DOKUZ EYLUL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CHROMATOGRAPHIC AND SPECTROSCOPIC DETERMINATIONS OF SOME MYCOTOXINS AND METALS USING NOVEL LIQUID EXTRACTION METHODS IN VARIOUS FOOD PRODUCTS

by
H. Mine ANTEP

March, 2013
İZMİR

CHROMATOGRAPHIC AND SPECTROSCOPIC DETERMINATIONS OF SOME MYCOTOXINS AND METALS USING NOVEL LIQUID EXTRACTION METHODS IN VARIOUS FOOD PRODUCTS

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by
H. Mine ANTEP

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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"CHROMATOGRAPHIC AND SPECTROSCOPIC DETERMINATIONS OF SOME MYCOTOXINS AND METALS USING NOVEL LIQUID EXTRACTION METHODS IN VARIOUS FOOD PRODUCTS"** completed by **H.MİNE ANTEP** 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 Doctor of Philosophy.

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CHROMATOGRAPHIC AND SPECTROSCOPIC DETERMINATIONS OF SOME MYCOTOXINS AND METALS USING NOVEL LIQUID EXTRACTION METHODS IN VARIOUS FOOD PRODUCTS

ABSTRACT

In this study, totally five different mycotoxins in several kinds of foodstuffs were determined by chromatographic analysis using three different extraction technologies. Thin-layer chromatography with densitometry and high performance liquid chromatography were used in optimization steps and / or in sample analysis. Besides this, several major and trace elements in wine and beer samples were detected using atomic absorption / emission spectrometer.

5-hydroxymethylfurfural was extracted from five grape vinegar and seven fruit wine samples by liquid-liquid extraction and extracts were directly applied to TLC-scanner with UV detection. The 5-hydroxymethylfurfural in all studied samples was detected and ranged from 0.59 to 33.10 mg per L. The limits of detection and quantification of this method were 0.045 and 0.125 μg per mL, respectively. For robustness, within and between-day repeatability of the method were calculated as percentage of 4.5 and 8.6.

For ochratoxin A and zearalenone analysis, a newly dispersive liquid liquid microextraction method was improved and applied to eight red and four white wine samples for ochratoxin A and thirteen beer samples for zearalenone. Under the optimum extraction conditions, the extraction recovery percentage and the enrichment factor were calculated as 63.9 and 34.5 for OTA and 83 and 43.3 for ZEN, respectively. The linearity of the DLLME method was employed in the concentration range of OTA in wines and ZEN in beer from 0.03 to1 ng per mL and from 0.4 to 120 ng per mL, respectively. The recovery of method was in the range of percentage between 63-109 for OTA and 71–108 for ZEN at 0.1 and 0.5 ng per mL, at 10 and 20 ng per mL spiking levels, respectively.

For tenuazonic acid and cyclopiazonic acid analysis in tomato juice samples, a novel cloud point extraction method was developed. The extraction recoveries as percentage were found as 39.9 and 94.6 for tenuazonic acid and cyclopiazonic acid, respectively. The linearity of the proposed method was in the concentration range 0.01-2 ng per mL for both mycotoxins. The recovery as percentage of this method was in the range of 84 to 98 for cyclopiazonic acid and 83 to 97 for tenuazonic acid at 0.05 and 0.1 ng per mL spiking levels.

In this study, Ca, Mg, Na, K, Fe, Cu, Zn and Pb were also studied in grape wine and beer samples. For this, atomic absorption spectrometer equipped with flame and graphite furnace atomization and, atomic emission spectrometer were used for all wine and beer samples after acid digestion using nitric acid and hydrogen peroxide. The accuracy of the method was confirmed by spiking at two levels to real samples for studied each metal ion.

Keywords: Mycotoxin, liquid-liquid extraction, dispersive liquid liquid microextraction, cloud point extraction, metal analysis, HPLC, HPTLC, AAS.

ÇEŞİTLİ GIDA ÜRÜNLERİNDEKİ BAZI MİKOTOKSİNLERİN VE METALLERİN FARKLI SIVI EKSTRAKSİYON METODLARI KULLANILARAK KROMATOGRAFİK VE SPEKTROSKOPİK TAYİNLERİ

ÖZ

Bu çalışmada, çeşitli gıdalardaki toplam beş farklı mikotoksin, kromatografik analiz ile üç farklı ekstraksiyon metodu kullanılarak tayin edildi. Optimizasyon ve / veya örnek analizlerinde densitometrik ince tabaka kromatografisi ve yüksek performanslı sıvı kromatografisi kullanıldı. Bunun yanında, şarap ve biralardaki bazı temel ve eser elementin tayini atomik absorpsiyon / emisyon spektrometresi ile gerçekleştirildi.

5-hidroksimetilfurfural beş üzüm şarabından ve yedi meyve şarabından etil asetat kullanılarak sıvı-sıvı ekstraksiyonu ile ekstrakte edildi ve ekstraktlar TLC-tarayıcısına uygulandı ve UV dedektöründe 286 nm' de.tayin edildi. Çalışılan tüm örneklerde 5-hidroksimetilfurfural miktarı litrede 0,59-33,10 mg olarak belirlendi. Yöntemin gözlenebilme ve tayin sınırları sırasıyla mili litrede 0,045 ve 0,125 µg bulundu. Yöntemin dayanıklılığı için gün içi tekrarlanabilirlik için yüzde 4,5, günler arası tekrarlanabilirlik için değeri yüzde 8,6 olarak belirlendi.

Okratoksin A ve zeralenon analizi için yeni bir ekstraksiyon metodu olan dispersif sıvı sıvı mikroekstraksiyonu geliştirildi ve okratoksin A için sekiz kırmızı ve dört beyaz şarap örneğine, zeralenon için onüç bira örneğine uygulandı. Bu optimum ekstraksiyon koşulları altında, ekstraksiyon geri kazanımı ve zenginleştirme faktörü OTA için sırasıyla yüzde 63.9 ve 34,5, ZEN için yüzde 83 ve 43,3 olarak hesaplandı. DLLME metodunun doğrusallığı şaraplardaki OTA ve biradaki ZEN için sırasıyla mililitrede 0,03 ile 1 ng ve mililitrede 0,4 ile 120 ng arasında uygulandı. OTA ve ZEN için örneklere eklenen mililitrede 0,1 ve 0,5 ng, mililitrede 10 ve 20 ng derişimlerinde geri kazanım değerleri sırasıyla yüzde 63-109 ve yüzde 71–108 aralığında bulundu.

Domates suyundaki tenuazonik asit ve siklopiazonik asit analizi için yeni bir metot olan bulutlanma noktası ekstraksiyonu geliştirildi. Tenuazonik asit ve siklopiazonik asit ekstraksiyon geri kazanım değerleri sırasıyla yüzde 39,9 ve 94,6 olarak belirlendi. Hedeflenen metodun doğrusal çalışma aralığı her iki mikotoksin için mililitrede 0,01-2 ng olarak bulundu. Örneklere mililitrede 0,1 ve 0,05 ng eklenen standartlar için geri kazanım değerleri sırasıyla siklopiazonik asit için yüzde 84-98, tenuazonik asit için yüzde 83-97 aralığında hesaplandı.

Bu çalışmada ayrıca üzüm şarapları ve biralardaki Ca, Mg, Na, K, Fe, Cu, Zn ve Pb analizi yapıldı. Bunun için, tüm şarap ve bira örneklerinin analizinde nitrik asit ve hidrojen peroksit kullanılarak asit çözünürleştirilmesi yapıldıktan sonra alev ve grafit fırınlı atomlaştırıcı ile birleştirilmiş atomik absorpsiyon spektrometresi ve atomik emisyon spektrometresi kullanıldı. Metodun doğruluğu için gerçek örneklere her bir metal iyonunun iki farklı derişiminde eklemeler yapıldı.

Anahtar sözcükler: Mikotoksin, sıvı-sıvı ekstraksiyon, dispersif sıvı sıvı mikroekstraksiyonu, bulutlanma noktası ekstraksiyonu, metal analizleri, HPLC, HPTLC, AAS.

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CHAPTER ONE
INTRODUCTION

1.1 The Mycotoxins

Mycotoxins are toxic secondary metabolites produced by fungi, such as *Fusarium, Aspergillus* and *Penicillium* species. Their growth is affected from the climatic conditions such as moisture, temperature and storage and transport conditions. The mycotoxins can cause severe nephrotoxic, neurotoxic, carcinogenic, immunosuppressive and estrogenic effects. The relatively less amount contamination by mycotoxins can cause diarrhea in animals and humans and also reducing feed and lossing weight in animals (Zollner & Mayer-Helm, 2006).

Within more than 30.000 different mycotoxin species, some mycotoxins such as aflatoxins, ochratoxin A, fumonisins, tenuazonic acid, cyclopiazonic acid, patulin, deoxynivalenol, zearalenone, hydroxymethyl furfural, nivalenol etc. have been discovered so far by demonstrating differentiation in structure. Most of them have significant thermal and chemical stability. They can or cannot only partly be removed by food processing or by other suitable decontamination procedures.

Cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruit, particularly apples are affected from mycotoxins. They can also be found in beer and wine because of use of contaminated grapes, barley and other cereals in their production. By meat and other animal products like egg, milk and cheese, mycotoxins can influence human body due to eating contaminated feed (Turner, Subrahmanyam, & Piletsky, 2009).

Because of the potential health risks to humans and animals, the presence of mycotoxin have been controlled and adopted in regulatory limits by many autorities (Krska, & Molinelli, 2007). The quantity survey for monitoring and controlling mycotoxin levels have been arranged by authorities in many countries. For this reason

determination of mycotoxins is important because of its toxicity and causes economic losses in the world seriously.

1.1.1 Hydroxymethylfurfural (HMF)

5-Hydroxymethylfurfural (Figure 1.1) is comprised by an acid catalysed degradation reducing sugars or using Maillard reaction. While reducing sugars reacts with amino acids or proteins, HMF is formed (Mouron, 1981). The pH, concentration of reagents, temperature and reaction time is important for improving the Maillard reaction. This reaction takes place in foods heating and storage. It is also effective on the taste and appearance of food. Besides, this reaction also generates in human body and affects many physiological functions (Ledl, & Schleicher, 1990). The amount of HMF in foods is directly related to the heat applied during processing of carbohydrate-rich products. Another source of HMF is represented by ingredients used in the formulation such as caramel solutions or honey.

In several literatures, HMF intake by humans has been given. A daily intake of HMF as 150 mg/person or 2.5 mg kg^{-1} body weight by Ulbricht, Northup, & Thomas (1984) and 30 to 60 mg/person or 0.5-1 mg kg^{-1} body weight by Janzowski, Glaab, Samimi, Schlatter, & Eisenbrand (2000) were reported. Environmental Protection Agency recommends acute oral LD_{50} as 2.5 g $kg^{-1}$for males and 5.0 g kg^{-1} for females in rats (US EPA, 1992). A weak genotoxic and mutagenic ability of HMF in vitro studies have been demonstrated by Janzowski et al. (2000).

The studies concerning determination of HMF in food products such as tomato paste, coffee and dried fruits, (Murkovic, & Pichler, 2006), sugars and honey (Gaspar, & Lopes, 2009), vinegar and wine (Cocchi et al., 2011; Alcazar, Jurado, Pablos, Gonzalez, & Martin, 2006), commercial fruit jams (Rada-Mendoza, Olano, & Villamiel, 2002) and bread (Teixido, Nonez, Santos, & Galcera, 2011) have been done by mostly HPLC with different type of detectors.

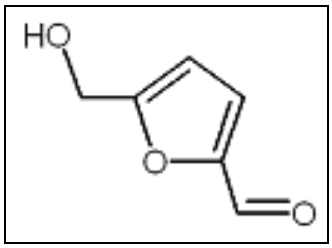

Figure 1.1 Structure of HMF

For HMF, The International Federation of Fruit Juice Processors (IFFJP) has recommended the maximum concentration in fruit juices and concentrates as 5–10 mg L^{-1} and 25 mg kg^{-1}, respectively (Frank, 1974). However, the Codex Alimentarius of the World Health Organisation and the European Union have also established a maximum HMF quality level in honey as 50 mg kg^{-1} (EC, 2001).

1.1.2 Zearalenone (ZEN)

Zearalenone is a major mycoestrogen. It is chemically described as 6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-B-resorcyclic acid lactone (Figure 1.2). It is produced by *Fusarium* species which are commonly found in soil fungi in temperate and warm countries. They are mostly effective in the contamination of cereals such as corn, oat, barley, wheat, rice and sorghum (Bennett, & Klich, 2003). ZEN has derivatives like α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL). Zearalenone and its derivatives are also found in malt, beer and flour (Kuzdralinski, Soarska, & Muszyriska, 2013).

Fusarium species have been implicated in several human outbreaks of mycotoxicosis that causes symptoms as nausea, vomiting, and diarrhea in some countries (Hussein, & Brasel, 2001). Under available storage conditions for fungal growth and mycotoxin formation, the level of ZEN can be increased such as at comparatively cold temperatures (Richard, 2007). It has been known that ZEN has oestrogenic activity. It attributes to the oestrogen receptors in mammals and causes ostrogenic effects. The toxicity of this mycotoxin is low (Muri, Van der Voet, Boon, Klaveren, & Bruschweiler, 2009).

The European Union committee recommended the maximum level of ZEN in animal feed as 0.1 mg kg^{-1} (EU, 2006). In literature, there are several studies concerning high performance liquid chromatography methods in combination with ultraviolet, fluorescence or mass spectrometric detection and thin layer chromatography methods using fluorescent silica gel plates with reflectance-absorbance mode for the analysis of ZEN in corn, animal feed, beer, alcoholic beverages (Briones-Reyes, Gomez-Martinez, & Cueva-Rolon, 2007; De Saeger, Sibanda, & Van Peteghem, 2003; Maragou, Rosenberg, Thomaidis, & Koupparis, 2008; Odhav, & Neicker, 2002)

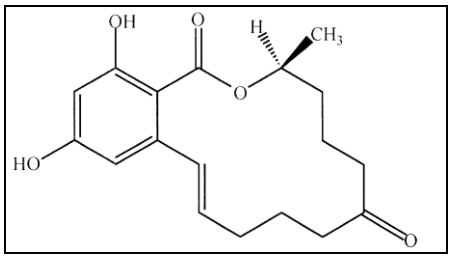

Figure 1.2 Structure of ZEN

1.1.3 Ochratoxin A (OTA)

Ochratoxins are a group of secondary metabolites produced by *Penicillium* and *Aspergillus.* They are composed of a polyketide derived from dihydroisocoumarin which is linked to 7-carboxy group of L-β-phenylalanine by an amide bond except ochratoxin α (Figure 1.3). There are five ochratoxins; ochratoxin A, its methyl ester; ochratoxin C, its ethyl ester; 4-hydroxyochratoxin A, its methyl ester; ochratoxin B, its ethyl ester and ochratoxin α, missing the phenylalanine part (Ringot, Chango, Schneider, & Larondelle, 2006).

It has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC monographs, 1993). It is known as a kidney toxin, but its high concentrations can damage the liver (Richard, 2007).

Ochratoxin A can breed on barley, soy products, raisins and coffee in varying amounts, of course at low levels. Neverthless, it may accumulate in humans or

animals fluid or tissues because of consuming contaminated food. (Skaug, Wignand, & Stormer, 2001). The European Union regulations for ochratoxin A are 5 μg kg^{-1} for raw cereal grains, 3 μg kg^{-1} for all products derived from cereals and 10 μg kg^{-1} for dried vine fruit (FAO, 2004) No regulations has been found for United States. Recently, the European Food Safety Authority established a tolerable weekly intake (TWI) of 120 ng kg^{-1} body weight (EC, 2006).

Nowadays, occurrence of OTA has been reported using liquid chromatography combined with fluorescence or mass spectrometric detection in grape juice, dried wine fruits (Ng, Mankotiat, Pantazopoulog, Neil, & Scott, 2004; Stefanaki, Foufa, Tsatsou-Dritsa, & Dais, 2003), cocoa products (Goryacheva et al., 2006), nuts (Saito, Ikeuchi and Kataoka, 2012), spices (Bonvehi, Manzanares, & Vilar, 2004) and black table olives (El Adlouni, Tozlovanu, Naman, Faid, & Pfohl-Leszkowicz, 2006), beer (Rubert, Soler, Marin, James, & Manes, 2013), cereal (Campone, Piccinelli, Celano, & Rastrelli, 2012).

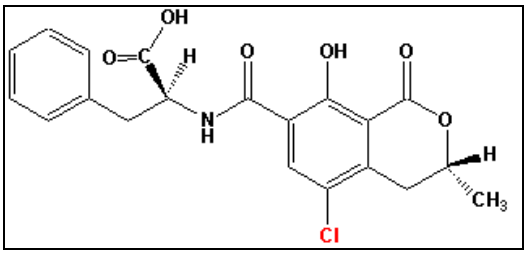

Figure 1.3 Structure of OTA

1.1.4 Cyclopiazonic and Tenuazonic Acids (CPA & TEA)

Cyclopiazonic acid (Figure 1.4), toxic-indole tetramic acid, is known as a secondary metabolite produced by several species of *Aspergillus* and *Penicillium* fungi (Luk, Kobbe, & Townsend 1977; Ohmomo, Sugita, & Abe, 1973). CPA is classified as a neurotoxin because of its effectiveness on the central nervous system in animals (Pier, Belden, Ellis, Nelson, & Maki, 1989; Lomax, Cole, & Dorner, 1984). CPA causes degenerated changes in liver, kidney, salivary glands and skeletal muscle in experimental and farm animals (Morrisey, Norred, Cole, & Dorner, 1985; Dorner, Cole, Lomax, Gosser, & Diener, 1983).

