specific substrates and comprise extraction and clean-up steps to reduce or eliminate unwanted matrix components (Krska & Molinelli, 2007).

Sample clean-up for mycotoxin analysis has undergone substantial development, and some methods now target a selected mycotoxin or mycotoxin class. Immunoaffinity columns (IACs) for all major mycotoxins are available commercially and MycoSep columns and other SPE materials in disposable plastic cartridges are available for purification of mycotoxin samples (Krska & Molinelli, 2007).

1.6.1 Solid Phase Extraction (SPE)

SPE is currently being used as a preconcentration or separation technique whenever complex matrices or low concentration analytes have to be analyzed. It is now the most common sampling technique in many areas of chemistry, including environmental, pharmaceutical, clinical food and industrial chemistry. The basic principle of SPE is the transfer of analytes from the aqueous phase to the active sites of the adjacent solid phase; it can also be termed solid-liquid extraction. The transfer is stimulated by the selection of appropriate optimal conditions in the system of three major components: water (liquid phase); analyte; and, sorbent. Either the analyte after sorption on the solid phase is desorbed with a suitable eluate or the analyte along with the sorbent is dissolved in a suitable solvent and further analyzed (Martinez, Cugat, Borrull, & Callul, 2000; Bruzzoniti, Sarzanini, & Mentássi, 2000).

A typical SPE sequence starts with the conditioning of the column, by activating it with a solvent and/or water. The aqueous sample is then applied and the analyte is trapped together with interfering compounds. Ideally most of the interferences are removed by a rinsing step, with the analyte staying on the column. Consequently the analyte is eluted with an organic solvent and further preconcentration takes place by evaporation with N_2 (Figure 1.3).

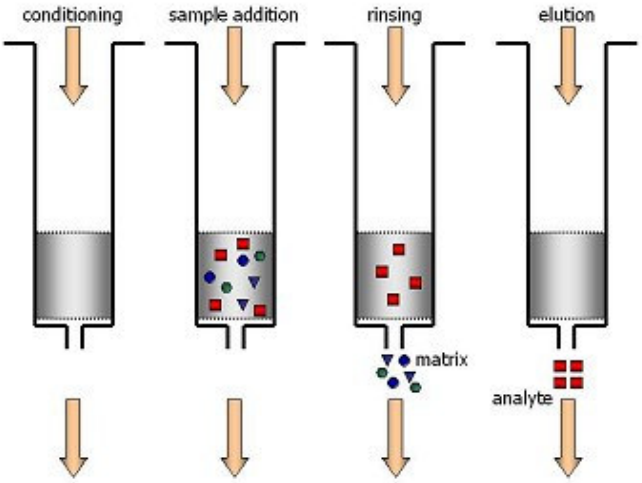


Figure 1.3 A typical SPE sequence

SPE is replacing LLE because of the several advantages that it offers, including: flexibility; low cost because of lower consumption of reagents; absence of emulsion; speed and simplicity; sampling in the field; safety with respect to hazardous samples; and, ease of automation (Pyrzynska, & Trojanowicz, 1999).

SPE represents a good elution for OTA in wines. It is an alternative to the use of immunoaffinity columns (Kuhn, Valenta & Rohr, 1995) especially if it can be used to apply the samples through ordinary stationary phases, such as octadecylsilane.

1.6.1.1 SPE columns

SPE for liquid sample become a widely used laboratory technique in the early 1980s with the introduction of disposable cartridges containing silica based chemically bonded sorbents of a suitable size for sample processing by gentle suction. Typical cartridge devices consist of short columns (generally an open syringe barrel) containing a sorbent with a nominal particle size of 50-60 µm, packed between porous plastic or metal frits (Figure 1.4). Low-volume cartridge or pre-column devices are the basis of on-line hyphenated systems (SPE-LC, SPE-GC) for integrates sample processing and separation that matured into robust practical systems in the mid-1990s (Masque, Marce, & Borrull, 1998; Louter, Vreuls, & Brinkman, 1999).

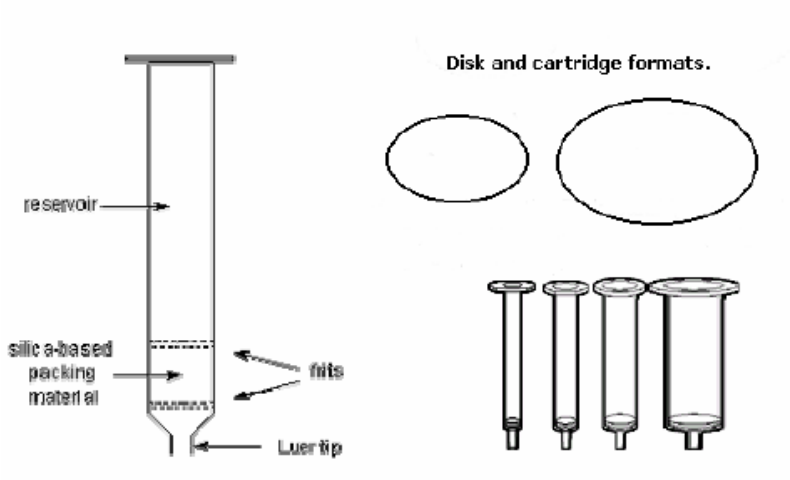


Figure 1.4 A solid-phase extraction column.

1.6.2 Immunoaffinity Clean-up (IAC)

Immunoaffinity columns (IACs) for clean-up purposes have become increasingly popular in recent years, because they offer high selectivity. They are easy to use for purification of samples that are contaminated with different mycotoxins. The analyte molecules (i.e. the mycotoxin) are bound selectively to the antibodies on the column after a preconditioning step. As matrix components do not interact with the antibodies, a rinsing step removes most of the possible interferences and the toxin can be eluted by antibody denaturation (Figure 1.5). Columns feature a higher recovery than standard liquid-liquid partitioning. Single analyte columns include those for aflatoxins, ZEA, OTA, fumonisins and deoxynivalenol (DON). Multifunctional columns for the simultaneous determination of OTA and ZEA are also available. The fact that columns can only be used once and their relative high costs are major disadvantages. Columns are commercially available (European Mycotoxin Awareness Network, 2000).

The use of IACs, either combined or not with solvent extraction techniques, is commonly used in food analysis, but has shown low recoveries in the analysis of wines (Goodman, 1996). Furthermore, its use can be an inconvenient for routine analysis of mycotoxins because they imply the use of specific stationary phases, which cannot be universally used and so increases expense.

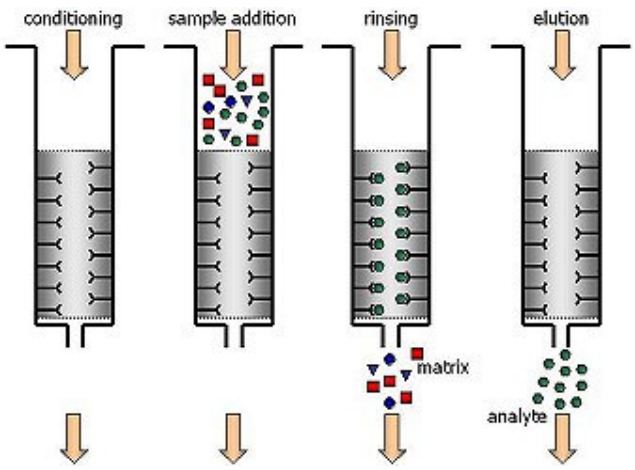


Figure 1.5 Basic principle of immunoaffinity columns

1.6.2.1 IAC Columns

The MycoSep™ multifunctional cleanup columns consist of adsorbents, which are packed in a plastic tube (Figure 1.6). A rubber flange, a porous frit and a one-way valve on the lower end ensure that the extract is forced through the packing material, when the column is inserted into the culture tube. On top of the plastic tube, the purified extract appears within seconds. No additional rinsing steps are required and almost all interfering substances are retained on the column, while the analyte does not show an affinity to the packing material. Columns are usually suitable for one analyte only and are available for a range of mycotoxins (European Mycotoxin Awareness Network, 2000).

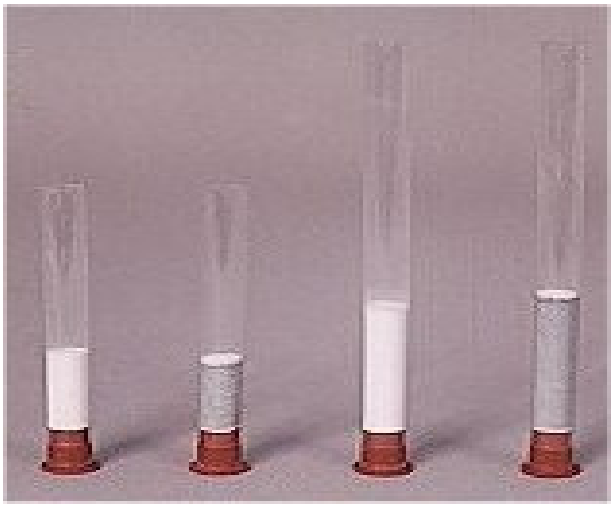


Figure 1.6 Romer MycoSep™ columns are solid phase cleanup columns for sample preparation prior to analysis with ELISA or instrumental methods (HPLC, GC, TLC, etc).

1.6.3 Liquid Phase Extraction (LPE)

Solvent extraction is a useful technique for the analysis of liquid samples, such as milk and apple juice. The method is based on the relative solubility of an analyte in two immiscible liquids and is used to remove interferences and concentrate the analyte prior to analysis. Examples of use of this technique include the analysis for patulin in fruit juices, aflatoxin M_1 in milk, OTA in wine.

Usually multiple extractions are necessary. In the end all fractions are pooled for further treatment (e.g. clean-up) (European Mycotoxin Awareness Network, 2000).

For mycotoxins the complexity of the samples requires a pretreatment step using techniques such as solvent extraction. But solvent extraction techniques are usually avoided nowadays because they involved long analytical times and the use of harmful solvents such as chloroform, toluene, acetonitrile, methanol.

1.7 Related Studies

There are a few articles about analyzing of OTA from vinegar samples in literature until now. So, literatures concerning OTA in wine and vinegar samples were given.

In 2000, Jornet and coworkers used reversed-phase high-performance liquid chromatographic method (RP-HPLC-FLD) to describe for the analysis of OTA at low μg L^{-1} levels in samples of artificially contaminated wines. The method involves SPE of samples using octadecylsilane cartridges (C18) and an additional preconcentration step prior to chromatography with isocratic elution and fluorimetric detection. The method was evaluated for accuracy and precision with relative standard deviations (RSD) lower than 10%. Recoveries of OTA added to commercial wines over the range 0.1–3.0 μg L^{-1} were higher than 80% in the assays. The performance of the octadecylsilane cartridge method tested compared very favourably with results of other published studies of OTA which use immunoaffinity columns (IAC) or solvent extraction techniques (Jornet, Busto & Guasch, 2000).

Leitner and coworkers have been compared different analytical methods for the determination of OTA in wine. Sample clean-up was based on SPE with (i) immunoaffinity or (ii) RP-18 sorbent materials applying different experimental protocols. The detection of OTA was accomplished with HPLC combined either with electrospray ionisation (ESI) tandem mass spectrometry (MS–MS) or FLD. The analytical results are discussed in view of the respective method validation data and the corresponding experimental protocols. In general, analytical data obtained with RP-18 SPE combined with LC–MS–MS detection and immunoaffinity extraction combined with FLD offered comparable good results in the sub-ppb concentration level indicating that high selectivity of either the sample clean-up or, alternatively the detection system are equally well-suited to guarantee an accurate OTA analysis in wine (Leitner et al, 2002).

Aboul-Enein, Kutluk and their friends determined the presence of OTA in some food materials by HPLC-FLD. The mobile phase consisting of Acetonitrile:water:acetic acid (99:99:2, v/v/v) was used for the resolution of the compound on a C18 Hypersil column. The retention time of OTA and diflunisal which was used as an internal standard were 11.7 and 12.8 min, respectively. The method is selective, reliable, reproducable with RSD of 1.70 and linear in the range of 2.5 $x10^{-8}$–1.5 $x10^{-8}$ M OTA. The limit of detection (LOD) and limit of quantification (LOQ) were $2.5x10^{-10}$ M corresponding to 0.1 ng mL^{-1} and 8.2 $x10^{-8}$ corresponding to 3.3 ng mL^{-1}, respectively. Recovery studies were 81.2 ± 1.9 %. The method was applied for analysis of OTA in wheat, corn, red pepper, cheese and wine. The proposed method can be used for the routine analysis of OTA in food and animal feed (Aboul-Enein, Kutluk, Altiokka & Tuncel, 2002).

In 2004, Dall'Asta et al. used RP-HPLC-FLD method for the detection of OTA in wine, by directly injecting the liquid in the chromatographic system without any extraction or clean-up. In this study, an alkaline mobile phase (NH_4Cl:CH_3CN 85:15 (v/v), 20 mM, pH 9.8) was used to obtain a distinct fluorescence enhancement. This improvement allows reaching, without an immunoaffinity clean-up or concentration, a detection limit of 0.05 ng mL^{-1}, which is similar to those commonly obtained after immunoaffinity purification and acidic elution. The method was statistically validated and directly applied to a series of wine samples (Dall'Asta, Galaverna, Dossena & Marchelli, 2004).

In 2005, determination of OTA in musts and wines was described using HPLC-FLD. They used SPE cartridges with octadecyl-silica stationary phase (C18) instead of IACs for sample clean-up and preconcentration. Utilizing 100-fold concentration, the LOD and LOQ of OTA in wine were 0.024 and 0.125 ng mL^{-1}, respectively. Mean recovery amounted to 83.5%. Confirmation of the presence of OTA was performed by quantitative conversion of OTA into its methyl ester with BF_3 /methanol. The method proved to be robust during the 2 weeks of routine measurements resulting in a mean recovery of 99.4% (std. dev. 2.4%, n=11) related to the first measurements on the control sample. They found the OTA was not

detectable in the Hungarian wine and must samples (Berente, Ágnes, H.-Otta, Záray, Lékó & Rácz, 2005).

In Spain, Herńandez and colleagues developed and apply two procedures for the analysis of OTA in grape musts (during the raisining process) and sweet wines with RP-HPLC-FLD. In grape must, the method involves the direct injection of the sample in a HPLC-FLD system without any kind of prior clean-up procedure. The complexity of the sweet wine samples requires a SPE clean-up on a C18 column which enables the OTA to be isolated from the matrix. The methods used were statistically validated. The validation also included the comparison of the slopes of the curve obtained with standards and the regression curves obtained by the addition of a standard. Two different studies of standard additions were conducted. One method was validated without sample preparation and it was applied to must samples. The other method was validated with SPE and it was applied to sweet wine samples. Recovery was always better than 89.69%. The LOD (S/N = 3) and LOQ (S/N = 10) were established at 0.22 and 0.77 μg L^{-1}, respectively. In general, the analytical data obtained provided good results at the sub-μg L^{-1} concentration level (Herńandez, García-Moreno, Durán, Guillén & Barroso, 2006).

In 2001, Markaki and colleagues described a method for the determination of OTA in red wine and vinegar using an acidic chloroform extraction, an IAC clean-up step, and a HPLC-FLD. The LOD was estimated at 0.002 μg L^{-1}. The mean recovery factors were found at 91.3 and 96.6% for wine and vinegar, respectively. Thirty-one samples of red wine originating from Mediterranean Sea countries and 15 samples of vinegar were examined for the presence of OTA. All red wine samples contained OTA. Seventy-two percent of these samples were found to he contaminated over 0.1 μg L^{-1}. Among them, nine samples contained ochratoxin A in the range of 0.5 to 3.4 μg L^{-1}, 12 samples in the range of 0.10 to 0.50 μg L^{-1}, and 9 samples in the range of 0.010 to 0.100 μg L^{-1}. All 15 vinegar samples showed the presence of OTA. The most contaminated ones were three balsamic vinegar samples containing 0.156 μg L^{-1}, 0.102 μg L^{-1}, and 0.252 μg L^{-1} of OTA. In the remaining 12 samples, OTA levels ranged from 0.008 to 0.046 μg L^{-1}. These data are in good agreement

with the hypothesis that wine originating from Southern countries might contain significant OTA concentration and showed the possible occurrence of traces of OTA in vinegar (Markaki, Delpont-Binet, Grosso & Dragacci, 2001).