Several studies about analysis of CPA in corn (Gallagher, Richard, Stahr, & Cole, 1978; Lee, & Hagler, 1991), peanuts (Urano et al., 1992), rice and poultry feed (Moldes-Anaya, Asp, Eriksen, Skaar, & Rundberget, 2009), milk (Oliveira, Rosmanınho, & Rosim, 2006), cheese (Zambonin, Monaci, & Aresta, 2001), tomato products (Da Motta, & Soares, 2001), dried figs (Heperkan, Somuncuoglu, Karbancioglu-Guler, & Mecik, 2012), feed mixed with wheat, peanut and rice (Moldes-Anaya et al., 2009) have been found in literature.

Up to now, there is no available regulatory standard for CPA because of its low occurrence in foods. The acceptable daily intake might be 10 μg kg^{-1}/day or 700 μg/day no observed effect level (NOEL) is accepted as 1 μg kg^{-1}/day for several kinds of animals. For human exposure, the maximum limit of CPA in cheese is 4 μg g^{-1} and the average individual consumes 50 g of cheese daily (EMAN, 2000).

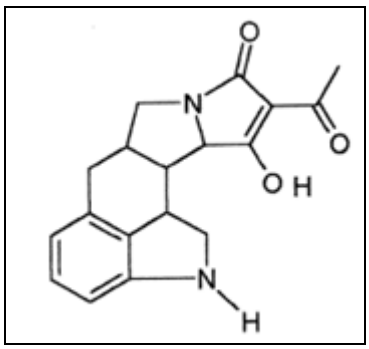

Figure 1.4 Structure of CPA

Tenuazonic acid ((5S,8S)-3-acetyl-5-sec-butyltetramic acid) is a toxic metabolite produced by *Alternaria spp., Phoma sorghina and Pyricularia oryzae* (Iwasaki, Muro, Nozoe, Okuda, & Sato, 1972; Steyn, & Rabie, 1976; Umetsu, Kaji, Aoyama, & Tamari, 1974). TEA is considered to be of the highest toxicity amongst the *Alternaria* mycotoxins (Weidenbörner, 2001). It inhibits protein biosynthesis (Carrasco, & Vazquez, 1973). It is biologically active. It acts as antitumor and has antiviral and antibiotic activities (Shephard, Thiel, Sydenham, Vleggaar, & Marasas, 1991; Weidenbörner, 2001). *Alternaria spp*. have been commonly infesting a broad range of agricultural products, including wheat (Azcarate, Patriarca, Terminiello, &

Pinto, 2008; Li, & Yoshizawa, 2000) and barley (Sanchis, Sanclemente, Usall, & Vinas, 1993).

Recent studies in the analysis of TEA were carried out with tomato products, cereals and beer using HPLC with ultraviolet detection or mass spectrometric detection (Da Motta, Soares, 2001; Siegel, Rasenko, Koch, & Nehls, 2009; Siegel, Merkel, Koch, & Nehls, 2010).

The LD_{50} value for TEA is 162 and 115 mg kg^{-1} bodyweight for male and female mice, respectively. In recent articles, Alternaria mycotoxin levels in Argentinian wheat as 2.3 mg kg^{-1}, similar quantity in Chinese wheat has been reported (Azcarate, Patriarca, Terminiello, & Pinto, 2008; Li, & Yoshizawa, 2000).

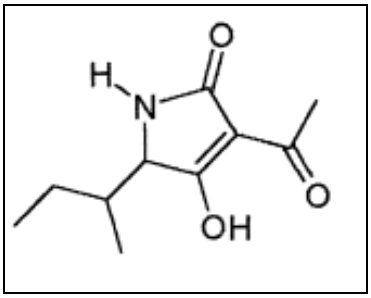

Figure 1.5 Structure of TEA

1.2 Extraction and Preconcentration Methods of Mycotoxins

1.2.1 Liquid Liquid Extraction

Liquid liquid extraction (LLE) is a traditional separation process, containing two phases as aqueous and organic phase. They are immiscible or partially immiscible within each other. By LLE, the compounds are separated with respect to their solubilities in two different immiscible liquids. This procedure is performed using a separatory funnel. Nonpolar solvents such as hexane, cyclohexane and benzene are used to remove nonpolar contaminants. The procedure is also effective for toxins and works well in small-scale preparations (Bauer, & Gareis, 1987). This technique is time consuming, and depends on type of matrix and type of compounds being

determined. Disadvantages are possible loss of sample by adsorption onto the glassware and spending a large amount of organic solvents that causes environmental contamination.

Liquid liquid extraction has been a traditional method for mycotoxins for a long time. In 2000, Da Motta and Soares applied the procedure for the simultaneous determination of TEA and CPA in tomato products (Da Motta and Soares, 2000). Additionally, liquid liquid extractions of multi mycotoxin from soil and commercial baby foods have been studied (Rubert, Soler, & Manes, 2012; Spanjer, Rensen, & Scholten, 2008). In many studies, LLE as clean-up method has been used for separation of solutes from analytical matrices before preconcentration by solid phase extraction or immunoaffinity columns. With this technique, OTA was firstly extracted using chloroform. After the evaporation step, the residue was redissolved in phosphate buffered saline solution and transferred to the immunafinity column. The elution of OTA was completed using methanol/acetic acid (Zimmerli, & Dick, 1996). In addition, Oasis HLB cartridges or Myco separation columns and anion exchange columns have been used for the purification of mycotoxins after their liquid liquid extractions (Lattanzio, Solfrizzo, & Visconti, 2006; Pussemier et al., 2006).

1.2.2 Dispersive Liquid Liquid Microextraction

One of the most recent modalities of microextraction is dispersive liquid liquid microextraction (DLLME) which is a miniaturized LLE that uses microliter volumes of organic solvents. This technique was first introduced by Assadi and co-workers (Rezaee et al., 2006). In this extraction technique a binary mixture of a water miscible solvent, named as disperser, and a solvent having high density and very low water solubility, referred as extractant, is used to extract and concentrate especially organic compounds from various analytical matrix (Figure 1.6). Acetone, methanol and acetonitrile are normally considered as disperser, and several chlorinated solvents possessing high density such as chloroform, dichloromethane are used as extractant.

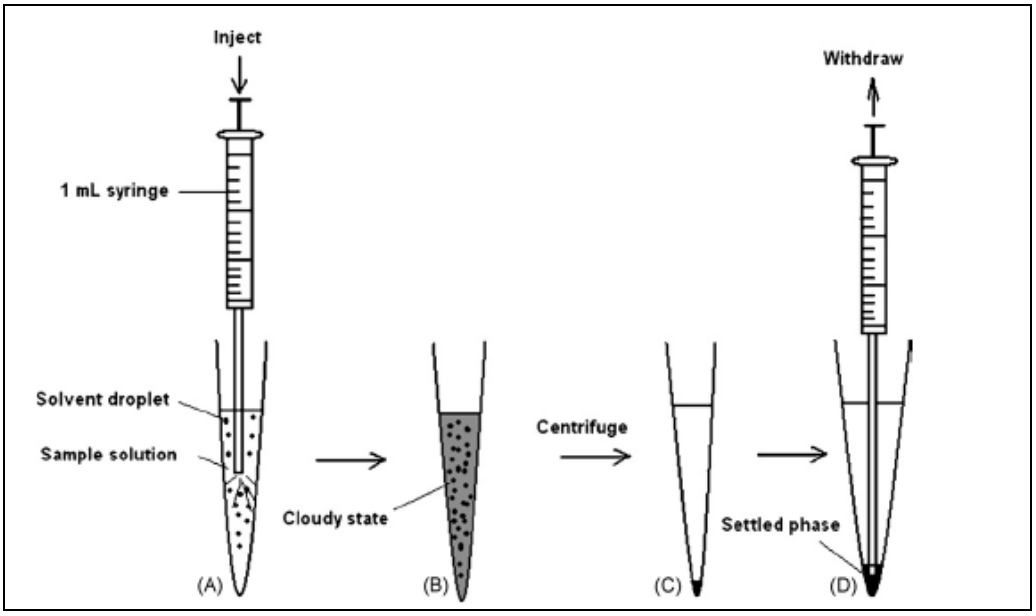

Figure 1.6 Dispersive liquid–liquid micro extraction procedure (A) Injection of disperser containing extractant into an aqueous sample solution, (B) dispersion of disperser and extractant, (C) centrifugation and (D) injection of settled phase using a syringe (Nagaraju, & Huang, 2007).

In DLLME, when adding of the extraction mixture to the aqueous sample quickly, a cloudy state consisting of fine droplets of the extractant confirmed and dispersed in the aqueous phase. After centrifugation of the turbid dense mixture, drops of extractant settle at the bottom of the test tube. So, a high enrichment factor depending upon the type and volume of extractant and dispersive solvent is ensured by getting settled phase.

The extraction efficiency not only depends on the type of extraction and dispersive solvent and also depends on the other extraction parameters as salt effect and the equilibrium time. A good extraction with high efficiency in DLLME is succeeded in the given condition steps:

- The extraction solvent must have higher density than water and less solubility in water. The first one provides the successful separation of extraction solvent from aqueous part after centrifugation. The second one leads to the higher extraction efficiency for the solute.

- The dispersive solvent must be sufficiently soluble in extraction solvent and also be miscible with water. These properties provide the dispersion of extractant as fine particles in the aqueous solution and formation of turbidity in solution. Besides, the extraction efficiency is increased by supplying large surface area between extractant and aqueous phase.

- Equilibration time defined as the interval time from injecting the extraction mixture to centrifugation is the other important parameter. Generally, the extraction time is short because of the fast transition of the solute from aqueous phase to extraction phase.

- High ionic strength provides the less solubility of organic molecules (solute and extractant) in aqueous phase. This causes high recovery, but large volume of settled phase and low enrichment factor (Xiao-Huan, Qiu-Hua, Mei-Yue, Guo-Hong, & Zhi, 2009).

As mentioned above, DLLME method is rapid, low cost, ease of operation and ensures high enrichment factor. This method has been successfully applied for the analysis of a various organic and/or inorganic compounds in aqueous samples. Some of analyzed compounds using DLLME can be summarized as cholesterol in milk, egg yolk and olive oil using of carbon tetrachloride as extractant and ethanol as disperser, organosulfur pesticides in environmental and beverage samples using carbon tetrachloride and methanol and triazine herbicides in water using chlorobenzene and acetone, antibiotics in mineral and run-off waters using chloroform and acetonitrile (Daneshfar, Khezeli, & Lotfi, 2009; Nagaraju, & Huang, 2007; Herrera-Herrera; Hernandez-Borges; Borges-Miquel; Rodriguez-Delgada, 2013). Recent advances include coupling DLLME with single-drop, microwave-assisted, ultrasound-assisted solvent extraction, using liquid base as extractant, low-density solvent based DLLME combined with spectrometric or chromatographic techniques have been grown up (Andruch, Kocurova, Balogh, & Skilikova, 2012;

Piazarro, Saenz-Gonzalez, Perez-del-Notario, & Gonzalez-Saiz, 2012; Bidari, Ganjali, Norouzi, Hosseini, & Assadi, 2011; Padro, et. al., 2013).

There are only a few studies concerning preconcentration of mycotoxins using DLLME in literature. Determination of ochratoxin A in wine samples by capillary high performance liquid chromatography using chloroform and acetonitrile was studied by Arroyo-Manzanares and co-workers. The dynamic range was between 0.02 and 4 μg L^{-1} and the enrichment factor is 5 (Arroyo-Manzanares, Gamiz-Gracia, & Garcıa-Campana, 2012). Also, the analysis ochratoxin A in cereals was studied by pH-controlled DLLME-HPLC-FLD method using carbon tetra chloride and methanol and by liquid chromatography coupled to positive electrospray ionization tandem mass spectrometry (Campone, Piccinelli, Celano, & Rastrelli, 2012; Campone, Piccinelli, & Rastrelli, 2011). Another study about analysis of aflatoxins in cereal products by DLLME using methanol and chloroform was succeeded by Campone et. al. (Campone, Piccinelli, Celano, & Rastrelli, 2011). Patulin analysis in the presence of HMF in apple juice was studied by DLLME-micellar elektrokinetic capillary chromatography using propanol as disperser and chloroform as extractant (Victo-Ortega, Lara, Garcia-Campana, & del Olma-Iruela, 2013).

1.2.3 Cloud Point Extraction

Cloud point extraction (CPE) is a new alternative extraction technique which was first developed by Watanabe and Tanaka for preconcentration of zinc ion using 1-(2pyridylazo)-2-naphthol (Watanabe, & Tanaka, 1978). In CPE, phase separation is achieved by formation of surfactant micellar after changing temperature or adding salt to an aqueous solution (Figure 1.7).

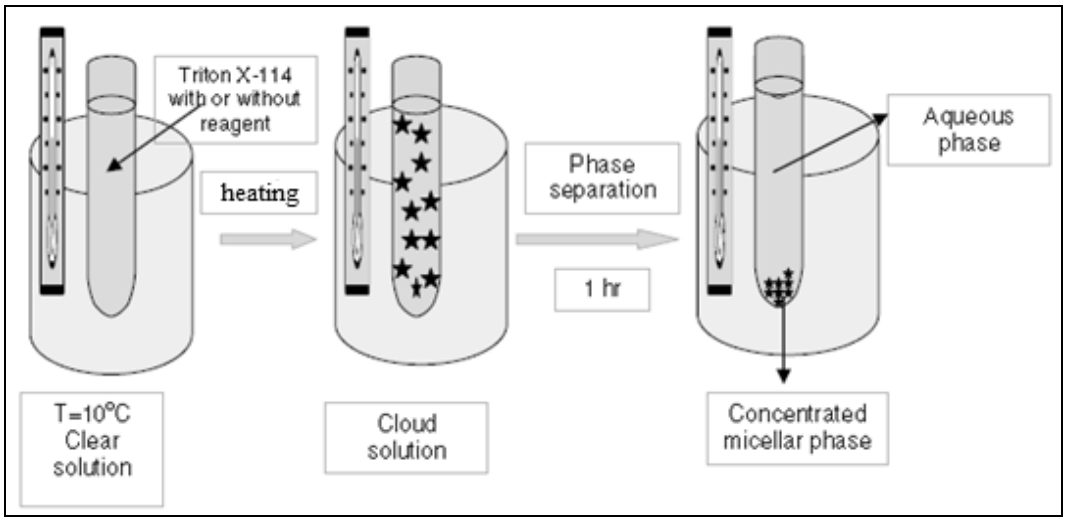

Figure 1.7 Cloud point extraction procedure

Surfactants are amphiphilic molecules that contain a polar or hydrophilic group in the head and hydrophobic group in the tail. The tail part is generally a long hydrocarbon chain on the form of linear or branched or aromatic rings while the head is ionic or strongly polar groups. In aqueous solutions, the tail and the head group behave as hydrophobic and hydrophilic, respectively. As can be summarized in Table 1.1, the surfactans are classified according to the tail structure as non-ionic, cationic, anionic, and amphoteric (zwitterionic). The hydrophobic tails tend to form aggregates called micelles (Xie, Paau, Li, Xiao, & Choi, 2010).

Cloudy formation is a typical physical change in the homogeneous solutions of amphiphilic substances. By amphiphilic substances, aqueous solution is separated two phases which are surfactant-poor and surfactant-rich at a definite temperature named as cloud point temperature (CPT) (Mukherjee, Susanta, Dash, Patel & Mishra, 2011). Below the cloud point temperature, water molecules surrounds the all surfactant molecules by forming H-bonds with the polar head groups of ionic-surfactants and the ethylene oxide units of non-ionic surfactant. But above the cloud point temperature, the increase in entropy causes dehydration of the polyoxyethylene chains and destroying the H-bonding with water molecules. The attraction between surfactant molecules is occurred by van der Waals forces and they aggregate by forming micelles and eventually, the phase separation is carried out.

In aqueous solution surfactants can aggregate to form micelles. For this situation, the required minimum concentration of surfactant is called as the critical micelle concentration (CMC) which depends on its molecular structural formula. Micelles are not static structures and are affected from experimental conditions such as ionic strength, counterions, temperature, etc. Micelles are stable and regenerated. However, they can be degraded by dilution with water because of lowering the surfactant concentration below its CMC. Below the CMC, the surfactant is predominantly in a nonassociate monomer form. But above the CMC, these monomers associate forming micelles spontaneously due to the diminished solubility of the surfactant in water (Silva, Roldan, & Gine, 2009).

Cloud point extraction is an inexpensive extraction technique because of using very less amount of surfactant, eco-friendly, less laboratory residues and environmentally friendly. The used surfactants are nontoxic, nonvolatile, and less flammable.

Table 1.1 Classification and characteristics of surfactants

Surfactant	Characteristic	Example	Density (g/mL)	CPT (°C)	CMC (mM)
Cationic	The hydrophilic group carrying a positive charge such as the quaternary ammonium halides ($R_4N^+Cl^-$)	Cetyl trimetyl ammonium bromide (CTAB)	0.9	-	0.92
Anionic	The hydrophilic group carrying a negative charge such as carboxyl ($RCOO^-$), sulfonate (RSO_3^-), or sulfate ($ROSO_3^-$)	Sodium dodecyl sulfate (SDS)	0.9	>100	7-10
Nonionic	The hydrophilic group has no charge but derives its water solubility from highly polar groups such as polyoxyethylene or polyol groups	Triton X-114	1.06	23-25	0.2-0.35
		Triton X-100	1.07	64-65	0.17-0.30
		Brij 35	1.05	60	-
		Genapol X-080	1.05	41-45	0.02-0.06
		Tween 20	1.07	95	0.059
		Tween 80	1.06-1.09	65	0.012
Zwitterionic	Its molecules present both the anionic and cationic groups and, depending of pH, its prevalence the anionic, cationic, or neutral species	*N*-dodecyl-*N*,*N*-dimethylbetaine (C_{12}-Bet)	-	-	1.25

Nowadays, scientists have developed this technique using different micelles and solvents for organic and inorganic analytes. In many studies CPE was for determination of metal contents in water samples using Triton X-100, Triton X-114 and PONPE 7.5 (Xiao, Chen, Wu, & Miao, 2007), in biological samples as human saliva using PONPE 7.5, in cereals using Triton X-114 (Luconi, Olsina Fernández, & Silva, 2006; Lemos et al., 2008). Additionally, CPE has been applied to determine the organic substances such as phatalate esters (Wang et al., 2007), synthetic azo dye as allura in food samples (Pourreza, Rastegarzadeh, & Larki, 2011), aesculin and aesculetin in Cortex fraxini (Shi, Zhu, & Zhang, 2007). But until now, a few studies have been published concerning toxic compounds such as ergotamine in pharmaceticals and biological fluids as human urine using PONPE 7.5 (Wang, Fernandez, & Gomez, 2013). Only one study for the analysis of mycotoxin using surfactant has been found in literature. It is concerning the determination of OTA in wine using decanoic acid as surfactant in CPE following HPLC with fluorescence (Garcia-Fonseca, Ballesteros-Gomez, Rubio, & Perez-Bendito, 2008).