1.8 Objective of This Work

Determination of OTA is important because of its toxicity and mycotoxicoses causes economic losses in the world seriously. Vinegar is used widely in also Turkey export to many countries. And only there are a few articles in literature, especially not studied in Turkey. For these purposes, the development of analytical methodologies for determination of OTA in vinegars becomes essential.

The aim of this study was to determine the amount of OTA in vinegar samples using a SPE method. To achieve this goal we aimed at: (1) controlling of working excitation and emission wavelengths for OTA in spectrofluorimetry (2) Optimization for HPLC (i) determination of oven temperature; (ii) selection of mobile phase ratio (iii); control of flow rate; (3) determination the linear range and plotting a calibration graph for OTA standard; (4) studying direct injection and SPE procedures in HPLC for liquid samples; (5) spiking to known volumes of samples for accuracy (6) controlling of precision with between-day and within-day analysis applying the proposed method to determine the amounts of OTA in grape vinegars (Figure 1.7).

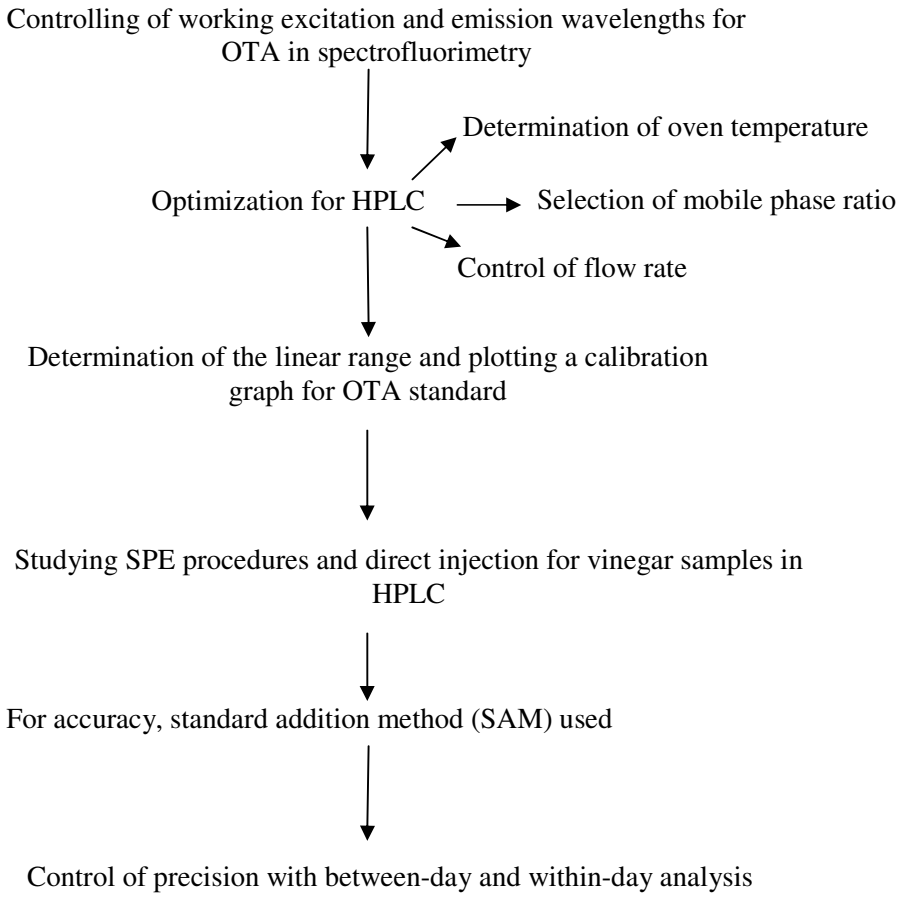


Figure 1.7 Flowchart of this study

CHAPTER TWO

EXPERIMENTAL METHODS AND MATERIALS

2.1 Preparing the OTA Standard Solutions

A stock solution (approximately 200 mg L^{-1}) was prepared by solving 5 mg of OTA (Sigma–Aldrich) in 250 mL methanol (Panreac) and stored at -20 $^{\circ}$C.

A series of OTA working standards from 1.0 to 10.0 ng mL^{-1} was prepared by evaporation of known volumes of the stock solution under N_2 stream, followed by dissolution in LC mobile phase. They were used to calibrate the LC detector response.

Varian Cary Eclipse Spectctrofluorimeter (Figure 2.1) was used to determine the emission and excitation wavelengths for working solutions of OTA.



Figure 2.1 Varian Cary Eclipse Spectctrofluorimeter

2.2 Procedure for Optimization of HPLC for Separation

The instrument used was an Agilent Technologies 1100 series liquid chromatographic system equipped with a fluorescence detector (model G1321A) at 333 nm (excitation) and 458 nm (emission), controlled by Chemstation 3D software (Figure 2.2). This system includes Agilent 1100 series quaternary pump (model G1311A). ACE C18 reverse phase (4x250 mm, 5µm) HPLC analytical column was used to separate OTA from the matrix. Injections were performed 25 and 500 µL syringes which were from Hamilton to HPLC apparatus comprising the following injection system with 20 µL injection loop. The separations were achieved by isocratic elution.

The optimized parameters for the proposed procedure in HPLC were of oven temperature, mobile phase ratio and flow rate.



Figure 2.2 The Agilent Technologies 1100 series HPLC system

2.2.1 Optimization of Oven Temperature

A suitable mobile phase water/Acetonitrile (ACN)/acetic acid (HAc) (49/49/2, v:v:v) was preferred depending on the literatures. 5.0 mg L^{-1} concentration of OTA standard solution was injected to HPLC at 1.0 mL min^{-1} flow rate and the effect of oven temperature was studied from 25 to 50 $^{\circ}C$.

2.2.2 Optimization of Mobile Phase Ratio

OTA working solutions as 2.5 mg L^{-1} was injected into the HPLC system at a flow rate of 1.0 mL min^{-1} and oven temperature at 50 $^{\circ}C$. A mobile phase as water/ACN/HAc at different ratios was tested to get the best retention time.

2.2.3 Optimization of Flow Rate

After optimization of mobile phase ratio, the retention time at different OTA concentration was controlled in water/ACN/HAc (48.5/50.5/1, v:v:v) mobile phase at 50 $^{\circ}C$ oven temperature while the flow rate of mobile phase was increased from 1.5 to 2.0 mL min^{-1}.

2.3 Sampling and storage

Nine grape vinegar samples (Doganay, Fersan, Kemal Kükrer, Kühne, Taris, Taris-Balsamic, Sok, Ege Baglari and Kipa) were purchased from the market. The last three ones were produced by Fersan.

All vinegar samples were stored in their original bottles, darkness at +4 $^{\circ}C$ because of the storage conditions of the samples showed influence on the results of analyze for OTA to exhibit natural fluorescence in wine.

2.4 Sample Preparation

All samples were filtered through from 0.45 µm filter paper (Millipore Millex-HV, Hydrophilic PVDF) and added to vials.

2.4.1 Direct Procedure

All filtered vinegar samples were added to vials and evaporated to dryness under N_2 stream. Residues were redissolved in HPLC mobile phase and analyzed duplicate.

For accuracy standard addition method (SAM) were used. 0.80 mL and 1.50 mL of vinegar samples were studied at first to accuracy. But no change was obtained. Then, 0.8 mL of vinegar samples was filtered through the 0.45 µm filter paper and used at further studies. Different volumes of 50 ng mL^{-1} standard OTA solution were added to samples until the final concentrations of these samples were 2.50, 5.0, 7.50 ng mL^{-1}. After the final volumes of samples were completed to 1.0 mL with HPLC grade methanol (CH_3OH), samples were evaporated to dryness under N_2 stream and analyzed with HPLC in duplicate. Before the analysis, all dried samples were redissolved in 1.0 mL mobile phase.

2.4.2 SPE Procedure

Figure 2.3 shows the vacuum manifold technique performed the SPE procedure with AccuBond ODS-C18 (500mg/3 mL) and ODS-C18-(200 mg/3 mL) cartridges (Agilent Technologies) in this study. Best sorption of OTA was achieved with the C18 cartridge (500 mg), and therefore, this was used for the SPE method. Different parameters for the extraction of OTA for vinegar samples were tested such as effect of the pH in conditioning of column for extraction efficiency, amount of sorbent, volume of sample, type of elution solvent and effect of sample dilution. Best results were obtained with the following procedure proposed. An aliquot of 10 mL of vinegar was passed through the cartridge which was washed with 5 mL CH_3OH, 5 mL Milli-Q water and equilibrated with 2 mL Milli-Q water before. Prior to sample

loading, the cartridge was conditioned with CH_3OH/water (60:40) at H=3 with 0.1 M HCl. It was then passed through the C18 cartridge. After air-drying of the cartridge for 30 min in the vacuum manifold, elution of OTA was carried out with 2 mL of methanol. The eluted extract was evaporated to dryness under N_2 stream, redissolved in HPLC mobile phase and analyzed in duplicate.

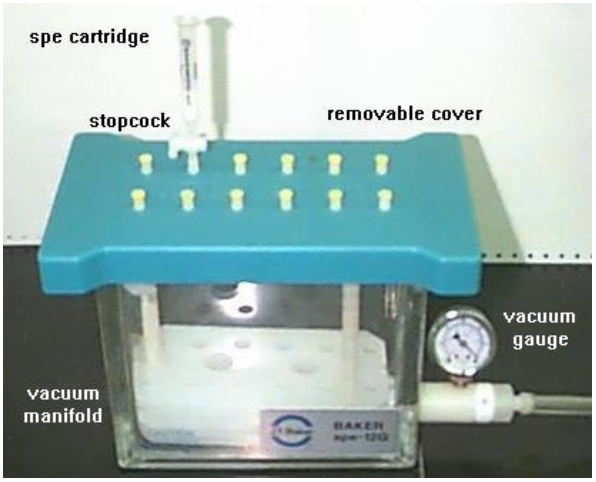


Figure 2.3 A vacuum manifold system

2.5 Statistical Analyze for Validation of the Method

The samples employed in this study are of great chemical complexity and are difficult to reproduce accurately. They are very susceptible to presenting the matrix effect: decrease or increase of the absorbance signal due to the presence of other components in the sample. Thus, the method of standard additions was used in this validation of the method.

The limit of detection (LOD) (3 s) was calculated using the following relation.

$S_m = \overline{S_{bl}} + k\,\overline{s_{bl}}$

$S = \overline{S_{bl}} + mc$

$LOD = c_m = (S_m - \overline{S_{bl}}) / m$

Where:

S_m: Minimum distinguishable analytical signal,

$\overline{S_{bl}}$: The average signal of blank solution,

$\overline{s_{bl}}$: The standard deviation of blank solution,

k: The constant which is 3,

m: The slope of the calibration curve.

The limit of quantitation (LOQ) (10 s) was calculated as the lowest concentration for which acceptable data of recovery and precision were obtained.

Once the procedures were optimized, the study of the repeatability was undertaken. The within and between-day repeatability were studied. The within-day repeatability was performed with different vinegar samples in triplicate. Each sample was spiked with a known amount of OTA to reach a concentration within the range of the linear response (5.0 ng mL^{-1}). The between-day repeatability was also performed with standard OTA solutions at (3.0 ng mL^{-1}).

CHAPTER THREE

RESULTS AND DISCUSSIONS

3.1 Selection of wavelength

Before the HPLC analyses determination of emission and excitation wavelengths for OTA solution was determined by spectrofluorimetry at 5 mg L^{-1} (Figure 3.1). The maximum excitation and emission wavelengths were determined as 333 and 458 nm, respectively.

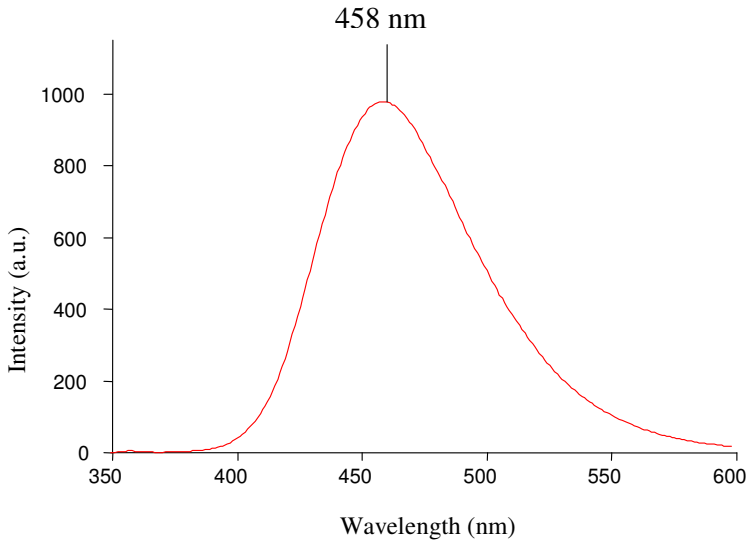


Figure 3.1 Fluorescence spectrum of OTA, 5 mg L^{-1}.

3.2 Optimization Procedure for OTA in HPLC

A successful use of HPLC for determining the amount of OTA in grape vinegars depended on the choice of the right combination of operating conditions: the type of analytical separation column, flow rate of the mobile phase, the mobile phase composition and temperature of the column. This choice in turn required a basic understanding of the various factors that control HPLC separations. So oven temperature, mobile phase composition and flow rate were optimized before this analyze in this study.

3.2.1 Optimization of Oven Temperature

Temperature effects in HPLC are not as significant as in gas chromatography. Volatile solvents are not allowed to rise to higher temperatures too much, and the stability of the attached bonded ligands on the adsorbent surface may be influenced by the high temperature. So, the main temperature range is from ambient temperature to 60 or 70 $^{\circ}$C (Kazakevich & McNair H, 1996-2002).

Stabilization of the column under the elevated temperature usually leads to the stabilization of the retention times. Origin of this effect is not well understood yet. Possible explanation is that the solvent viscosity decreased and more uniform stabilized temperature with absence of local temperature fluctuations due to the solvent friction lead to the more uniform adsorption-desorption process (Kazakevich & McNair, 1996-2002).

Another effect is the increase of the column efficiency. At the elevated temperature viscosity of liquids decrease and the diffusion coefficient increase. From the Van Deemter equation the second term will increase which will lead to the decrease of the efficiency at the very low flow rates (which is not important). The last term will decrease which will lead to the increasing of the efficiency at the common flow rates. It also widens the flow rate range with optimum efficiency (Kazakevich. & McNair., 1996-2002).

To determine the good resolution of OTA from the matrix different temperatures were studied from 25 to 50 $^{\circ}$C in water/ACN/HAc (49/49/2, v:v:v) at 1.0 mL min^{-1} flow rate. Table 3.1 shows the variation in retention time and peak symmetries depending on the oven temperature. For 5.0 mg L^{-1} concentration of OTA standard solution the increasing of the temperature was decreased the retention time. The peak symmetries became the best at 50 $^{\circ}$C besides the getting shorter retention times.

Table 3.1 Optimization of oven temperature

Oven temperature (°C)	Retention Time (min)	Symmetry
25	10.9	0.744
30	10.7	0.758
35	10.3	0.864
40	9.7	0.905
45	9.4	0.910
50	9.1	0.927

3.2.2 Optimization of Mobile Phase Ratio

The mobile phase must be chosen according to its chromatographic properties: it must interact with a suitable stationary phase to separate a mixture as fast and efficiently as possible. As a general rule, a range of solvents is potentially able to solve any particular problem, so selection must be based on different criteria such as viscosity, purity, inert with respect to sample compounds, toxicity and price.