1.3 The Metals

Heavy and toxic metals are natural components of the Earth's crust and cannot be degraded or destroyed. They enter the bodies by food, drinking water and air at a small extent. As trace elements, some heavy metals as copper, selenium, zinc are essential to maintain the metabolism of the human body. However, they can be toxic at higher concentrations. Lead pipes, intaking in food chain and air particulates in emission sources can cause heavy metal poisoning (Food info, 2007).

Toxic metals are dangerous because they can accumulate in human body. It means that an increase in the concentration of a chemical in a biological organism in time. Compounds are taken up and stored faster than their metabolization and excretion so can be accumulated in plants, aimals and humans (Food info, 2007).

Classification of trace elements based on current acceptance by the scientific community is given in Table 1.2. Among the given elements, some of them are essential but some of them are toxic. The essential and toxic species are listed in Table 1.3.

Table 1.2 Classification of trace element (Frieden, 1981)

Classification	**Elements**
Bulk structural elements	Carbon (C), Hydrogen (H), Oxygen (O), Phosphorus (P), Sulfur (S).
Macroelements	Calcium (Ca), Chlorine (Cl), Potassium (K), Sodium (Na).
Trace elements	Copper (Co), Iron (Fe), Zinc (Zn).
Ultratrace elements	Arsenic (As), Boron (B), Fluorine (F), Iodine (I), Selenium (Se).
Metals	Cadmium (Cd), Chromium (Cr), Cobalt (Co), Lead (Pb), Manganese (Mn), Molybdenum (Mo), Nickel (Ni), Tin (Sn), Vanadium (V).

Table 1.3 Classification of trace metals as plant (Adriano, Mcleod, & Ciravolo, 1986)

Trace element	**Essential**	**Toxic**
Boron (B)	Yes	Yes
Cobalt (Co)	Yes	Yes (low)
Copper (Cu)	Yes	Yes
Manganese (Mn)	Yes	Yes
Molybdenum (Mo)	Yes	Yes
Selenium (Se)	Yes	Yes
Vanadium (V)	Yes	Yes
Zinc (Zn)	Yes	Yes

1.4 Thin Layer Chromatography (TLC) and TLC-densitometry

Thin layer chromatographic applications have been used for analysis and quality control of food products in the wide range of laboratories because of its easy uses, simple, rapid and inexpensive separation technique.

High performance thin layer chromatography (HPTLC) is now a modern TLC technique with some improvements of classical TLC equipments. It has quality of sorbents with smaller particle sizes. By these improvements, HPTLC ensures a good separation efficiency, shorter analysis time, faster separation (Sherma, & Fried, 1986). In Table 1.4, some differences between TLC and HPTLC are given (Poole, & Schuette, 1984).

With the difference of TLC system, scanner equipment is attached to HPTLC system for quantitative determinations for food and drug analysis. This can lead to comparable results with other chromatographic techniques in terms of the simplicity of operation, the separation and the quantification of standards and samples on the same plate at the same time, the usage of less organic solvents and shorter analysis time.

Table 1.4 Differences between HPTLC and TLC (Poole, & Schuette, 1984)

	HPTLC	**TLC**
Layer of Sorbent	100 μm	250 μm
Efficiency	High due to smaller particle size generated	Less
Separations	3 - 5 cm	10-15 cm
Analysis Time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed phase modes	Silica gel, Alumina, Kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Scanning	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

Many applications have been found in literature for determination of different substances in several matrices on pharmaceutical analysis (Ali, Ali, Sultana, Baboota, & Faiyaz, 2007; Machale, Gatade, & Sane, 2011), environmental analysis (Morlock, Schuele, & Grashorn, 2011), food and agricultural analysis (Abjean, & Lahogue, 1997; Lautie, & Stankovic, 1996), etc. Analysis of diazepam in diazepam tablets and analysis of sucralose after solid phase extraction and lutein in environmental samples were examples of recent studies concerning HPTLC densitometry (Machale, Gatade, & Sane, 2011; Morlock, Schuele and Grashorn, 2011; Rodic, Simonovska, Albreht & Vovk, 2012).

Additively, some works were summarized on determination of different mycotoxins in different matrices. In 2002, Odhav and Neicker analysed OTA, ZEN and citrinin in brewed beers using silica gel and fluorescent silica gel TLC plates (Odhav, & Neicker, 2002). Pittet and Royers studied the detection of OTA in green coffee (Pittet, & Royer, 2002). Another study described by Shephard and Sewram was about the analysis of fumonisin B1 in ground maize samples using reversed-phase HPTLC (Shephard, & Sewram, 2004).

1.5 Aim of The Study

Mycotoxins are secondary toxic metabolites. They are produced by microfungi. They can cause disease and death in human and animals. Because of the difficulty of removing of mycotoxins from food matrices, their measurements must have been studied and their regulation limits must have been controlled in their legal limits.

Trace metal analysis in wines, beers and food samples is important for determining of legal limits for export purposes, controlling for quality and flavor of wine and showing the health effects on human. And also trace metal analysis may interest to identify the origin of samples and composition of wines.

Using of TLC-scanner provide lots of advantages such as the usage of less amounts of organic solvent, not time consuming studies, not required expensive sample pre-treatment and also identifying and determination of several analytes in a single analytical step only one plate. In addition, it is simple, economic and fast technique for optimization step. Besides TLC-scanner, using of HPLC is one of the most popular techniques to detect the mycotoxins because of its sensitivity and lower detection limits.

Atomic absorption spectrometry and/or atomic emission spectrometry techniques have been an essential technique for the analysis of major and trace elements at high and low concentrations in numerous samples.

In this study, major aim is to determine the mycotoxins levels in some kind of food matrices using different liquid extraction techniques before their chromatographic analysis. The proposed mycotoxins, liquid extraction methods and chromatographic analysis are summarized as;

A- Analysis of 5-hydroxymethylfurfural in some Turkish vinegar and wine samples using liquid-liquid extraction method prior to analysis with TLC-scanner and high performance liquid chromatography,

B- Analysis of ochratoxin a in some kinds of wine samples using dispersive liquid-liquid microextraction method prior to analysis with TLC-scanner and high performance liquid chromatography,

C- Analysis of zearalenone in some kinds of beer samples using dispersive liquid-liquid microextraction method prior to analysis with TLC-scanner and high performance liquid chromatography,

D- Analysis of cyclopiazonic acid and tenuazonic acid in tomato juice samples using cloud point extraction method prior to analysis with high performance liquid chromatography.

Besides this, another aim of this study is to analyze some major and trace elements in wine and beer samples. The determination of Ca(II), Mg(II), Na(I), K(I), Fe(II), Zn(II), Cu(II) and Pb(II) metal ions was carried out atomic absorption/emission spectroscopy after acid digestion system.

CHAPTER TWO
MATERIAL AND METHODS

2.1 Reagents, Solvents, and Preparation of Standard Solutions

All mycotoxins standards (HMF, OTA, ZEN, CPA, and TEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored in a freezer at -20 °C. Stock solutions of 1000 mg L^{-1} metal ions such as Na(I), K(I), Ca(II), Mg(II) were prepared by solving of their nitrate salts and 500 mg L^{-1} Cu(II) was prepared by solving of its sulphate salt in 100 mL of 2 % (v/v) nitric acid solution. Standard atomic absorption stock solutions (Inorganic Ventures, Virginia, U.S.A.) as 1000 mg L^{-1} were used for Zn(II), Pb(II) and Fe(III). Calibration and working solutions were prepared from their stock solutions by diluting with 2% (v/v) nitric acid solution. Acetonitrile (ACN), methanol (MeOH), 1,2-dichloroethane ($C_2H_4Cl_2$), tetrachloroethylene (C_2Cl_4), trichloroethylene (C_2HCl_3), methylene chloride (CH_2Cl_2), chlorobenzene (C_6H_5Cl), carbon tetrachloride (CCl_4) and chloroform ($CHCl_3$) were supplied from Merck (Darmstadt, Germany). Formic acid and nitric acid were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Toluene and ethyl acetate were purchased from Riedel de Haën (Seelze, Germany). All other chemicals and solvents were reagent grade or HPLC grade and were used without further purification. Ultrapure water was used throughout the experiments (Milli-Q system; Millipore, MA, U.S.A.).

A stock solution (200 mg L^{-1}) of HMF was prepared in ethyl acetate (EtAc). Working standard solutions (0.5-20 μg mL^{-1}) of HMF were prepared by evaporation of known volumes of the stock solution under a stream of N_2 then solving in chloroform for HPTLC, in water (pH adjusted to 4 with acetic acid) for HPLC.

Zearalenone stock standard solution (200 mg L^{-1}) was prepared in acetonitrile (ACN). Working standard solutions of ZEN (8-400 μg mL^{-1} for HPTLC and 5-2000 ng mL^{-1} for HPLC) were prepared by diluting with ACN.

A stock standard solution of OTA (200 mg L^{-1}) was prepared in methanol (MeOH). Working standard solutions (4-100 μg mL^{-1} for HPTLC and 1-20 ng mL^{-1} for HPLC) of OTA were prepared by diluting with MeOH.

Stock standard solutions of CPA and TEA were prepared in MeOH at 200 mg L^{-1} and 400 mg L^{-1}, respectively. All working standard solutions (0.020-10 μg mL^{-1} for TEA and 0.010-20 μg mL^{-1} for CPA) of CPA and TEA were prepared by diluting with MeOH. Also, each working standard solution was prepared prior to analysis.

Phosphate buffer at pH=2 was prepared by mixing 5.7 mL of 1 mol L^{-1} phosphoric acid solution and 0.5712 grams of potassium dihydrogen phosphate monohydrate salt and diluting to 100 mL with distilled water. When necessary, pH adjustment was done by adding 0.1 M sodium hydroxide solution.

2.2 Apparatus

All of the pH adjustments were done using Selecta pH 2001 equipped with calomel glass electrode. For cloud point and dispersive liquid-liquid microextraction studies, Nüve NF200 model centrifuge was used. The Yellow line MSC basic heater/stirrer equipped with TC2 IKA-WERKE thermo couple was used for all heating and stirring steps. The Bandelin SONOREX ultrasonic bath was used in degassing of beer samples, and cloud point extraction and dispersive liquid-liquid microextraction methods. The Heidolph REAXtop model vortex was used for mixing of the mycotoxin standards.

2.3 Samples

Vinegar, wine, beer and tomato juice samples were purchased from local stores in Izmir and stored in the dark until analysis. All samples were stored in their original bottles at 4°C before analysis. All vinegar, wine and beer samples were filtered using 0.45 μm filter disk before the analysis (Millipore Millex-HV, Hydrophilic PVDF, MA, U.S.A.). Lids of beer bottles were opened the day before analysis and degassed

in an ultrasonic bath for 30 min to remove foaming. All given informations of the all samples were taken from the labels of bottles (Table 2.1-2.4).

Table 2.1 Special features of vinegar samples

Vinegar	**Raw Material**	**Acidity (%)**	**Region**
Vinegar 1	Grape	4-5	Izmir, Aegean
Vinegar 2	Grape	4-5	Izmir, Aegean
Vinegar 3	Grape	4-5	Izmir, Aegean
Vinegar 4	Apple	5-6	Izmir, Aegean
Vinegar 5	Balsamic	NG	Izmir, Aegean

NG: not given

Table 2.2 Special features of wine samples

Wine	Raw material	Color	Alcohol Content (%)	Region	Manifacture Year
Wine 1	Grape	Red	NG	Izmir, Aegean	2007
Wine 2	Apple	Yellow	NG	Izmir, Aegean	2007
Wine 3	Sour cherry	Dark red	NG	Izmir, Aegean	2007
Wine 4	Bilberry	Red	NG	Izmir, Aegean	2007
Wine 5	Peach	Dark yellow	NG	Izmir, Aegean	2007
Wine 6	Pomegranate	Red	NG	Izmir, Aegean	2007
Wine 7	Melone	Yellow	NG	Izmir, Aegean	2007
Wine 8	Grape	Red	13.5	Argentina	2008
Wine 9	Grape	Red	14	Tekirdag, Marmara	2008
Wine 10	Grape	Red	13.5	Manisa, Aegean	2007
Wine 11	Grape	Red	12	Aegean	2008
Wine 12	Grape	Red	12.7	Canakkale, Marmara	2007
Wine 13	Grape	Red	11.5	Izmir, Aegean	2007
Wine 14	Grape	Red	12	Tekirdag, Marmara	2008
Wine 15	Grape	Red	12	Bursa, Marmara	2009
Wine 16	Grape	Red	15	Trakia, Marmara	2010
Wine 17	Grape	Red	12	Denizli, Aegean	2007

Table 2.2 Continue

Wine	Raw material	Color	Alcohol Content (%)	Region	Manifacture Year
Wine 18	Grape	White	12	Aegean	2008
Wine 19	Grape	White	12	Tokat, Black Sea	2002
Wine 20	Grape	White	12.5	Denizli, Aegean	2009
Wine 21	Grape	White	13	Import	2007
Wine 22	Grape	White	13	Denizli, Aegean	2007
Wine 23	Grape	White	12	Denizli, Aegean	2007

Table 2.3 Special features of beer samples

Beer	Raw material	Alcohol content (%)	Region
Beer 1	Barley	4.7	Izmir, Aegean
Beer 2	Barley	3	Istanbul, Marmara
Beer 3	Barley	5	Istanbul, Marmara
Beer 4	Barley	5	Istanbul, Marmara
Beer 5	Barley	6.1	Canakkale, Marmara
Beer 6	Barley	5	Izmir, Aegean
Beer 7	Barley	5	Denmark
Beer 8	Barley	5	Izmir, Aegean
Beer 9	Barley	5	Izmir, Aegean
Beer 10	Barley	5	Istanbul, Marmara
Beer 11	Barley	4.9	Istanbul, Marmara
Beer 12	Wheat	5	Istanbul, Marmara
Beer 13	Wheat	5.5	Istanbul, Marmara

Table 2.4 Special features of tomato juice samples

Tomato Juice	Raw material	Region
Tomato juice 1	100% tomato	Izmir, Aegean
Tomato juice 2	Tomato	Istanbul, Marmara
Tomato juice 3	Tomato	Izmir, Aegean

2.4 Extraction Procedures for Mycotoxins

2.4.1 LLE for HMF

Liquid-liquid extraction was applied to vinegar and wine samples by modifying the AOAC International Official Method used for analysis of patulin in apple juice (Scott, 1974). Briefly, after filtering the unspiked/spiked wine/vinegar samples through 0.45 μm pore size filter paper (Millipore Millex-HV, Hydrophilic PVDF) 2.5 mL of filtered samples were extracted three times with 5 mL of EtAc by shaking for

5 min. The three extracts were combined in a 25-mL volumetric flask, diluted to volume with EtAc, dried over 0.5 g anhydrous sodium sulfate, and evaporated to dryness under a stream of N_2. The residues were then redissolved in 1 mL $CHCl_3$ for TLC-scanner or in water (pH adjusted to 4 with acetic acid) for HPLC.

2.4.2 DLLME for ZEN

A five mL of unspiked/spiked beer sample was put on a 15 mL of polyethylene tube having conical bottom. A disperser solvent as 0.25 mL of ACN containing 75 µL of $CHCl_3$ as extraction solvent was added rapidly into the sample, and the mixture was shaken by hand for 1 min. After that the cloudy solution formed, the resulting solution was centrifuged for 5 min at 4000 rpm and the dense phase was settled in the bottom of the polyethylene tube. Then, the settled phase was removed using a 100 µL microsyringe and applied to the HPLC for quantification of the studied samples.

In optimization of parameters of extraction method, test solutions of ZEN were used. Test solutions of ZEN at concentration of 0.2 ng μL^{-1} were prepared by adjusting pH around 3.8-4.8 using 0.1 M HCl. The TLC-scanner was used for optimization of the proposed extraction method.

2.4.3 DLLME for OTA

A five mL of filtered unspiked/spiked wine sample was placed in a 15-mL screw capped test tube with conic bottom. A 1.00 mL of ACN (disperser solvent) containing 100 µL of chloroform (extraction solvent) was rapidly injected into the wine sample, and the mixture was gently shaken for 1 min. After that the cloudy solution formed, the resulting solution was centrifuged at 4000 rpm for 5 min and the extraction solvent was sedimented in the bottom of the conical test tube. Then, the sedimented phase was transferred to another test tube using a 100 µL microsyringe to the HPLC for quantification of the studied samples.

In optimization of parameters of extraction method, test solutions of OTA were used. Test solutions of OTA at concentration of 0.2 ng μL^{-1} were prepared by adjusting pH around 3.5-4.0 using 0.1 M HCl. The TLC-scanner was used for optimization of the proposed extraction method.