As a general rule, the mobile phase should not be detector-active, i.e. it should not absorb in the same region used for the analytes. Otherwise, it is very possible that unwanted baseline effects and extra peaks will show up in the chromatogram.

In this study, water/ACN/HAc composition was selected to separate the OTA peak from the matrix because of commonly usage of this composition in literature (Herńandez, García-Moreno, Durán, Guillén & Barroso, 2006). Also, the flow rate of mobile phase and oven temperature was selected as 1.0 mL min^{-1} and 50 °C, respectively. Several ratios were studied for 0.25 mg L^{-1} OTA standard solution but the retention time was not changed significantly when the compositions were 48.5/50.5/1 or 49/50.5/0.5. So following studies were completed with in the 48.5/50.5/1 (v:v:v) range (Table 3.2).

Table 3.2 Optimization of mobile phase ratio (water/ACN/HAc)

Mobile Phase Ratio (v:v:v)	Retention Time (min)
49/49/2	6.0
49.5/49.5/1	6.0
49/50/1	5.6
48.5/50.5/1	5.4
49/50.5/0.5	5.4

3.2.3 Optimization of Flow Rate

In order to obtain a good resolution between OTA peak and other peaks, flow rate of the mobile phase was gradually increased from 1.0 to 2.0 mL min^{-1} in water/ACN /HAc (49.5/50.5/1, v:v:v) mobile phase at 50 $^{\circ}$C.

As shown in Table 3.3, when the flow rate was increased the retention time became shorter. But the shorter retention time was not preferred. Because at higher flow rate, OTA peak was not seemed clearly and some analytical problems were observed with HPLC. The OTA peak was not separate from the other interference peaks. The repeatability of chromatograms for OTA was not seemed well. The fluctuation also observed on the baseline. The higher flow rate also affected the instrument which was the blockage of frits and retaining some interferences substances on the column. Also the lower flow rates affected the whole time of analysis such longer analyses. In this case, the best flow rate was determined as 1.5 mL min^{-1} at the optimized best mobile phase ratio and oven temperature.

Table 3.3 Optimization of flow rate

Concentration ($mg\ L^{-1}$)	Flow rate ($mL\ min^{-1}$)	Retention Time (min)
5	1.0	6.1
5	1.5	6.1
2.5	1.5	6.1
0.5	1.5	6.0
0.25	1.5	5.9
0.25	1.7	4.5
0.15	2.0	3.8
0.125	2.0	3.8
0.115	2.0	3.8
0.105	2.0	3.8

As a result of these experiments optimum chromatographic conditions were;

Mobile phase : Water/ACN/HAc (48.5/50.5/1, v:v:v)

Flow rate : 1.5 $mL\ min^{-1}$

Oven temperature : 50 $^{\circ}C$

3.3 Calibration Curve of OTA

In order to verify the linearity of the response of OTA at the specified wavelengths for the working concentration, standard solutions of OTA were prepared and injected. Different standards of OTA were prepared in both HPLC grade methanol and HPLC mobile phase of this study. When the solutions were prepared in methanol, the solvent peak was seemed in the first minute at the negative direction of baseline. But, when the water/ACN/HAc was used instead of methanol, solvent peak was seen normally and no fluctuations were also seen in baseline. Then, dilution solvent was preferred as mobile phase of HPLC method after volatilization of samples or standard solutions.

The calibration graph was constructed by plotting the OTA peak area against five levels of OTA concentration which ranged from 1.0 to 10.0 ng mL^{-1}. The calibration curve had the regression equation of y = 0.1188 x + 0.0524 where y was the peak area obtained by use of fluorescence detection, x was the concentration of OTA in the standard solutions. The curve also was linear within the given range, with the regression coefficient of 0.9973 (Figure 3.2).

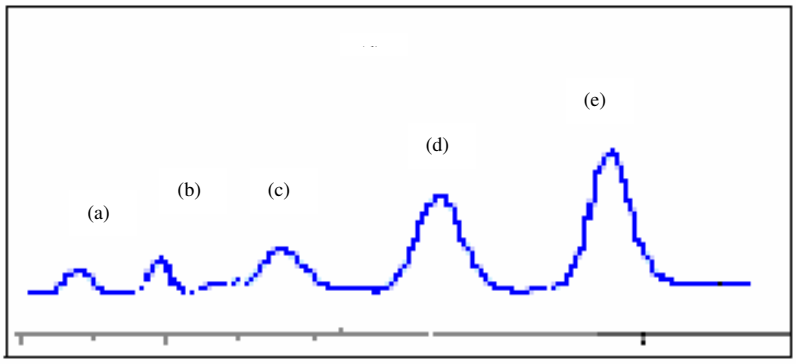


Figure 3.2 Chromatograms of calibration standards. (a) 1.0 ng mL^{-1}; (b) 2.5 ng mL^{-1} (c) 5.0 ng mL^{-1} (d) 7.5 ng mL^{-1} (e) 10.0 ng mL^{-1}

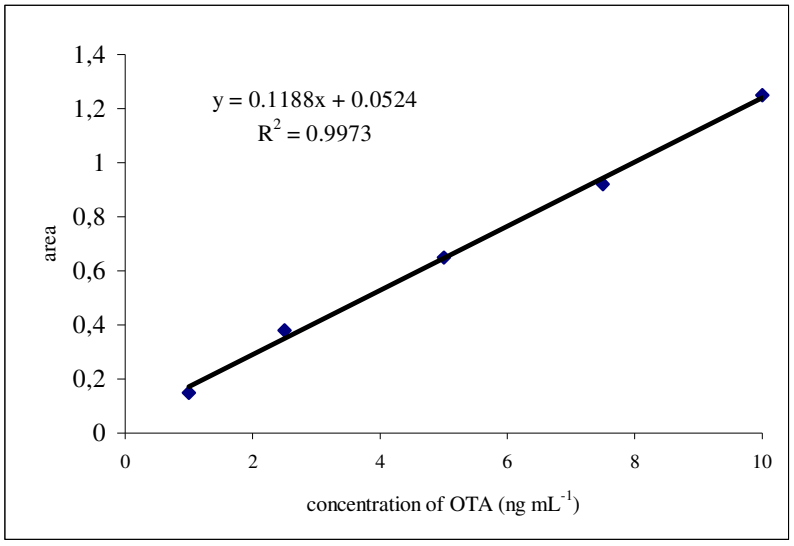


Figure 3.2 Calibration graph of OTA solution

3.4 Determination of OTA in Vinegars

A range of highly sensitive and sophisticated analytical methods has been developed for the determination of mycotoxins in several agricultural commodities and matrices. However, the determination step is usually preceded by a number of operations such as sampling, sample preparation, extraction and clean-up.

It should be noted that sample preparation is the time determining step in the whole analytical procedure with 2/3 of the total analysis time. It is the primary source of errors and differences in the results obtained by different laboratories.

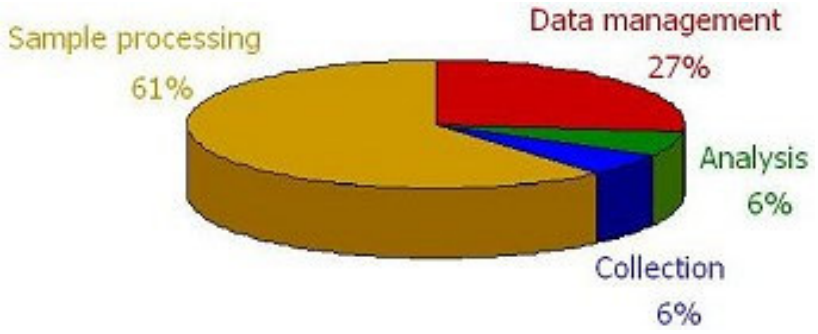


Figure 3.3 The distribution of time that spend on sample analysis
(R.E. Majors, 1991, LC/GC 4, 2, 10-14).

Sample preparation procedures play a key role and are dependent on the physicochemical properties of the sample and the toxin that should be determined. Clean-up procedures are used for the removal of interfering compounds such as lipids, carbohydrates and proteins. In order to examine the efficiency of the chosen method, spiking experiments are recommended: A defined amount of standard is added to the sample and the obtained recovery is compared with the added amount (European Mycotoxin Awareness Network, 2000).

For determination of OTA, studies were focused on a pretreatment step with extraction on silica gel or immunoaffinity columns or liquid extraction with organic solvents. But the direct determination of OTA is also available in some studies.

So in this study direct and solid phase preconcentration steps were applied to all vinegar samples.

3.4.1 Direct Procedure

In the present work, we proposed to detect OTA without any pre-treatment which involves the direct injection of vinegar samples in a HPLC-FLD apparatus. The proposed direct injection method was applied to the analysis of all vinegar samples and compared with SPE.

All vinegar samples (1 mL) were directly injected in the HPLC system after filtering on a 0.45 µm filters, evaporating under N_2 stream and redissolving in HPLC mobile phase because of the same effects in Section 3.3. Under these conditions the OTA peak was separated from other peaks in less then five minutes as shown in Figure 3.4.

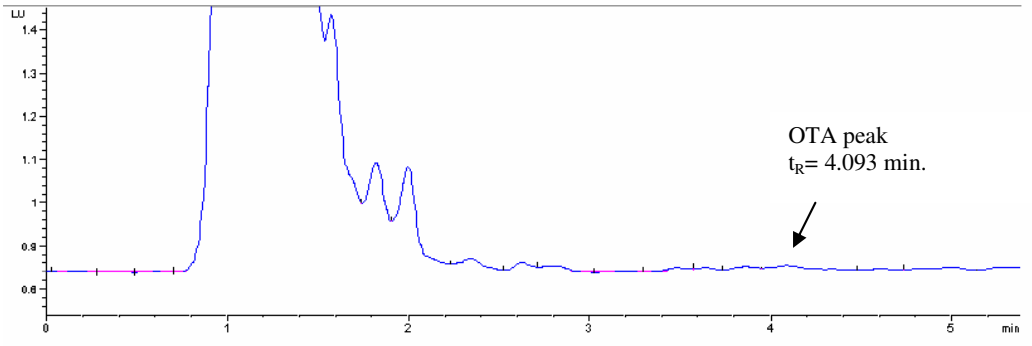


Figure 3.4 Chromatogram of direct injection for vinegar sample.

Because of the total amount of OTA was at very low limits in vinegars, SAM was used to check the accuracy for the procedure. Prepared OTA standard solution (50 ng mL^{-1}) was added to all samples at 0.02, 0.05, 0.10 and 0.15 mL. The enhancement in the fluorescence intensity was observed after the addition of standard OTA solution but some deviations at retention time and fluctuations at baseline were observed only at 0.02 mL of this standard OTA solution. So in the following studies three levels of standard addition from 0.05 to 0.15 mL were used and the final

volume was completed to 1 mL with CH_3OH. Figure 3.5 shows the chromatograms of the vinegar sample after standard addition.

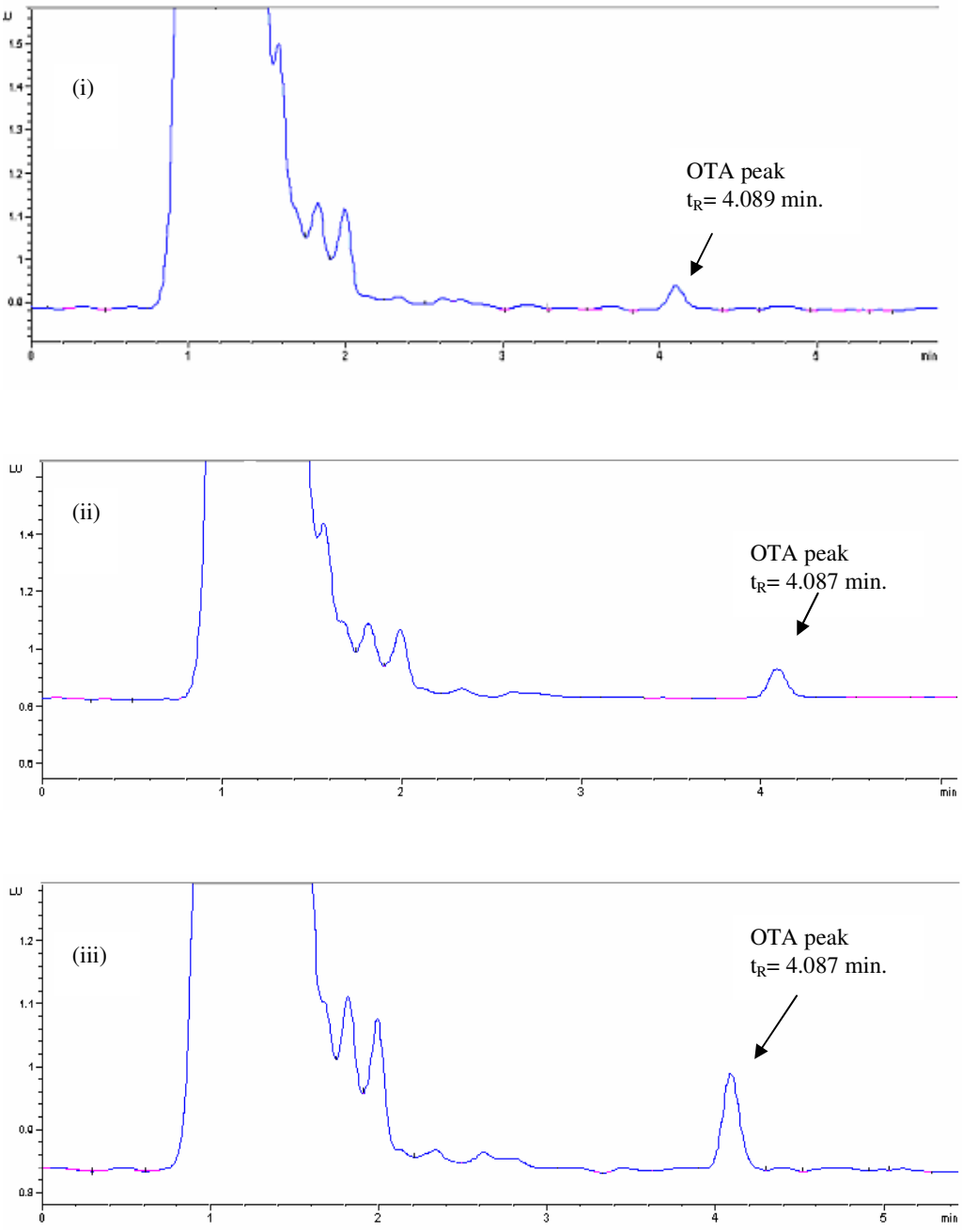


Figure 3.5 Chromatograms of after standard addition for vinegar sample. (i) 2.50 ng mL^{-1}, (ii) 5.0 ng mL^{-1}, (iii) 7.50 ng mL^{-1}

3.4.2 SPE procedure

For mycotoxin extraction methods different cartridges with different amounts of sorbent masses have been used such as octadecylsilica (C18-200/500 mg), styrene-divinylbenzene (200 mg), modified divinylbenzene (200 mg), etc. But in lots of studies, octadecylsilica cartridges (500 mg) are widely used to isolate the OTA from wines depending on the literatures (Jornet, Busto & Guasch, 2000; Herńandez, García-Moreno, Durán, Guillén & Barroso, 2006). So in this study C18 cartridges with 200 and 500 mg sorbent masses were tested and compared for sorption of OTA from synthetic OTA solutions and vinegar samples.

To perform the SPE of OTA with commercially available cartridges, several variables were studied. These were effect of the pH in conditioning of column for extraction efficiency, amount of sorbent, volume of sample, type of elution solvent and effect of sample dilution.