2.4.4 CPE of CPA&TEA

Cyclopiazonic acid and tenuazonic acid was firstly extracted from tomato juice samples using liquid-liquid extraction method described by Da Motta, & Soares, (2000). Shortly, five grams of tomato juice sample was put to a 50 mL of reaction flask and mixed with 15 mL of methanol for 3 min using a magnetic stirrer. Later, the resultant mixture was filtered using a glass funnel. Then the residues left in the flask were washed with the additional 5 mL of MeOH and filtered again. The volume of collected filtrate was recorded for future calculations. The collected methanolic extract was transferred to a 100 mL of separating funnel and extracted with 4 mL of hexane by gently shaking for 1 min. After the complete phase separation, the hexane phase was removed. To prevent emulsion formation, 5 mL of water was added and the solution of pH was arranged to 2 by adding concentrated HCl solution a few drops. Afterwards, the recent methanolic extract was shaked with 4 mL of chloroform twice. The chloroform extract was washed with 3 mL of water after separation methanolic phase. The obtained chloroform extract was evaporated under N_2 stream after recording its volume for future calculations and then redissolved with 3 mL of pH=2 phosphate buffer for getting ready cloud point extraction.

In cloud point extraction, the tomato juice sample extract was mixed in turn with 2 mL of 4% (w/v) Triton X-114 solution and 2 mL of 1% (w/v) KNO_3 solution and its final volume was completed to 10 mL with water. The final mixture was heated at 50 °C for 30 min, centrifuged at 4000 rpm for 15 min and finally put in an ice bath for 30 min. The upper aqueous phase was discarded using a long-needled syringe. A surfactant-rich phase was diluted with methanol to reduce the viscosity and then analyzed by HPLC.

2.5 Dissolution procedure for metal ions

A 10 mL of filtered wine/beer sample was introduced into a PTFE beaker and 2 mL of concentrated nitric acid and 2 mL of 30 % (v/v) hydrogen peroxide were added. The mixture was digested by heating until dryness. Then the residues were dissolved in 25 mL of 1M HNO_3 solution (Dos Santos, Brandao, Portugal, David, & Ferreira, 2009). Two replicated digestions were made for each sample and analysed by AAS.

2.6 Analysis of Mycotoxins

2.6.1 HMF, OTA and ZEN by TLC

In TLC-scanner analysis, silica gel $60F_{254}$ HPTLC plates as 20 cm × 10 cm (Merck, Germany) plates were used. Samples and standards as 1 μL were applied to the plates as 4-mm bands, 0.7 cm from the side edge and 1.0 cm from the bottom, by use of a CAMAG Linomat V semi-automatic sample applicator (Wilmington, NC, USA). Three pairs of duplicate samples were applied to each plate. Chromatograms were developed in ascending mode, to a distance of 5 cm, at room temperature (22–25°C) using 20 cm × 10 cm CAMAG twin-trough chamber previously equilibrated with toluene–EtAc–formic acid (90%) 6:3:1 (v/v) as mobile phase for HMF and toluene–EtAc–formic acid 6:3:1 (v/v) for OTA and ZEN and vapor for 20 min before insertion of the plate (Odhav, & Naicker, 2002). After development, the plates were dried at room temperature. Suitable detection mode was performed at suitable wavelength for each mycotoxin with a CAMAG TLC Scanner III densitometer and controlled by CATS version 4.X software. The applied mobile phases, the detection modes and the wavelengths and the retardation factor (hR_F, $R_F x100$) for HMF, OTA and ZEN were tabulated in Table 2.5. During detection, D_2 light source or Hg lamp and K 400 secondary filter were used.

Table 2.5 Chromatographic conditions in TLC-scanner for HMF, OTA and ZEN

Mycotoxin	**Detection mode**	**Wavelength (nm)**	**hR_F (x±s)**
HMF	Absorbance	286	38±3
OTA	Fluorescence	333	62±3
ZEN	Absorbance	277	74±2

The HPTLC chromatograms and densitograms of standard HMF, OTA and ZEN were given in Figure 2.1-2.6, respectively. The calibration curves of these studied mycotoxins for HPTLC were established by injecting standard solutions at least five calibration levels and correltion coefficients were obtained as seen in Table 2.6.

Table 2.6 Instrumental calibration data for HMF, OTA and ZEN

Mycotoxin	**Linear working range (µg mL^{-1})**	**Number of Calibration Levels**	**Linear regression equation**	**Correlation coefficient**
HMF	0.5-20	10	y = 39.776x – 12.399	0.9968
OTA	4-100	10	y = 84.457x + 70.202	0.9985
ZEN	4- 20	9	y = 44.465x + 322.01	0.9902

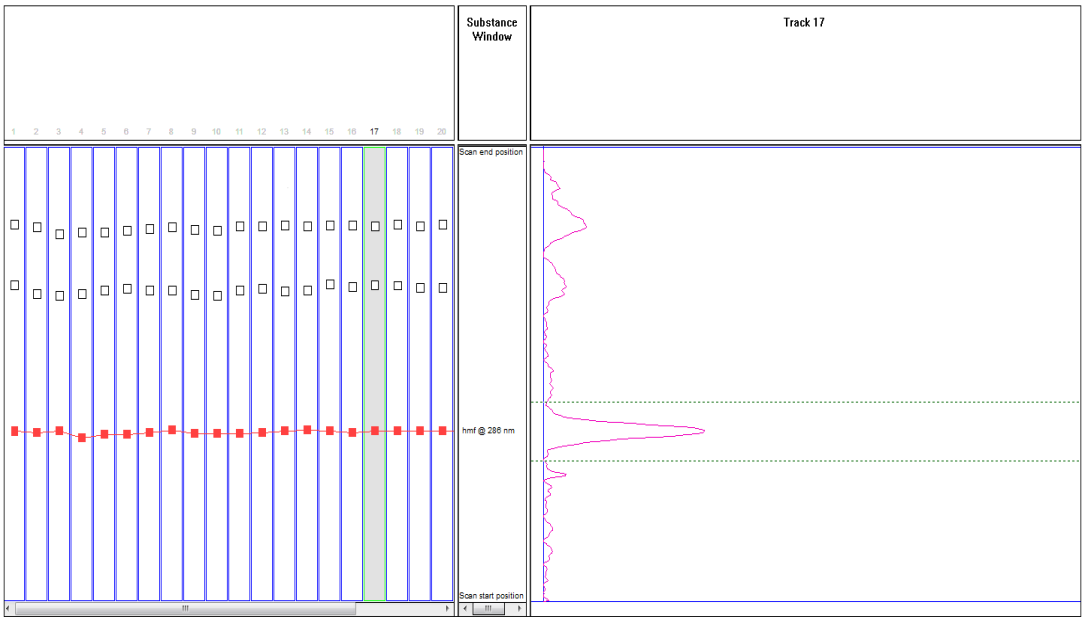

Figure 2.1 The HPTLC chromatogram of standard HMF

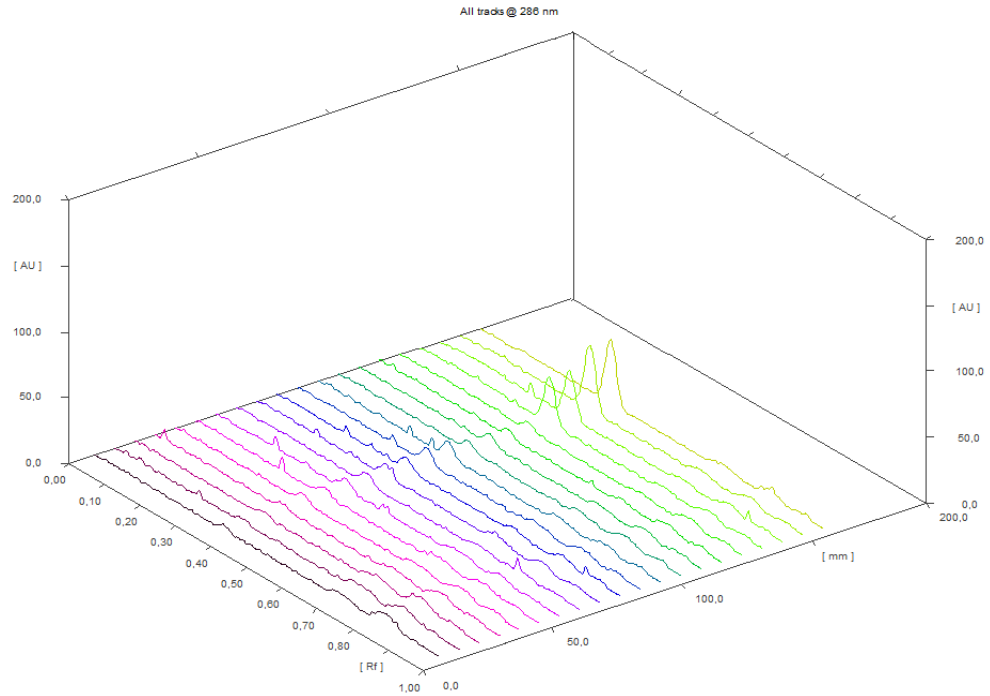

Figure 2.2 The HPTLC densitogram of standard HMF

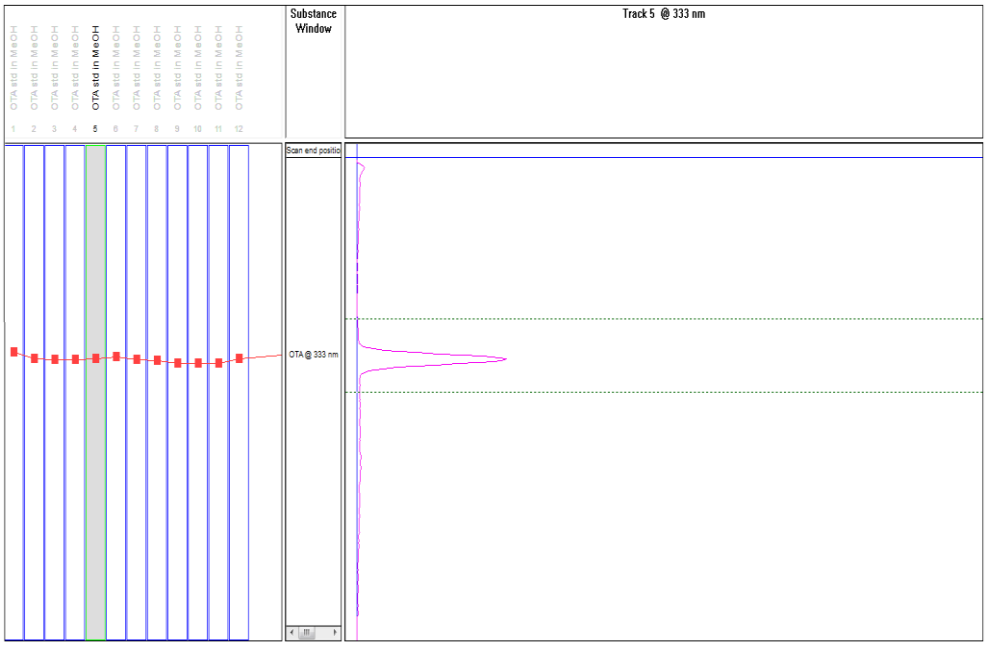

Figure 2.3 The HPTLC chromatogram of standard OTA

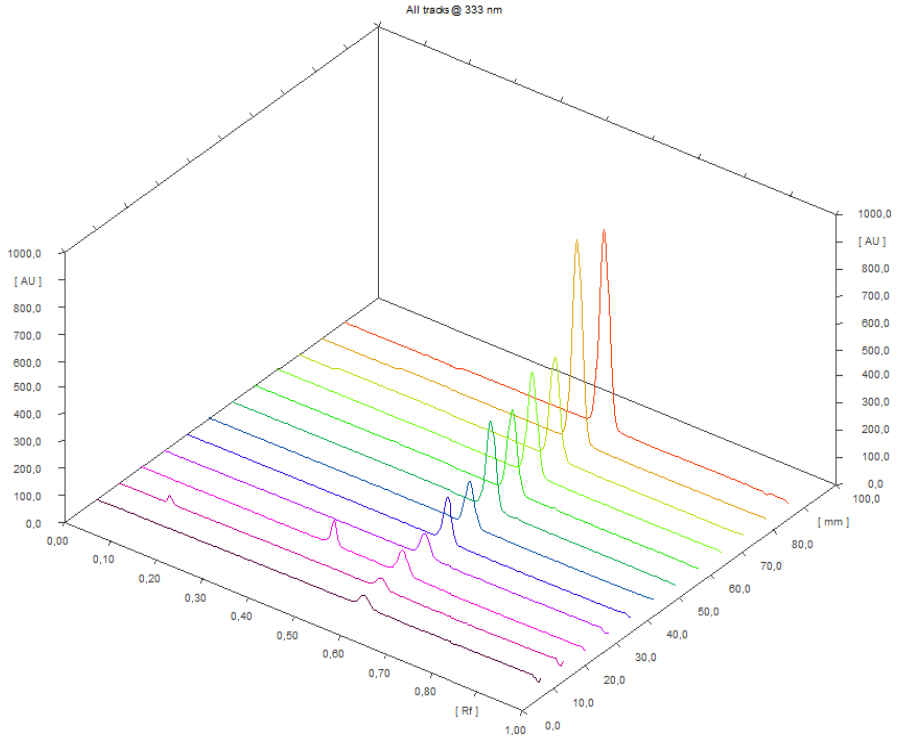

Figure 2.4 The HPTLC densitogram of standard OTA

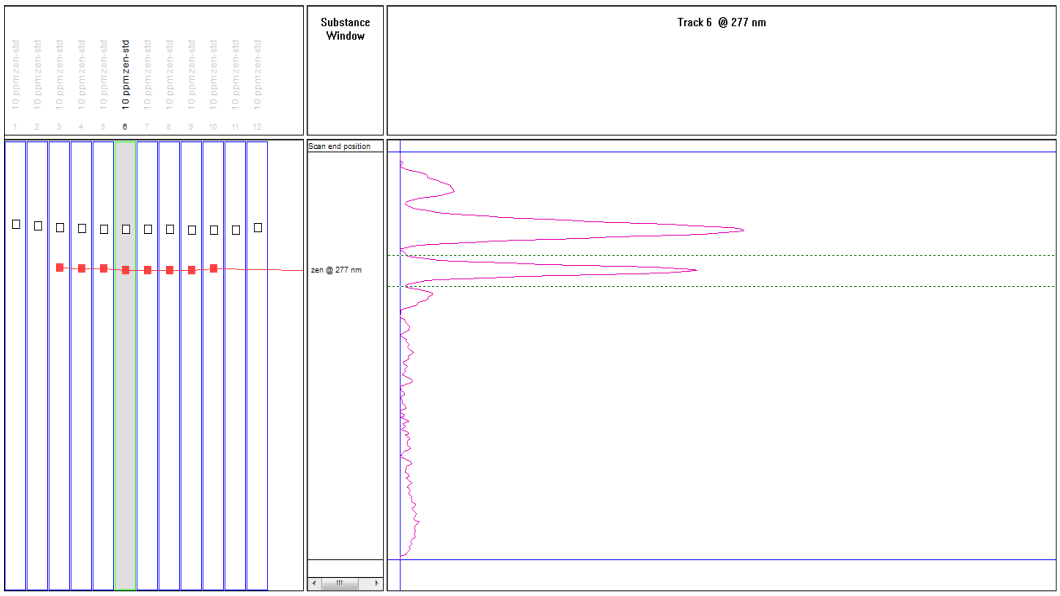

Figure 2.5 The HPTLC densitogram of standard ZEN

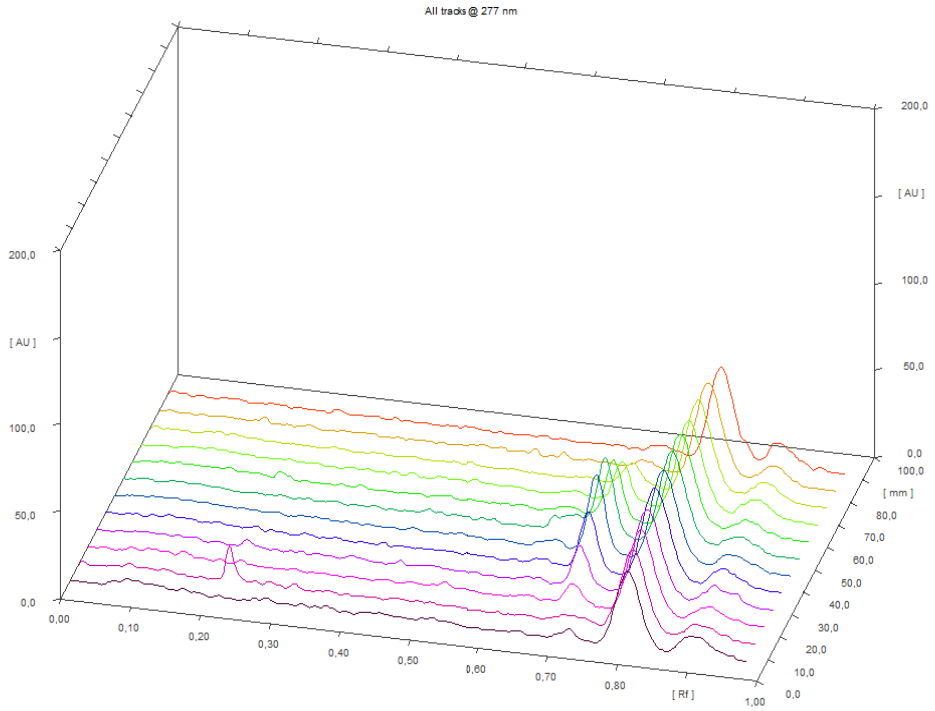

Figure 2.6 The HPTLC densitogram of standard ZEN

2.6.2 HMF, OTA, ZEN, CPA and TEA by HPLC

The Agilent 1100 model HPLC system (Waldbronn, Germany) in Chemistry Department, DEU consists of an online vacuum degasser (G1322A), a column oven (G1316A), a quaternary pump (G1311A), diode array detector (G1315B) and fluorescence detector (G1321A) with manuel injection was used. Also the Agilent 1100 model HPLC system (Waldbronn, Germany) in Environmental Engineering, DEU including a G1379A degasser, a quaternary pump (G1311A), a column oven (G1316A), diode array detector (G1315B) with automatic injection system (G1316A) was used in chromatographic studies. The separation was carried out using analytical column hypersyl gold C18 (Thermo, 250 x 4,6 mm, 5 μm) for HMF, OTA and ZEN, ODS-2 hypersyl C18 (Thermo, 250 x 4,6 mm, 5 μm) for CPA&TEA. A Hamilton stainless steel manual injector as 100 μL was used. Each sample was injected two/three times. The injection volume of samples was 20 μL. Chemstation 3D software was used to control the chromatograms and the process signals. The mobile phase, the elution type, the detector type and the wavelength (λ) and the retention time (t_R) were summarized in Table 2.7.