In the washing of the cartridge, CH_3OH /water mixture was used as 60:40 (v/v) ratio. pH effect was controlled in conditioning of cartridge (200/500 mg) for OTA methanolic solutions having different volumes and concentrations (Table 3.4). The OTA solutions were loaded on the column after conditioning with CH_3OH/water (60:40) without and with pH adjustment. The sorption efficiencies were 30.8 % (C18-500 mg) and 9.9 % (C18-200 mg) without any pH adjustment. In this case, to increase the sorption efficiency of OTA using only a cartridge of C18-500 mg, the conditioning solvent was acidified with 0.1 M HCl at pH=3 (natural pH of vinegars) and the sorption was increased to 74.2%.

Analytical performance of the SPE, selection of the elution solvent was also important. The use of pure Acetonitrile to elute OTA from the SPE cartridge resulted in very poor recoveries (Jornet, Busto & Guasch, 2000). But this was solved by use of CH_3OH. In addition, when Acetonitrile was used as the eluent in SPE the required volume was 5 mL but for CH_3OH 2 mL of eluent was enough for complete desorption of OTA from the cartridge. Since CH_3OH showed the best results in the elution of the OTA standard, it was used as the eluent in further studies.

Table 3.4 SPE procedure for OTA solutions

Sorbent mass of SPE column (mg)	**Conditioning**	**Sample**	**Elution**	**Sorption efficiency of OTA (%)**	**Desorption efficiency of OTA (%)**
C18-200	I) 5 mL methanol+5 mL water, II) 2 mL water, III) 2 mL methanol/water (60:40)	10 mL 1 mg L^{-1} OTA solution in methanol	2 mL methanol	9.9	24.0
C18-500	I) 5 mL methanol+5 mL water, II) 2 mL water, III) 2 mL methanol/water (60:40)	10 mL 5 ng mL^{-1} OTA solution in methanol		30.8	61.5
	I) 5 mL methanol+5 mL water, II) 2 mL water, III) 2 mL methanol/water (60:40) (pH=3 with 0.1 M HCl)	10 mL 5 ng mL^{-1} OTA solution in methanol		74.2	95.7

In Table 3.5, the detail of the SPE procedure of OTA for vinegars was summarized. Aliquots of vinegar were analyzed to get good limits of determination for OTA. The 10 mL of vinegar samples were loaded the C18-500 mg column after conditioning procedure. The percentage of sorption efficiency in real samples was increased when compared to standard OTA methanolic solution with no pH adjustment from 74.2 % to 84.9. This may be caused the nature pH of vinegar samples.

Dilution effect was also studied in vinegar samples. The decrease was observed on sorption efficiency from 84.9 % to 54.2 % when the sample was diluted at 1:1 (v/v) ratio with Milli-Q water. But no significant changes were calculated on desorption efficiency. In this study, for only balsamic vinegar the dilution was required because this type of vinegar was very dense and blockage the column.

Table 3.5 SPE procedure for OTA in vinegar samples.

Sorbent mass of SPE column (mg)	**Conditioning**	**Sample**	**Elution**	**Sorption efficiency of OTA (%)**	**Desorption efficiency of OTA (%)**
C18-500	I) 5 mL methanol+5 mL water, II) 2 mL water, III) 2 mL methanol/water (60:40) (pH=3.5 with 0.1 M HCl)	10 mL vinegar	2 mL methanol	84.9	59.2
		10 mL vinegar (dilution with water, 1:1, v/v)		54.2	61.8

3.5 Analytical Figures of Merit

Under the optimum conditions, calibration range, detection limit, the limit of quantitation and repeatability for the chromatographic method for OTA were analyzed in vinegar samples.

The calibration study is one of the most important steps in HPLC studies for all kinds of matrix to determine the linear working range. The calibration curve was plotted the concentration of OTA against the peak area and the linearity was obtained over the 1.0 and 10.0 ng mL^{-1} range with the regression coefficient of 0.9973.

The statistical calculations were based on the triplicate injections and readings for each standard solution and vinegar samples such as 0.26 ng mL^{-1} (based on 3s) and 1.0 ng mL^{-1} (based on 10s) for detection and quantitation limit, respectively. For within-day repeatability, a known amount of standard OTA solution was spiked to vinegar sample to reach a concentration within the range of the linear response (5.0 ng mL^{-1}). The between-day repeatability was also performed with standard OTA solutions at (3.0 ng mL^{-1}). The relative standard deviation was calculated as 3.44 % and 5.22 % for within-day and between-day repeatability. Table 3.6 summarized the statistical data in this study.

Table 3.6 Summary of analytical statistical data

Linear working range (ng mL^{-1})	1.0-10.0
Detection limit (ng mL^{-1})	0.26
Quantitation limit (ng mL^{-1})	1.0
Repeatibility for within- and between-day (%)	3.44 and 5.22
Linear working equation	y=0.1188x+0.0524
Regression coefficient (R^2)	0.9973

In this study, the average retention factor (k') and the number of theoretical plates (N) as a function of retention times of whole analyze and dead time on the column were 4.21, which was in the optimum range of 1 to 10, and 8830.

3.6 Analysis of Vinegar Samples

Determination of OTA in grape vinegars was achieved by direct injection and after SPE preconcentration steps. All the results are shown in Table 3.7. In direct injection method all samples were analyzed to HPLC dublicate. All samples were calculated in the linear working range except one. The calculated results for amount of OTA in grape vinegars were between the range of 1.24 and 2.25 ng mL^{-1}. The amount of OTA in Kemal Kükrer vinegar sample was below limit of quantitation and the most contaminated vinegar sample containing 2.25 ng mL^{-1} of OTA was Tariş, Balsamic type. The SPE method was also applied successfully to the vinegar samples. The obtained results showed that the precisions of two methods, direct and SPE, were very closely to each other. The results of two direct methods and SPE method were found in agreement with each other (t-test, 95%). The OTA amount of studied vinegar samples was found in the range of 1.08 and 2.16 ng mL^{-1}.

Table 3.7 The results of OTA determination in vinegar samples

Vinegar	Concentration of OTA (ng mL^{-1})		
	Direct	**SPE**	**Direct (SAM)**
Tariş-Balsamic	2.25 ± 0.24	2.16 ± 0.52	2.77 ± 1.27
Doganay	1.83 ± 0.83	1.54 ± 0.57	1.91 ± 0.81
Ege Bağları	1.24 ± 0.00	1.08 ± 0.12	1.41 ± 0.16
Fersan	1.79 ± 0.30	1.6 ± 0.43	1.67 ± 0.64
Kühne	1.29 ± 0.06	0.89 ± 0.01	1.43 ± 0.04
K.Kükrer	ND*	ND	ND
Kipa	2.21 ± 0.06	2.02 ± 0.03	2.68 ± 0.34
Şok	1.79 ± 0.30	1.19 ± 0.12	1.62 ± 0.15
Tariş	2.23 ± 0.09	1.78 ± 0.02	2.19 ± 0.03

*ND: not detected (below the limit)

CHAPTER FOUR

CONCLUSION

Ochratoxin A (OTA) is a carcinogenic, genotoxic, teratogenic, immunotoxic and nephrotoxic secondary metabolite. Human diet is the major to intake of contaminated foods with OTA. Besides the serious health effect to human, contaminated foods with OTA decreases the quality of foods or feed and causes several economic losses in the world. Grape vinegars are use widely in Turkey and also export to Europe so the determination of OTA contamination becomes essential. There are only a few reports about determination of OTA in literature, especially not studied in Turkey. For these purposes, the development of analytical methodologies for determination of OTA becomes essential.

In this study, RP-HPLC-FLD determination of OTA for vinegars was purposed after selection of wavelengths for OTA solution by spectrofluorimetry. In this case some parameters were optimized in HPLC analyses such as oven temperature, mobile phase ratio and flow rate. Table 4.1 shows the best optimized parameters for OTA analysis. From the chromatograms, the average retention factor (k') and number of theoretical plates (N) were determined. k' was found as 4.21 which was in the optimum range of 1 to 10 and N was 8830.

Table 4.1 The best optimum conditions in HPLC

Oven Temperature ($^{\circ}$C)	**Mobile Phase & Ratio (v/v/v)**	**Flow Rate (mL min^{-1})**	**Retention Time (min)**
50	Water/Acetonitrile/Acetic Acid (48.5/50.5/1)	1.5	4.2-4.3

The direct injection and SPE methods were successfully applied to all selected grape vinegars. In Table 4.2, the studied extraction method and extraction efficiency on C18-500 mg silica cartridges were summarized.