Table 2.7 Chromatographic conditions in HPLC for HMF, OTA, ZEN, CPA and TEA

HPLC conditions	**Mycotoxin**			
	HMF	**OTA**	**ZEN**	**TEA & CPA**
Mobile phase (v/v)	ACN:water (99:1)	Water:ACN:HAc (48.5:50.5:1)	Water:ACN (48:52)	MeOH:water (75:25) containing 300 mg $ZnSO_4$. H_2O/L
Elution type	Isocratic	Isocratic	Isocratic	Isocratic
Detector type	DAD	FLD	FLD	DAD
λ (nm)	276	333(ex), 458(em)	235(ex), 450(em)	280
Flow rate (mL/min)	1.0	1.5	1.5	1.0
Column Temperature (°C)	30	50	40	30
Retention time (min)	18.4	4.1	5.9	4.8, 6.9
Reference	Gokmen, & Acar, 1999	Kurtbay, 2007	Bankole et al., 2010	Da Motta and Soares, 2000

The HPLC chromatograms of standard HMF, OTA, ZEN and CPA and TEA were given Figure 2.7-2.10, respectively. The calibration curves of these studied mycotoxins for HPLC were established by injecting at least five standard solutions. The linear working range and correlation coefficients were given in Table 2.8.

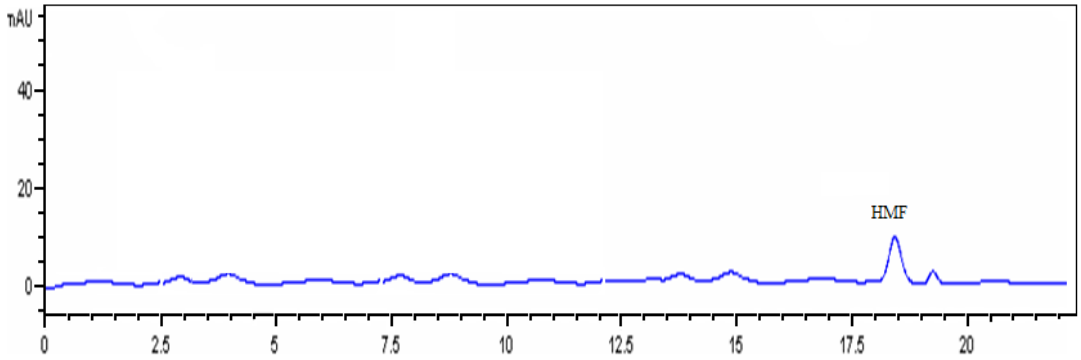

Figure 2.7 HPLC chromatogram of standard HMF

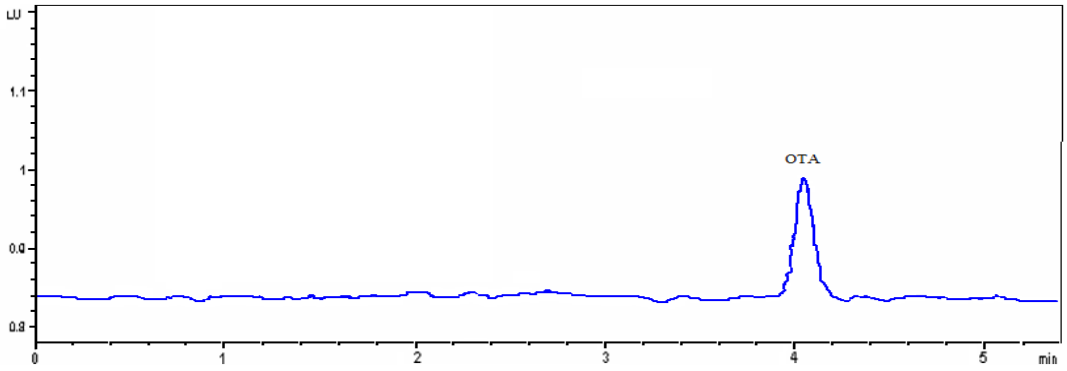

Figure 2.8 HPLC chromatogram of standard OTA

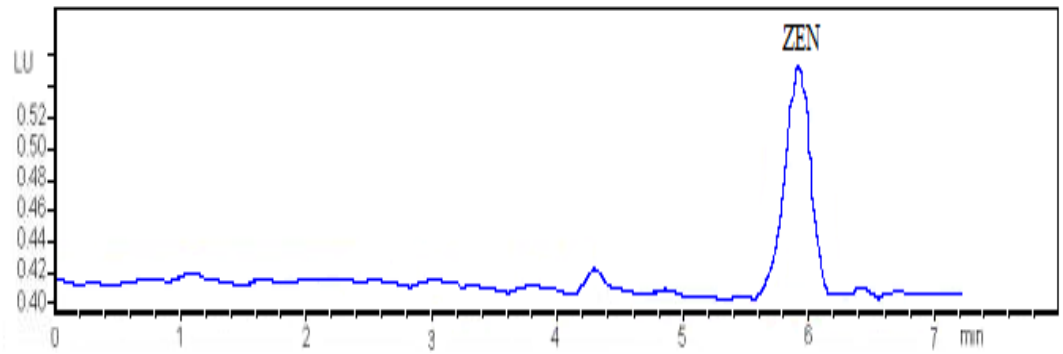

Figure 2.9 HPLC chromatogram of standard ZEN

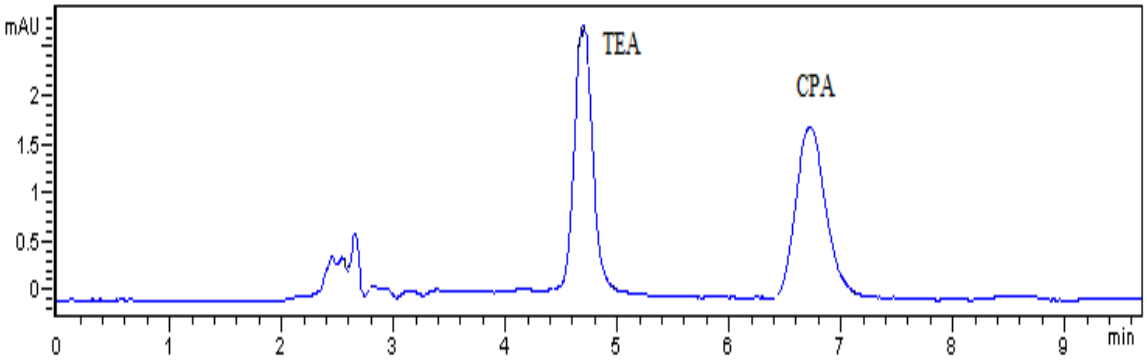

Figure 2.10 HPLC chromatogram of standards TEA and CPA

Table 2.8 Instrumental calibration data for HMF, OTA, ZEN, TEA and CPA

Mycotoxin	**Linear working range (µg mL^{-1})**	**Number of Calibration Levels**	**Linear regression equation**	**Correlation coefficient**
HMF	0.25-20	7	y =77.26x + 46.527	0.9977
OTA	1-10	6	y = 0.6932x – 0.5280	0.9975
ZEN	5-20	9	y = 0.0184x + 0.0033	0.9968
TEA	0.2-10	10	y = 14.944x - 0.5069	0.9981
CPA	0.010- 20	11	y = 43.416x + 5.0482	0.9984

2.7 Analysis of Metals by AAS

Mostly PerkinElmer AAnalyst 700 flame atomic absorption spectrometer (FAAS) attached to a PE HG-500 graphite furnace (with a PE AS 800 automatic injector) equipped with unielemental hollow cathode lamps in Chemistry Department and rarely Analytic Jena model Novaa 300 flame atomic absorption spectroscopy instrument in Mining Engineering Department, DEU and Perkin Elmer model Optima 7000 DV ICP-OES in Environmental Engineering Department, DEU were employed for metal analyses. A deuterium lamp continuum background corrector for spectral interferences was used. The conditions for the lean air–acetylene, air-argon and air-N_2O related to the fuel and the oxidant flow rate settings, the vertical burner position and the sample uptake rate, were adjusted to achieve the maximum sensitivity for flame and furnace operation. An air–acetylene flame was used with an

acetylene flow rate of 2.0 L min^{-1}, an air flow rate of 17.0 L min^{-1}. As shown in Table 2.9 the given analytical wavelengths with the spectral band passes and lamps currents were used according to the recommended conditions by handbook of the instrument used. Also the graphite furnace temperature program is gathered in Table 2.10. The calibration curves of these studied metal ions for FAAS, AES and GFAAS were established by reading at least five standard solutions. The linear working range and correlation coefficients were given in Table 2.11.

Table 2.9 Instrumental conditions of FAAS and AES

Metal Ion	**Wavelength (nm)**	**Slit (nm)**	**Lamp Current (mA)**
Na(I)*	589.0	0.2	-
K(I)*	766.5	0.2	-
Ca(II)	422.7	0.7	10
Mg(II)	285.2	0.7	10
Fe(II)	248.3	0.7	25
Cu(II)	324.8	0.7	25
Zn(II)	213.9	0.7	25
Pb(II)	283.3	0.7	10

*AES conditions

Table 2.10 Graphite furnace temperature programmes

Step	**Temperature (°C)**				**Ramp time (s)**	**Hold Time (s)**			
	Fe	**Cu**	**Zn**	**Pb**		**Fe**	**Cu**	**Zn**	**Pb**
1	100	100	100	100	5	20	20	20	20
2	140	140	140	140	15	15	15	15	15
3	1400	1000	700	700	10	20	20	20	20
4*	2400	2300	1800	1800	0	5	5	5	0
5	2600	2600	2600	2600	1	5	5	5	5

Argon flow rate 250 mL min^{-1}

* Reading step

Table 2.11 Instrumental calibration datas of metal ions

Metal Ion	**Linear working range (µg mL^{-1})**	**Number of Calibration Levels**	**Linear regression equation**	**Correlation coefficient**
Na(I)	0.1-5	6	y = 3077x +1112.5	0.9988
K(I)	0.25-25	7	y = 874.67x – 160.93	0.9969
Ca(II)	1- 25	7	y = 0.0286x + 0.02	0.9968
Mg(II)	1-25	7	y = 0.0521x + 0.0079	0.9999
Fe(II)	0.1-5	7	y = 0.0306x + 0.0004	0.9989
Cu(II)	5-50	5	y = 0.0149x + 0.0456	0.9945
Zn(II)	0.01- 0.5	7	y = 0.2777x + 0.0156	0.9949
Pb(II)	0.005-0.05	5	y = 0.012x + 0.1215	0.9950

CHAPTER THREE
RESULTS AND DISCUSSION

3.1 Analysis of HMF in vinegar and wine samples

3.1.1 Performance characteristics of LLE method using TLC-scanner

In validation of LLE method for HMF in vinegar and wine samples using TLC-scanner, the spot of HMF of the standard HMF solution and of the LLE extract were compared. For this, external and internal standards were applied to HPTLC plate. The external standard spot on plate contains only standard analyte. However, the internal standard spot on plate contains sample and analyte together. The chromatographic behavior of HMF was given in Figure 3.1. During the repeated experiments, hR_F value of HMF was in the range of 38 to 41. The analytical parameters such as limit of detection limit (LOD), limit of quantitation limit (LOQ), within-day and between-day repeatability under HPTLC conditions were defined. LOD as 0.045 μg mL^{-1} and LOQ as 0.125 μg mL^{-1} were calculated using regression plot. Repeatability values, relative standard deviation percentage (RSD %), were found as 4.5% for within-day repeatability and below 8.6% for between-day repeatability for 5.0 ng μL^{-1} spiked vinegar sample by five parallel determinations.

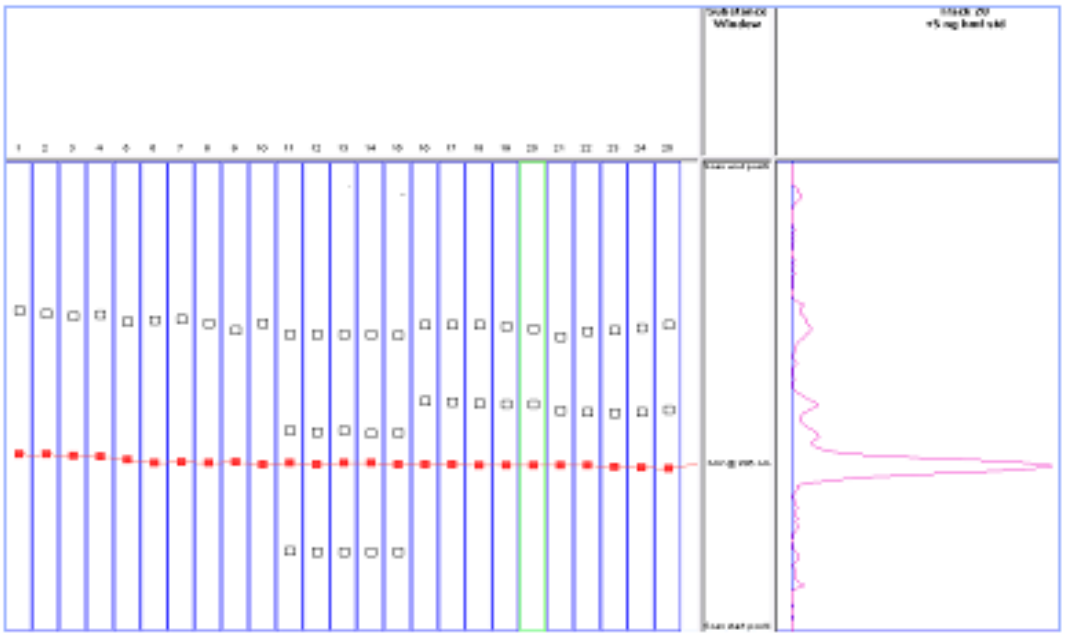

Figure 3.1 The HPTLC plate of HMF as external standards on 1-10 lines, apple vinegar sample on 11 and 12 lines, grape vinegar samples on 16, 17, 21 and 22 lines, internal standards on 13-15, 18-20 and 23-25 lines and the absorption spectrum of internal standard at 20th line.

3.1.2 Amount of HMF in Vinegar and Fruit Wine Samples

In the determination of HMF by TLC-scanner, the liquid-liquid extracts of vinegar and fruit wine samples were directly used. The clear spot was obtained on silica gel HPTLC plate, so no-clean up procedures for removing any interfering substances were needed. HMF was analyzed in the sample extracts using external calibration by three parallel determinations. In recovery experiments, standard HMF as 2 and 5 µg mL^{-1} was spiked to liquid-liquid sample extracts and it was found in the range of 95 to 112%. In Table 3.1, the results of HMF analysis of grape, apple, and balsamic vinegar and grape, apple, sour cherry, bilberry, peach, pomegranate, and melon wine samples were tabulated. In all studied vinegar and fruit wine samples, HMF was seen. The highest HMF was observed at balsamic vinegar because of long fermentation process during its production from the concentrated must of grapes (Theobald, Muller, & Anklam, 1998). The highest HMF in pomengranate wine and the lowest HMF in lemon and bilberry wine were indicated within the studied fruit wine samples. Storage conditions used for fruit juices can lead to increase of HMF level (Lo Coco, Novelli, Valentin, & Ceccon, 1997; Bortolotti, 1993). Especially

storage, use of concentrated must, heat treatment and time of ripening might cause an increase in the amount of HMF.

Table 3.1 Amount of HMF in fruit wines and vinegars (n = 3)

Sample	Added (µg mL^{-1})	Founded (µg mL^{-1})	R (%)
	-	1.6 ± 0.4	-
Grape wine	2	3.7 ± 0.1	104
	5	7.4 ± 0.0	113
	-	1.8 ± 0.2	-
Apple wine	2	3.9 ± 0.6	104
	5	7.8 ± 0.3	115
	-	5.3 ± 0.4	-
Sour cherry wine	2	7.2 ± 0.1	98
	5	9.8 ± 0.1	95
	-	0.5 ± 0.3	-
Bilberry wine	2	2.9 ± 0.2	116
	5	6.7 ± 0.0	121
	-	3.8 ± 0.4	-
Peach wine	2	5.7 ± 0.0	98
	5	8.4 ± 0.4	96
	-	8.6 ± 0.6	-
Pomegrante wine*	2	10.3 ± 0.5	97
	5	13.2 ± 0.4	97
	-	0.6 ± 0.2	-
Melon wine	2	2.0 ± 0.1	77
	5	4.5 ± 0.0	81
	-	17.8 ± 0.3	-
Grape vinegar 1*	2	20.0 ± 0.2	101
	5	23.8 ± 0.2	104

* The dilution factor was 4 to 10

Table 3.1 Continue

Sample	Added (µg mL^{-1})	Founded (µg mL^{-1})	R (%)
	-	1.0 ± 0.0	-
Grape vinegar 2	2	3.1 ± 0.3	104
	5	6.8 ± 0.1	113
	-	1.3 ± 0.2	-
Grape vinegar 3	2	3.3 ± 0.7	100
	5	7.1 ± 0.1	112
	-	3.4 ± 0.5	-
Apple vinegar	2	5.2 ± 0.1	98
	5	8.2 ± 0.5	98
	-	33.1 ± 0.1	-
Balsamic vinegar*	2	34.1 ± 0.3	97
	5	44.5 ± 0.2	106

* The dilution factor was 4 to 10

3.2 Analysis of ZEN in beer samples

3.2.1 Optimization of DLLME using TLC-scanner

The optimization of DLLME include the investigating of parameters: type and volume of extraction solvent, type and volume of dispersive solvent, extraction time, and salting effect.

In the optimization procedure, extraction recovery (ER) and enrichment factor (EF) were calculated according to given equations below:

$$EF = (C_{sed}/C_0)$$

$$ER\ \% = EF \times (V_{sed}/V_{aq}) \times 100$$

where,

C_{sed} concentration of analyte in sedimented phase, C_0: initial concentration of analyte in aqueous sample, V_{sed}: volume of sedimented phase and V_0: volume of aqueous phase. The analyte concentration in the sedimented phase was calculated using the calibration curve in the range of 4 to 20 ng ZEN μL^{-1} in acetonitrile.

3.2.1.1 Type and Volume of Extraction Solvent

The extraction solvent should satisfy the some criteria: immiscibility with water, higher density than that of water and good solubility for analyte. In this extraction method, $C_2H_4Cl_2$ (1.245 g mL^{-1}), C_2Cl_4 (1.622 g mL^{-1}), C_2HCl_3 (1.460 g mL^{-1}), CH_2Cl_2 (1.326 g mL^{-1}), C_6H_5Cl (1.107 g mL^{-1}), CCl_4 (1.594 g mL^{-1} and $CHCl_3$ (1.498 g mL^{-1}) were chosen and compared in the extraction of ZEN in beer samples. A series of synthetic beer sample solution spiked with 0.2 ng μL^{-1} at pH 3.8-4.8 with 0.1 M HCl were studied by using 1 mL of ACN containing 50 µL of extraction solvents. While using C_2Cl_4, C_6H_5Cl and CCl_4 the dense cloudy state was occurred but then white sedimented precipitate was accumulated at the bottom of the test tube. Dichloro ethane formed cloudy state but the volume of sedimented phase was smaller than expected. It was probably due to its higher solubility in water. By CH_2Cl_2 no sedimented organic phase was occurred. The chloroform and C_2HCl_3 formed fine particles in cloudy state and after centrifuging, the organic solvent was separated from sample solution, finally settled in the bottom of test tube. In order to control the highest extractant recovery, both of solvents at different volumes (25, 50, 75, 100 µL) in 1 mL extraction mixture were tested. For 25 µL of these extractant solvents no cloudy state and no sedimented phase on the bottom of the test tube was observed. But while increasing the volume of extractant from 50 µL to 100 µL, the volume of sedimented phase was changed from 31 µL to 98 µL in $CHCl_3$ and 29 µL to 92 µL in C_2HCl_3. The average extraction recovery of $CHCl_3$ and C_2HCl_3 were compared by changing their volume from 50 to 100 µL in 1.0 mL extraction mixture for three replicates. The results revealed that chloroform has the highest extraction recovery and better repeatability (62.1±3.4) for 75 µL in comparison with trichloroethylene.

3.2.1.2 Type and Volume of Dispersive Solvent

In DLLME, the miscibility of disperser solvent with the extraction solvent and the aqueous sample solution is the main point for the selection of disperser solvent. Amyl alcohol, 1-propanol, acetone, ACN and tetrahydrofuran (THF) were chosen for this purpose. A series of spiked synthetic beer sample solutions were tested using 1.0 mL of dispersive solvent containing 75 µL of $CHCl_3$. By amyl alcohol no cloudy solution and no sedimented phase at the bottom of the test tube were observed. By THF, the higher volume of sedimented phase was obtained. The reason might be the high miscibility of THF in chloroform. The volume of sedimented phase was low when acetone and 1-propanol were used as disperser. ACN was the most effective dispersive solvent which caused formation of stable cloudy solution and sedimented phase. So, further experiments were focused on optimizing of the volume of ACN. For this, different volumes of extraction mixture (0.25, 0.50, 0.75, 1.00 mL) containing 75 µL of chloroform were prepared. According to the results shown in Figure 3.2, the extraction recovery was high at low volume of ACN but decreased with increasing dispersive solvent. At high volume of ACN, the solubility of ZEN in aqueous phase was increased, leading to decrease in extraction efficiency because of a decrease in distribution ratio. Based on these results, further studies were followed with 0.175 mL of ACN.

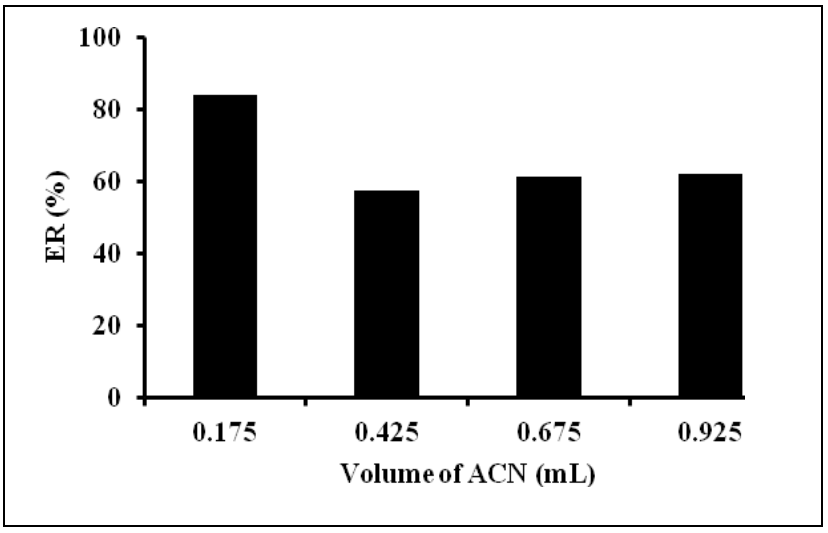

Figure 3.2 Effect of volume of ACN on extraction recovery. Extraction conditions: volume of synthetic beer solution, 5 mL; volume of chloroform, 75 µL; room temperature

3.2.1.3 Extraction Time

Extraction time is referred as the time passing from injection the extraction mixture until centrifuge. The effect of extraction time was examined up to 30 min. As given in Figure 3.3, the extraction recovery shows fluctuations within 30 min. But, it is clearly seen that the reasonable extraction was possible within a few seconds. So, it could be considered the transferring of ZEN to organic phase was fast.

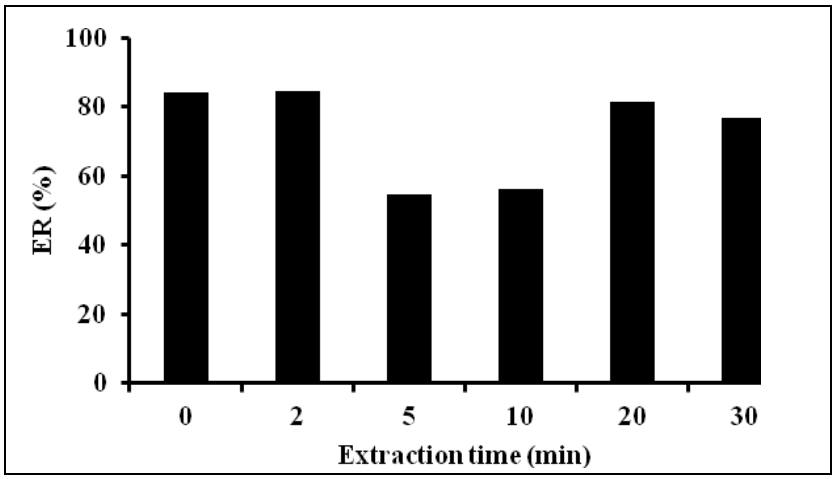

Figure 3.3 Effect of extraction time on extraction recovery of ZEN. Extraction conditions: volume of synthetic beer solution, 5 mL; volume of chloroform, 75 µL; volume ACN, 0.175 mL; room temperature.

3.2.1.4 Salting Effect

To control the ionic strength effect on the efficiency of the extraction, several KCl (0–10 %, w/v) aqueous solutions were added to spiked synthetic beer sample solution. As demonstrated in Figure 3.4, the sedimented phase volume was changed from 75 to 55 µL by increasing the ionic strength from 0% to 10%. It could be concluded that the presence of salt has no remarkable effect on the volume of sedimented phase. So, no salt was used in the determination of ZEN in beer samples.

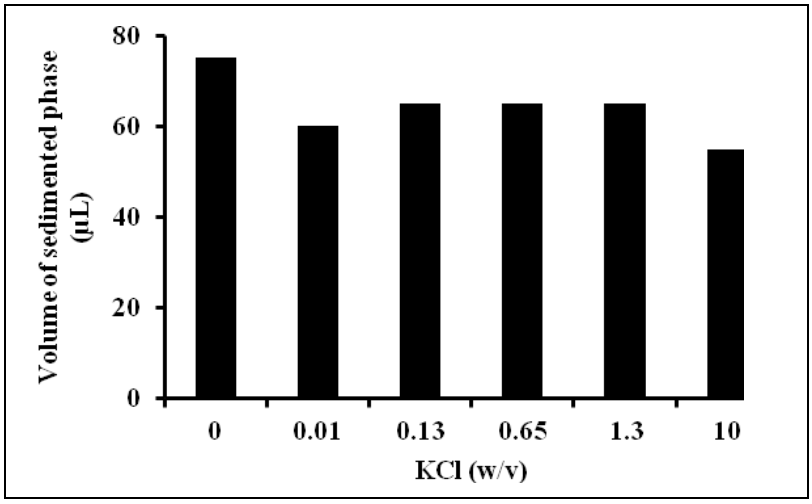

Figure 3.4 Effect of salt on the sedimented phase volume. Extraction conditions: volume of synthetic beer solution, 5 mL; volume of chloroform, 75 µL; volume ACN, 0.175 mL.

3.2.2 Performance Characteristics of DLLME Method Using HPLC

The performance characteristics in terms of linear dynamic range, range limit of detection and quantification and precision were checked to show the suitability of the proposed DLLME method for determination of ZEN in beer samples. The matrix-matched calibration curve using beer samples spiked with the standard ZEN solutions ranging from 0.4 to 120 pg μL^{-1}. Each concentration level was prepared and injected three times. A blank beer sample was also processed but no ZEN was detected. The slope and intercept values obtained by matrix-calibration curve were 0.795 and 0.187 with a regression coefficient (R^2) of 0.9979. The limit of detection and the limit of quantification were 0.12 µg L^{-1} (3x S/N) and 0.40 µg L^{-1} (10 x S/N),

respectively. The intra-day and the inter-day precision of the DLLME method as RSD was 4.83% and 6.63%, respectively at 3 ng mL^{-1}spiked level.

The enrichment factor for the DLLME method for ZEN was found as 43.3; and the extraction recovery of the proposed DLLME method was 83.0% for 0.2 ng μL^{-1} of ZEN concentration.

3.2.3 Amount of ZEN in Beer Samples

The DLLME-HPLC methodology was successfully applied to different brands of thirteen beer samples. The amount of ZEN in beer samples were tabulated in Table 3.2. The ZEN level in beer samples was ranged from 0.46 to 19.50 $\mu g\ L^{-1}$. The ZEN concentration in studied beer samples show similarity with European Beers (Kuzdralinski, Solarska, & Muszyriska, 2013). In order to show the validity of the proposed DLLME method, each beer sample was spiked with the following concentrations as 10 and 20 μg ZEN L^{-1}. As can be seen in Table 3.2, the ranges of recoveries were given limits by European Commisison. The acceptable limit range for recovery is established as from 70 to 110% (EU, 2006). Typical HPLC chromatograms of a standard ZEN and a spiked and unspiked beer sample were given in Figure 3.5.

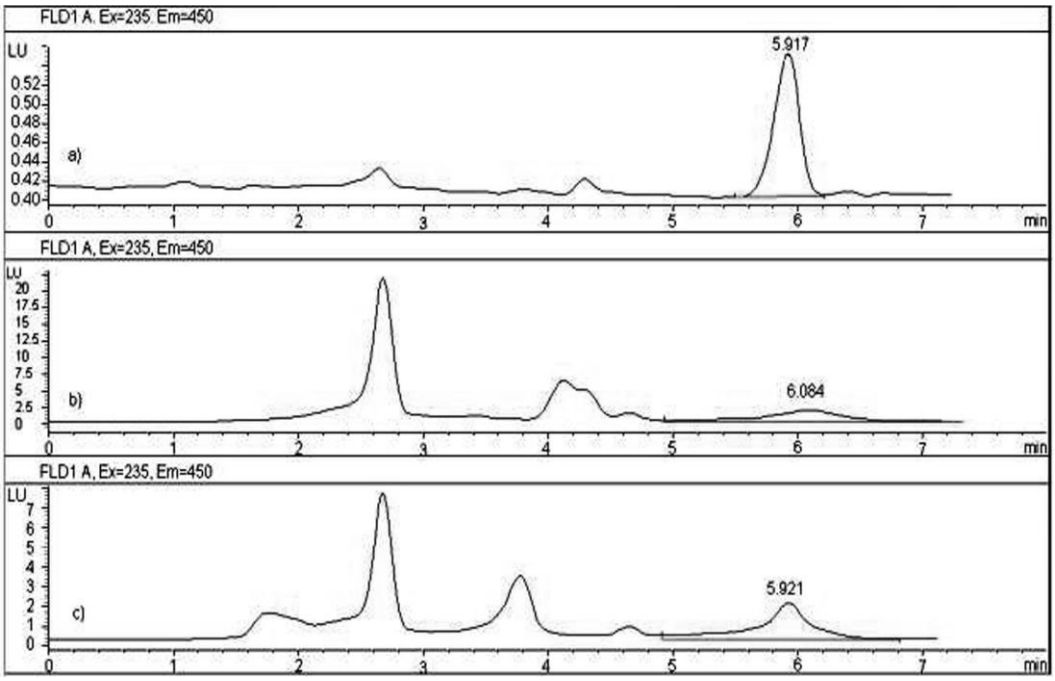

Figure 3.5 HPLC chromatogram of a) standard solution containing 5 pg ZEN μL^{-1}, b) an unspiked beer sample containing 0.60 pg ZEN μL^{-1}, c) a same beer sample spiked with 20 pg ZEN μL^{-1}.

Table 3.2 ZEN levels in beer samples analysed by the proposed DLLME-HPLC method

Sample	Added (ng mL^{-1})	Founded (ng mL^{-1})	R (%)
	-	0.46 ± 0.03	-
Beer 1	10	9.34 ± 0.4	89
	20	15.63 ± 0.12	76
	-	0.58 ± 0.04	-
Beer 2	10	9.16 ± 0.72	87
	20	17.32 ± 0.45	84
	-	ND	-
Beer 3	10	8.17 ± 0.21	82
	20	15.56 ± 0.34	78
	-	10.92 ± 0.54	-
Beer 4	10	14.85 ± 0.48	71
	20	23.09 ± 2.79	75

ND: not detected

Table 3.2 Continue

Sample	Added (ng mL^{-1})	Founded (ng mL^{-1})	R (%)
	-	19.5 ± 0.46	-
Beer 5	10	28.99 ± 0.83	98
	20	33.78 ± 0.81	86
	-	ND	-
Beer 6	10	8.69 ± 0.58	87
	20	18.83 ± 0.87	94
	-	0.59 ± 0.03	-
Beer 7	10	9.98 ± 0.61	94
	20	20.11 ± 0.87	98
	-	0.63 ± 0.02	-
Beer 8	10	9.98 ± 0.32	94
	20	18.15 ± 0.32	88
	-	14.81 ± 0.68	-
Beer 9	10	24.34 ± 0.71	98
	20	37.57 ± 1.32	108
	-	0.63 ± 0.04	-
Beer 10	10	10.72 ± 0.27	101
	20	21.17 ± 0.14	103
	-	0.56 ± 0.07	-
Beer 11	10	8.9 ± 1.06	84
	20	21.59 ± 3.90	105
	-	7.44 ± 0.52	-
Beer 12	10	16.30 ± 0.83	94
	20	22.50 ± 0.66	82
	-	15.59 ± 0.27	-
Beer 13	10	25.43 ± 0.33	99
	20	34.98 ± 0.47	98

ND: not detected

3.3 Analysis of OTA in wine samples

3.3.1 Optimization of DLLME using TLC-scanner

3.3.1.1 Type and Volume of Extraction Solvent

Under DLLME principles, $C_2H_4Cl_2$, C_2Cl_4, C_2HCl_3, CH_2Cl_2, C_6H_5Cl, CCl_4 and $CHCl_3$ were performed to determine the effect of solvents on extraction efficiency. Two phases were succeeded with all tested solvent except C_6H_5Cl and CH_2Cl_2. The reason might be the higher miscibility of these solvents with water. To check the extraction recovery, the synthetic wine sample solution spiked with 0.2 ng μL^{-1} at pH 3.5 with 4 were mixed with 1 mL of acetone containing 100 µL of extractant solvent. The obtained sedimented phase volume was changed from 40 to 100 µL with respect to the extraction solvent used. In Table 3.3, the extraction efficiencies of solvents were given. As a result, chloroform was selected as extractant for further experiments because of its highest extraction recovery.