Table 4.2 The optimized SPE parameters

Sample	**Conditioning**	**Sorption efficiency of OTA (%)**	**Desorption efficiency of OTA (%)**
Synthetic OTA solution (10 mL, 5 ng mL^{-1} in methanol)	I) 5 mL methanol+5 mL water II) 2 mL water III) 2 mL methanol/water (60:40), (pH=3)	74.2	100
Vinegar sample (10 mL filtered)		84.9	59.2

For accuracy, standard addition method was used the additions of standard solution as 0.05, 0.1 and 0.15 mL in 1 mL final volume.

In this study, all calibration solutions and vinegar samples were prepared by dissolving in the HPLC mobile phase after evaporation to drying under N_2 stream. The evaporation step was required to obtain the shorter retentions and reduce the solvent effect.

LOD and LOQ were 0.26 ng mL^{-1}, 1.0 ng mL^{-1}; relative standard deviation was 3.44 % and 5.22 % for within-day and between-day repeatability, respectively.

In this study, the amount of OTA in grape vinegars was found between the range of 1.24 and 2.25ngmL^{-1}. These levels were such different from Markaki et al. who had determined the amount of OTA in vinegar samples between the range from 0.008 μg L^{-1} to 0.046 μg L^{-1} for grape vinegars and between the range from 0.102 μg L^{-1} to 0.252 μg L^{-1} for balsamic vinegar samples (Markaki at al., 2001). These data are in good agreement with the hypothesis that vinegar samples might contain significant concentration OTA in vinegar.

In the future, amount of the other kinds of mycotoxins such as patulin (PAT), citrinin (CIT) will be analyzed in apple vinegars and also grape ones as a following study. For this purpose the HPLC parameters will be optimize for patulin and citrinin. Direct injection and extraction procedures will be also studied such as SPE for samples.

REFERENCES

Aboul-Enein, H.Y., Kutluk, O.B., Altiokka, G., Tuncel, M. (2002). A modified HPLC method for the determination of OTA by fluorescence detection. *Biomed.Chromatogr.,* 16, 470–474.

Alshana, U. (2004). *Separation and Quantitation of Some Platinum Group Metals by RP-HPLC.* Middle East Technical University. Graduate School of Natural and Applied Sciences, Ankara- Turkey.

Berente, B., Ágnes, M., H.-Otta, K., Záray, G., Lékó, L., Rácz, L. (2005). Determination of ochratoxin A in Hungarian wines. *Microchemical Journal,* 79, 103–107.

Brandburn, N., Jewers, K., Jones, B.D., Tomlins, K.I. (1989). Rapid method for the1 clean-up of extracts of cottonseed prior to aflatoxin determination by bi-directional HPTLC. *Chromatographia,* 28, 541-544.

Bruzzoniti, M.C., Sarzanini, C., Mentássi, E. (2000). Preconcentration of contaminants in water analysis. *J. Chromatog*r. A**,** 902, 289-309.

Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R. (2004). Reversed-phase liquid chromatographic method for the determination of ochratoxin A in wine. *J. Chromatog*r. A, 1024, 275–279.

European Mycotoxin Awareness Network (2000), http://193.132.193.215/eman2/wp2analy.asp, The European Commission, Quality of Life and Management of Living Resources Programme (QoL), Key Action 1 (KA1) on Food, Nutrition and Health, QLK1-CT-2000-01248.

Goodman, K. (1996). Risk assessment of ochratoxin A: an update. *Food Additives and Contaminants*, 13, 53-57.

Herńandez, M.J., García-Moreno, M.V., Durán, E., Guillén, D., Barroso, C.G. (2006). Validation of two analytical methods for the determination of ochratoxin A by reversed-phased high-performance liquid chromatography coupled to fluorescence detection in musts and sweet wines from Andalusia. *Analytica Chimica Acta,* 566, 117–121.

Jornet, D., Busto, O., Guasch, J. (2000). Solid-phase extraction applied to the determination of ochratoxin A in wines by reversed-phase high-performance liquid chromatography. *J. Chromatog*r. A, 882, 29-35.

Kazakevich, Y., McNair, H. M. (1996-2002). Basıc Lıquıd Chromatography, http://hplc.chem.shu.edu/HPLC/index.html.

Krska, R., Molinelli, A. (2007). Mycotoxin analysis: state-of-the-art and future trends. *Anal Bioanal Chem.,* 387:145–148.

Kuhn, I., Valenta, H., Rohr, K. (1995), Determination of ochratoxin A in bile of swine by high-performance liquid chromatography. *J. Chromatogr. A,* 668, 333.

Leitner, A., Zöllner, P., Paolillo, A., Stroka, J., Papadopoulou-Bouraoui A. , Jaborek S., Anklam E. , Lindner W. (2002). Comparison of methods for the determination of ochratoxin A in wine. *Analytica Chimica Acta*, 453, 33–41.

Lin, L., Zhang, J., Wang, P., Wang, Y., Chen, J. (1998). Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of Chromatography A*, 815, 3–20.

Louter, A.J.H., Vreuls, J.J., & Brinkman, U.A.T. (1998). On-line combination of aqueous-sample preparation and capillary gas chromatography. *J. Chromatog*r. A, 842, 391-426.

Markaki, P, Delpont-Binet, C, Grosso, F, Dragacci, S. (2001). Determination of ochratoxin A in red wine and vinegar by immunoaffinity high-pressure liquid chromatography. *Journal of Food Protection*, 64 (4), 533-537

Martinez, D., Cugat, M.J., Borrull, F., Callul, M. (2000). Solid-phase extraction coupling to capillary electrophoresis with emphasis on environmental analysis *J. Chromatogr.* A, 902, 65-89.

Masque, N., Marce, R.M., Borrull, F. (1998). New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from environmental water. *Trends Anal. Che*m., 1**7,** 384-394.

Matysik, G., Giryn, H. (1996). Gradient thin-layer chromatography and densitometry determination of Alternaria mycotoxins. *Chromatographia* 42: 555-558.

Pyrzynska, K., & Trojanowicz, M. (1999). Functionalized Cellulose Sorbents for Preconcentration of Trace Metals in Environmental Analysis. *Crit. Rev. Anal. Chem.*, 29, 313-321.

RHM TECHNOLOGY (2003). http://www.rhmtechnology.co.uk/science/mycotoxin_popup.pdf, RHM Technology- Premier Foods Group Ltd, The Lord Rank Centre, Lincoln Road, High Wycombe, Buckinghamshire, HP12 3QR, United Kingdom.

.

Skoog, D. A., James, J. L. (1992). *Principles of instrumental analysis, 4 th edition, Saunders College Pub.,* Fort Worth.

Tarım ve Köyişleri Bakanlığı- Koruma ve Kontrol Genel Müdürlüğü. Yetki Kanunu Türk Gıda Kodeksi Yönetmeliği, *Yayımlandığı R.Gazete 23.09.2002-24885 Tebliğ No 2002/63*

The Government of the Hong Kong Special Administrative Region. (2006). Centre for Food Safety, Food and Environmental Hygiene Department, Ochratoxin A in Food, *Risk Assessment Studies*, Report No. 23.

Valenta, H. (1998). Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. *J. Chromatography. A,* 815, 75-92.

Visconti, A., Lattanzio, V.M., Pascale, M., Haidukowski, M. (2005). Analysis of T-2 and HT-2 toxins in cereal grains by immunoaffinity clean-up and liquid chromatography with fluorescence detection. *Journal of Chromatography A,* 1075; 151–158.

Weingaertner, J., Krska, R., Praznik, W., Grasserbauer, M., Lew, H. (1997). Use of Mycosep multifunctional clean-up columns for the determination of trichothecenes in wheat by electrone capture gas-chromatography. *Fresenius J Anal Chem*., 357; 1206–1210.

Zóllner, P., Mayer-Helm, B. (2006). Review; Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. *Journal of Chromatography A*, 1136, 123-169