Table 3.3 Extraction efficiencies of the studied extractants

Extractant	**Extraction recovery (ER %)**
Chloroform, $CHCl_3$	60.0 ± 1.2
1,2-dichloroethane, $C_2H_4Cl_2$	36.0 ± 0.0
Trichloroethylene, C_2HCl_3	52.9 ± 5.4
Tetrachloroethylene, C_2Cl_4	18.5 ± 0.4
Carbon tetrachloride, CCl_4	38.5 ± 0.9

To determine the least extractant volume enough, extraction mixtures involving different volumes of $CHCl_3$ were prepared as 1 mL. The effect of volume of $CHCl_3$ on volume of sedimented phase was shown in Figure 3.6. When the volume of $CHCl_3$ was increased up to 200 µL, the sedimented phase volume was increased to 195 µL. The extraction recovery linearly increased by increasing volume of chloroform to 100 µL, but later diminished. Similar behavior was seen in enrichment

factor, which was decreased from 45.4 to 9.7. As a result, the high extraction recovery (60.0%) and the high enrichment factor (30.8) for chloroform was performed with a volume of 100 μL. So, the volume of $CHCl_3$ as 100 μL was preferred for further studies.

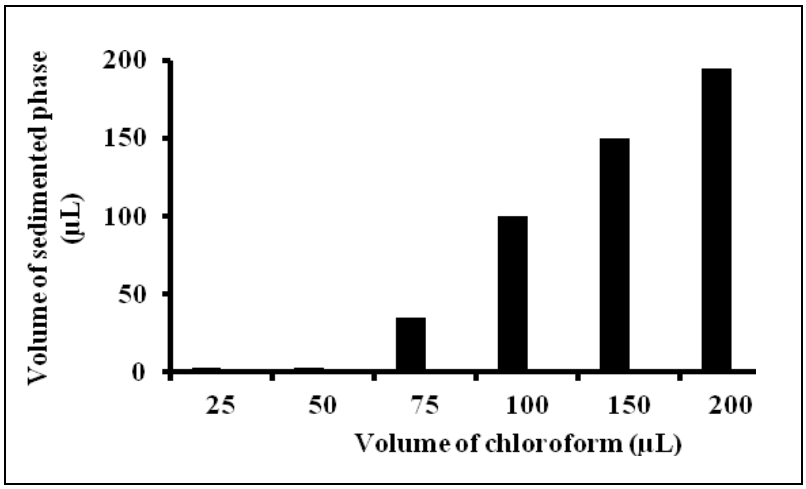

Figure 3.6 Effect of volume of chloroform on the volume sedimented phase. Extraction conditions: volume of wine sample solution, 5 mL; disperser solvent, acetone; volume of extraction mixture, 1 mL, room temperature

3.3.1.2 Type and Volume of Dispersive Solvent

Acetone, ACN, 1,4-dioxane, ethylene glycol, dimethyl sulphoxide (DMSO) and THF were tested as suitable dispersive solvent in this study. By THF, the sedimented phase volume was obtained twice of extractant volume. The reason might be the high molecular interaction of THF with chloroform. On the other hand, the sedimented phase was yielded less than 100 μL by using ethylene glycol. Solid particles were observed when DMSO was used. The obtained extraction recoveries were given in Table 3.4. As can be seen from Table 3.4, ACN was chosen as dispersive solvent for further studies.

Table 3.4 Extraction efficiencies of the studied dispersive solvents

Dispersive solvent	**ER (%)**
Acetone, C_3H_6O	60.0 ± 5.5
Acetonitrile, C_2H_3N	63.9 ± 2.6
1,4-dioxane, $C_4H_8O_2$	60.0 ± 0.4

The volume of dispersive solvent, ACN, was checked to get highest extraction recovery. The change in volume of ACN from 0.15 to 1.10 mL was lead to the change in sedimented phase from 65 to 100 µL. The recovery of extraction was slowly rose up by increasing the ACN volume up to 0.90 mL (Figure 3.7). For further studies, 0.90 mL of ACN was used.

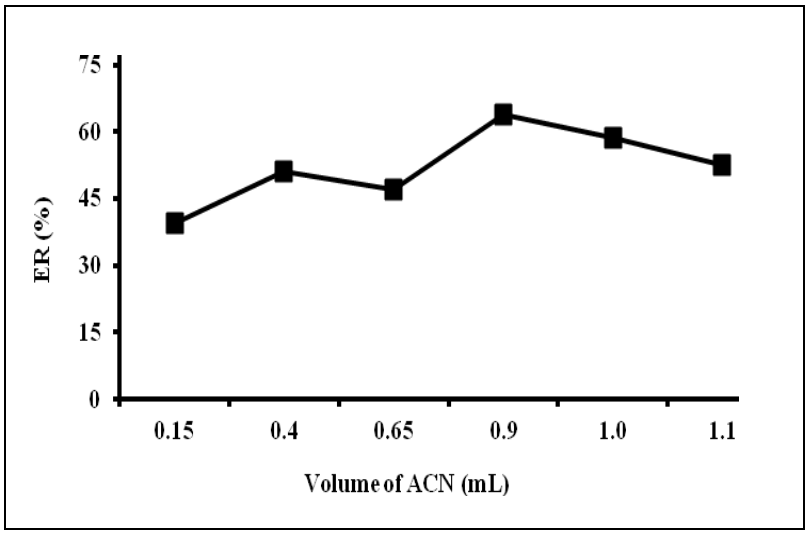

Figure 3.7 Effect of volume of ACN on the extraction recovery of OTA. Extraction conditions: volume of wine sample solution, 5 mL; volume of chloroform, 100 µL; sedimented phase volume range, 65-100 µL, room temperature.

3.3.1.3 Extraction Time

In DLLME for determination of OTA in wine samples, the time of extraction was studied for 30 min under optimized experimental conditions. The effect of extraction time versus the extraction recovery and enrichment factor was demonstrated in

Figure 3.8 and 3.9. Considering the obtained results, it could be stated that the required time to reach equilibrium was very short.

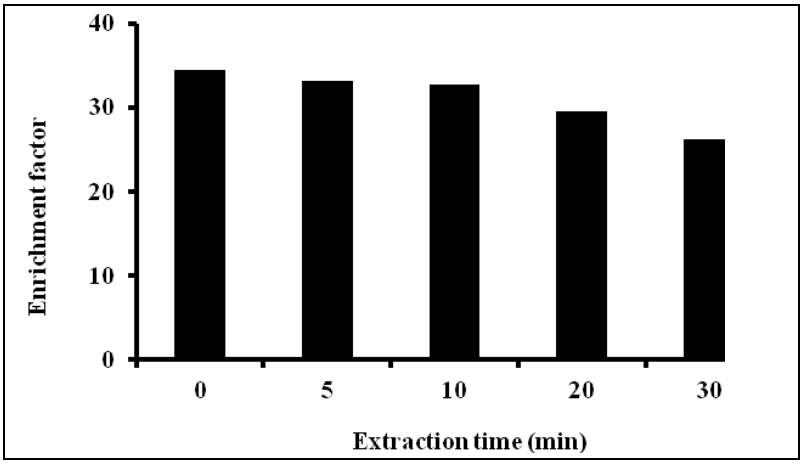

Figure 3.8 Effect of extraction time on the enrichment factor of OTA. Extraction conditions: volume of wine sample solution, 5 mL; volume of chloroform, 100 µL; volume of ACN, 900 µL, room temperature

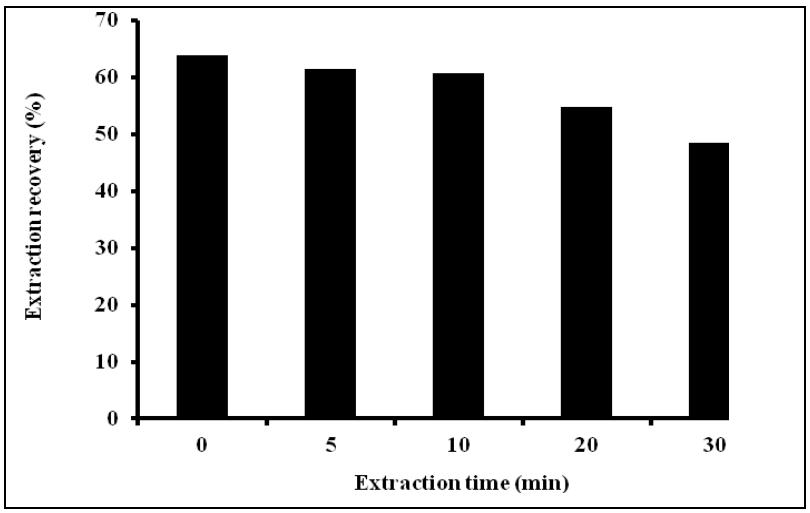

Figure 3.9 Effect of extraction time on the extraction recovery of OTA

3.3.1.4 Salting Effect

Several experiments were performed by adding KCl solution from 0 to 10%, w/v. The peak area of OTA was not changed so much by increasing salt amount as shown in Figure 3.10. This could be considered as the partitioning of OTA into organic phase was not effected from the presence of salt.

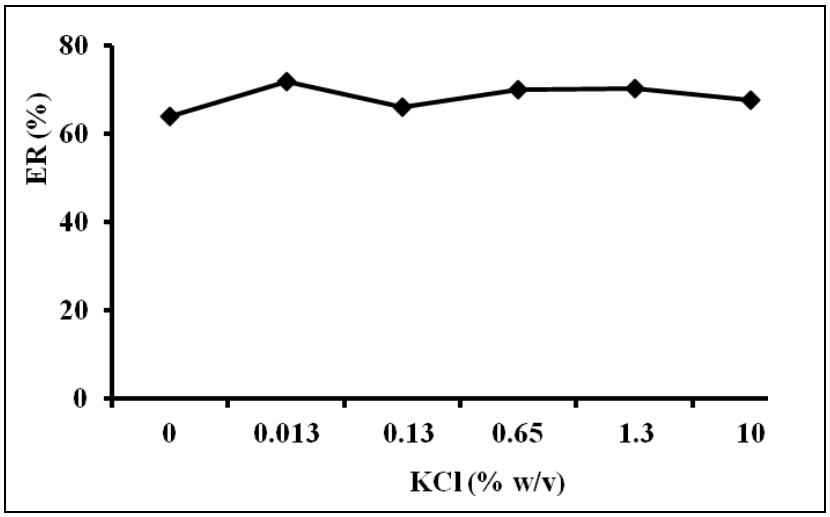

Figure 3.10 Effect of salt addition on the extraction recovery of OTA

3.3.2 Performance characteristics of DLLME method using HPLC

The analytical performance of the proposed DLLME method was exerted using matrix-matched standards. The dynamic linearity, repeatability, the detection and quantification limits, the enrichment factor and the extraction recovery were all studied under the optimized experimental conditions.

The matrix-matched calibration curve of wine samples fortified at five different concentration levels (0.1 to 5 pg OTA μL^{-1}) was linear with regression coefficient (R^2) of 0.9968 Each concentration level was prepared and injected three times. A blank wine sample was also processed but no OTA was detected. The precision of the proposed DLLME method in terms of RSD was < 4.7% for repeatability and 5.3% for reproducibility at 2.5 μg OTA L^{-1} for five replicate runs. The limit of detection as 3 x (S/N) was 0.009 μg L^{-1} and the limit of quantification as 10 x (S/N) was 0.027 μg L^{-1}. The enrichment factor and extraction recovery as percentage of the proposed DLLME method were 34.5 and 63.9%, respectively, at 0.2 ng OTA μL^{-1}.

3.3.3 Amount of OTA in Wine Samples

The determinations of OTA in all wine samples were first studied with TLC-scanner. As illustrated in Figure 3.11, the OTA level of naked wine samples was not

observed. Then, the amounts of OTA in samples were performed by HPLC because of its much better LOQ. The obtained results were summarized in Table 3.5. The OTA level in studied wine samples was below the permission limit of European Union. In order to check the accuracy of the proposed DLLME, 0.10 and 0.50 µg OTA L^{-1} standard solutions were added to wine samples. In Figure 3.12, the chromatograms of fortified and unfortified wine samples were shown. The wine samples were free from analytical matrices as seen from the HPLC chromatogram of naked wine sample. The obtained recovery values given in Table 3.5 indicated that the measured concentrations were in reasonable with agreement.

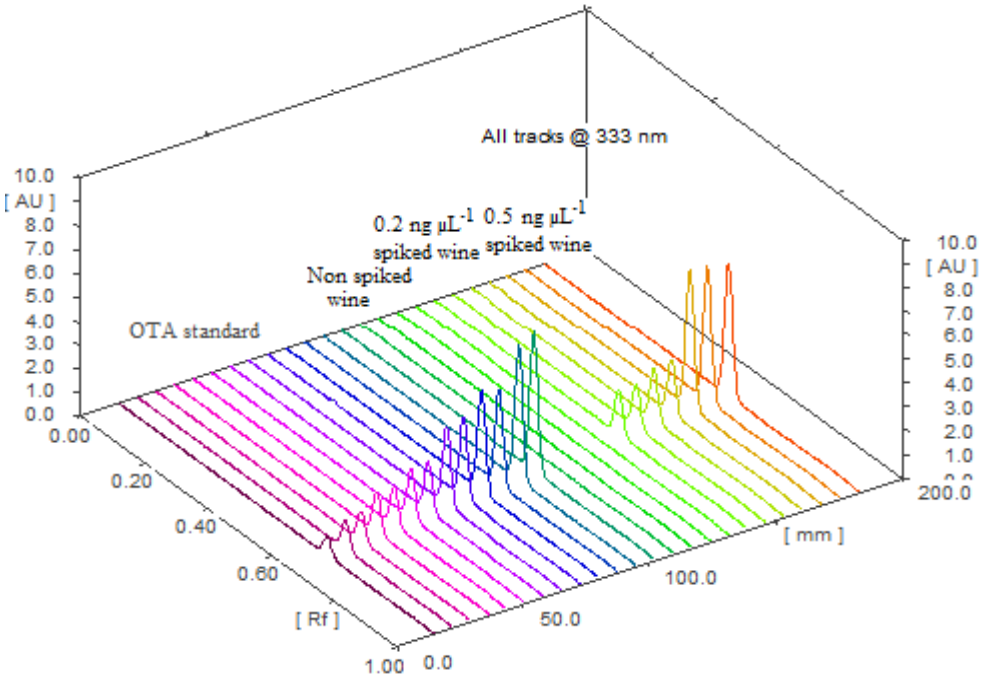

Figure 3.11 Densitograms of OTA standard and spiked and unspiked wine samples on three dimensional spectra.

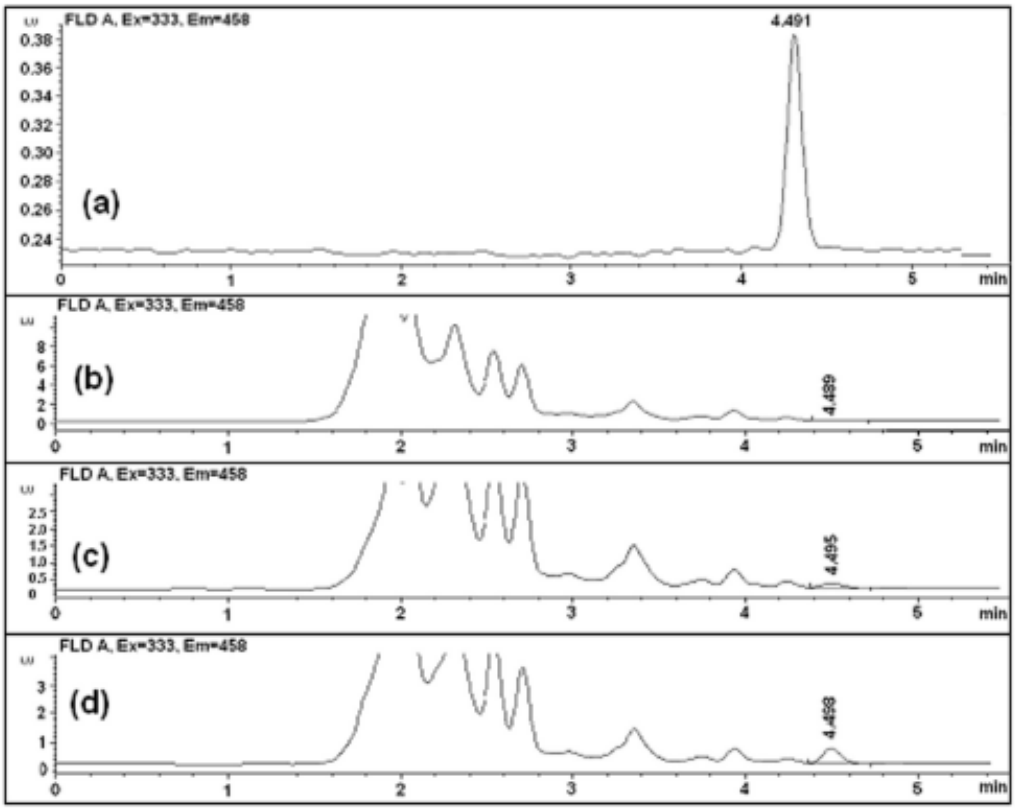

Figure 3.12 HPLC chromatogram of a) a standard OTA at 2.5 pg μL^{-1}, b) unspiked wine sample containing app. 0.03 pg OTA μL^{-1} after DLLME method, c) same wine sample spiked at a conc. 0.1 pg μL^{-1} after DLLME method and d) 0.5 pg μL^{-1}.

Table 3.5 OTA levels in wine samples analysed by the proposed DLLME-HPLC method

Sample	**Added (ng mL^{-1})**	**Founded (ng mL^{-1})**	**R (%)**
	-	0.08 ± 0.01	-
Wine 8	0.1	0.16 ± 0.02	86
	0.5	0.43 ± 0.02	74
	-	0.10 ± 0.06	-
Wine 9	0.1	0.12 ± 0.00	63
	0.5	0.50 ± 0.03	84
	-	0.05 ± 0.01	-
Wine 10	0.1	0.13 ± 0.00	85
	0.5	0.64 ± 0.01	103
	-	0.08 ± 0.00	-
Wine 11	0.1	0.14 ± 0.03	94
	0.5	0.58 ± 0.02	100

Table 3.5 Continue

Sample	Added (ng mL^{-1})	Found (ng mL^{-1})	R (%)
	-	0.19 ± 0.04	-
Wine 12	0.1	$0.29_5 \pm 0.01$	101
	0.5	0.44 ± 0.02	64
	-	0.10 ± 0.01	-
Wine 13	0.1	0.18 ± 0.01	101
	0.5	0.66 ± 0.01	97
	-	$003 \pm 0.00_5$	-
Wine 14	0.1	0.11 ± 0.01	84
	0.5	$0.49 \pm 0.00_5$	87
	-	$0.03 \pm 0.00_5$	-
Wine 15	0.1	0.12 ± 0.05	92
	0.5	$0.48 \pm 0.03_5$	91
	-	0.04 ± 0.01	-
Wine 16	0.1	0.13 ± 0.02	93
	0.5	0.59 ± 0.02	109
	-	0.10 ± 0.01	-
Wine 17	0.1	$0.13 \pm 0.00_5$	91
	0.5	0.51 ± 0.01	94
	-	$0.07 \pm 0.00_5$	-
Wine 18	0.1	0.16 ± 0.03	94
	0.5	0.58 ± 0.00	102
	-	0.07 ± 0.00	-
Wine 19	0.1	0.12 ± 0.00	73
	0.5	0.43 ± 0.03	76
	-	$0.04 \pm 0.00_3$	-
Wine 20	0.1	0.13 ± 0.01	93
	0.5	0.52 ± 0.02	96
	-	0.05 ± 0.00	-
Wine 21	0.1	0.12 ± 0.00	79
	0.5	0.51 ± 0.04	91

Table 3.5 Continue

Sample	Added (ng mL^{-1})	Found (ng mL^{-1})	R (%)
	-	0.04 ± 0.00	-
Wine 22	0.1	0.12 ± 0.01	85
	0.5	0.50 ± 0.00	80
	-	0.09 ± 0.03	-
Wine 23	0.1	0.19 ± 0.03	100
	0.5	0.57 ± 0.02	97

3.4 Analysis of TEA and CPA in tomato juice samples

3.4.1 Optimization of CPE

3.4.1.1 Effect of Surfactants

The interaction of an analyte and surfactant can be differently based on nature of the analyte and the surfactant. A polar molecule can bind with surfactant forming micelles by electrostatic interactions but non-polar molecule is partially solubilized or partititoning into hydrophobic micelle medium. Non-ionic and anionic surfactants (Triton X-100, Triton X-114, Brij 35, cetyl trimethylammonium bromide (CTAB), Genapol X-080, Tween 20, Tween 80 and sodium dodeceyl sulphate (SDS) were tested for preconcentration of TEA and CPA. Only, by Triton X-114 cloudy phenomena was occured and surfactant rich phase was observed.

3.4.1.2 Effect of pH

The ionizable organic molecules represent high extraction at pH values of their nonionized form, so they can easily partitioning to hydrophobic micelle medium. To optimize pH for extraction, phosphate buffer solutions as pH 2, pH 3.5 and pH 6 were prepared. As shown in Figure 3.13, the best optimum pH was chosen as 2. Above this value, a decrease was observed due to probably a deprotonation of CPA and TEA (pK_a of CPA and TEA is 2.97 and 3.5, respectively).

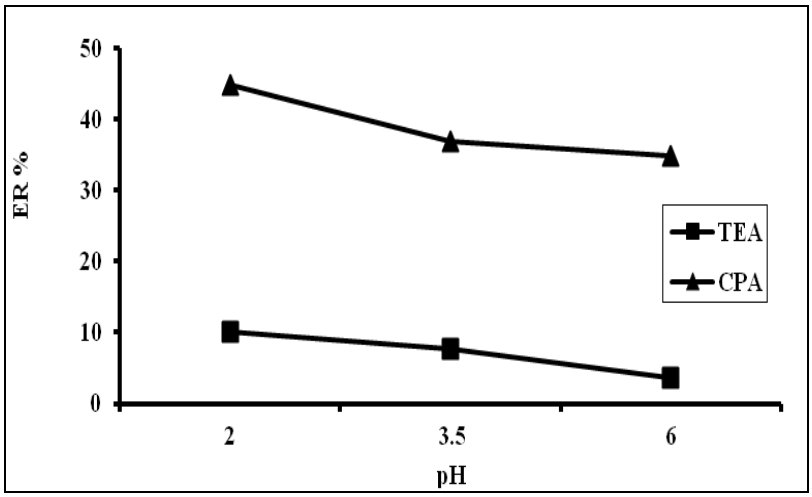

Figure 3.13 Effect of pH on CPA and TEA. Extraction conditions: synthetic sample solution, 2 mL; volume of Triton X-114(1%w/v), 2mL; T_{eq} 60 °C

3.4.1.3 Effect of Surfactant Concentration

The influence of Triton X-114 concentration on extraction of CPA and TEA was checked. The concentrations of Triton X-114 solutions varying from 0.2 to 10% (w/v) were tested. The extraction recoveries of both of them were shown in Figure 3.14. Their recoveries were increased up to 4% (w/v) and then dramatically decreased. Hence, 4% (w/v) Triton X-114 was used for further studies. This surfactant concentration was above critical micelle concentration ($0.35x10^{-3}$ M, 25 °C).

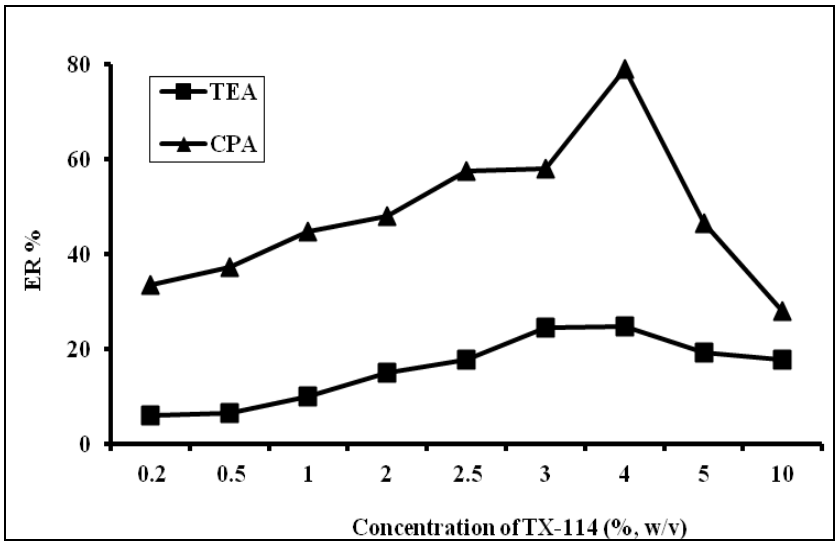

Figure 3.14 Effect of Triton X-114 on CPA and TEA. Extraction conditions: synthetic sample solution, 2 mL; pH, 2; T_{eq} 60 °C

3.4.1.4 Effect of Salting

The other optimizing parameter was salting effect. It assists the solubilization or partitioning to solute in organic phase. For this purpose, KNO_3, $NaNO_3$, NaCl, KCl, Na_2SO_4 and K_2SO_4 at a concentration of 1% (w/v) were studied. Also, the same CPE procedure was repeated without adding any salt. As seen in Figure 3.15, the best result was obtained by KNO_3, although getting comparatively high extraction recovery without salt. Later on, the amount of KNO_3 was performed at six different concentrations. For both of them, the maximum extraction recovery at 1% then the sharp decrease was observed (Figure 3.16). Nevertheless, the recovery value of TEA was around 40%. Therefore, the optimum salt and its concentration were selected as 1 % (w/v) KNO_3.

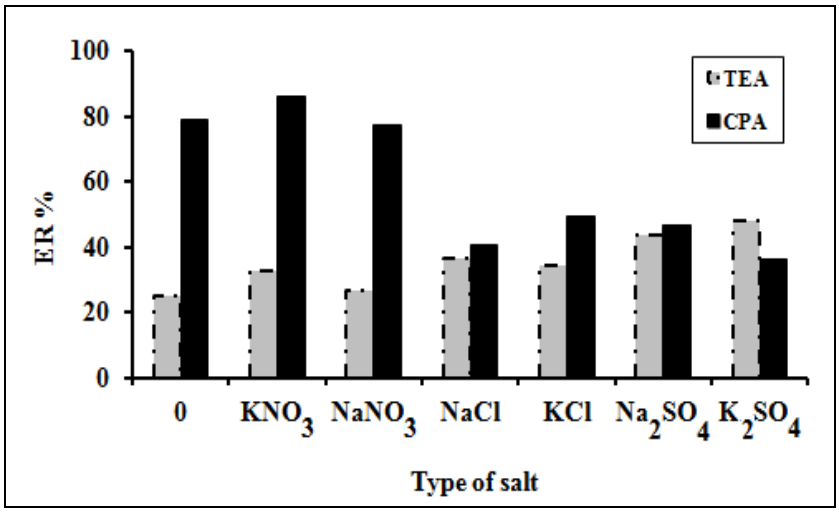

Figure 3.15 Effect of nature of salts on CPE and TEA extraction. Extraction conditions: synthetic sample solution, 2 mL; pH 2; volume of 4%w/v Triton X-114, 2 mL; volume of salt solution (1%,w/v, 2 mL; T_{eq} 60 °C

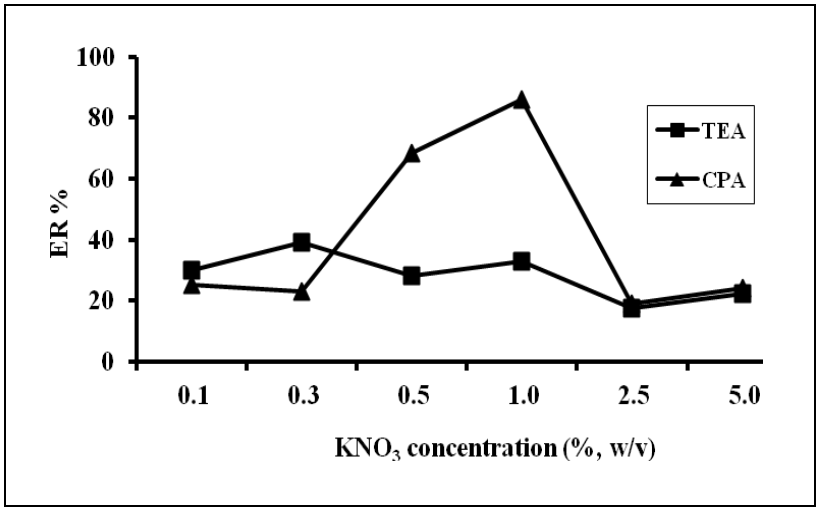

Figure 3.16 Effect of KNO_3 on CPE and TEA extraction. Extraction conditions: synthetic sample solution, 2 mL; pH 2; volume of 4%w/v Triton X-114, 2 mL; T_{eq} 60 °C

3.4.1.5 Effect of Temperature

In order to provide efficient cloud formation the equilibrium temperature was checked. Cloud point temperature is very important to form a cloudy state. The effect of temperature from 20 to 80 °C was studied (Figure 3.17). Cloudy state was not observed at 20 °C.

By increasing equilibrium temperature the extraction recovery was increased up to 50 °C and then decreased for CPA. However, the extraction yield of TEA was not affected from heat. The optimum lowest possible temperature was preferred as 50 °C for CPA and TEA.

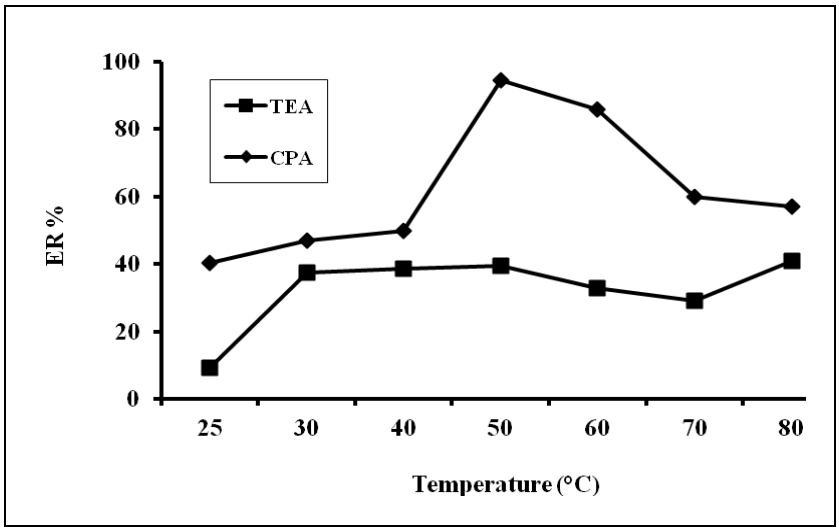

Figure 3.17 Effect of equilibrium temperature on CPA and TEA extraction. Extraction conditions: synthetic sample solution, 2 mL; pH 2; volume of 4%w/v Triton X-114, 2 mL; volume of 1%w/v KNO_3, 2 mL

3.4.1.6 Effect of Equilibration Time

For completeness of reaction and adequate separation of phases, the possible shortest equilibration time was optimized. For this, the equilibration time in the range of 5-60 min was studied. For CPA, the equilibration time was not effective up to 30 min., but the recovery for TEA was increased up to 30 min and then decreased. As a result, the optimal equilibration time as 30 min was selected.

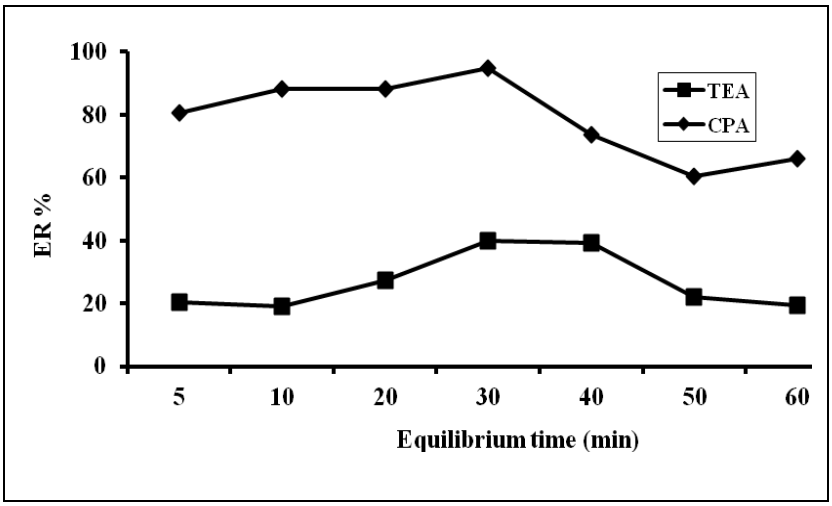

Figure 3.18 Effect of equilibration time on CPA and TEA extraction. Extraction conditions: synthetic sample solution, 2 mL; pH 2; volume of 4%w/v Triton X-114, 2 mL; volume of 1%w/v KNO_3, 2 mL; T_{eq} 50 ^{0}C

3.4.2 Performance Characteristics of CPE Method Using HPLC

Under the optimum conditions of CPE-HPLC method, the analytical performance of the proposed method was evaluted in terms of dynamic linearity, limit of detection and quantification, precision and the extraction recovery. The matrix-matched calibration curves of CPA and TEA after CPE method were linear in the range of 10 - 2000 pg μL^{-1} containing eigth concentrations with the correlation of cofficients (R^2) 0.9969 and 0.9956 for CPA and TEA. Each concentration was repeated three times. In a blank tomato juice sample no CPA and TEA was observed. LOD (3xS/N) and LOQ (10xS/N) were 0.6 pg μL^{-1} and 0.7 pg μL^{-1} for CPA and TEA and 7.6 pg μL^{-1}, 8 pg μL^{-1} for CPA and TEA, respectively. The precision of the proposed method as within-day and between-day were found as 1.8% and 2.5% for CPA, 11.6% and 12.1 for TEA, respectively at 0.1 ng μL^{-1} synthetic solution level for both of them. The extraction recovery of the proposed CPE method was 94.6% and 39.9% for 0.1 ng μL^{-1} of CPA and TEA concentration.

3.4.3 Amount of CPA and TEA in Tomato Juice Samples

By the proposed CPE-HPLC method, tomato juice samples were analyzed and the results were given in Table 3.6. The typical HPLC chromatograms of tomato juice sample blank and fortified tomato juice sample after the CPE method were given in Figure 3.19. To check the accuracy of the proposed method, tomato juice samples were fortified at two levels of standard CPA and TEA (50 and 100 pg μL^{-1}). The calculated recoveries of each level were also given in Table 3.6. They show high recoveries and they were acceptable limit according to current legislation in European commission (EU, 2006).

Table 3.6 TEA and CPA levels in tomato juice samples analysed by the proposed CPE-HPLC method

Sample	**CPA**			**TEA**	
	Added (ng mL^{-1})	**Founded (ng mL^{-1})**	**R %**	**Founded (ng mL^{-1})**	**R %**
	-	10.2 ± 0.02	-	7.30 ± 0.06	-
Tomato juice 1	50	58.4 ± 0.03	97	49.29 ± 0.32	86
	100	94.6 ± 0.05	86	88.65 ± 0.26	83
	-	4.7 ± 0.02	-	3.1 ± 0.01	-
Tomato juice 2	50	51.6 ± 0.05	94	49.4 ± 0.02	93
	100	102.4 ± 0.07	98	99.6 ± 0.04	97
	-	12.3 ± 0.05	-	13.8 ± 0.04	-
Tomato juice 3	50	60. 1 ± 0.06	96	54.8 ± 0.02	86
	100	94.7 ± 0.03	84	102.5 ± 0.08	90

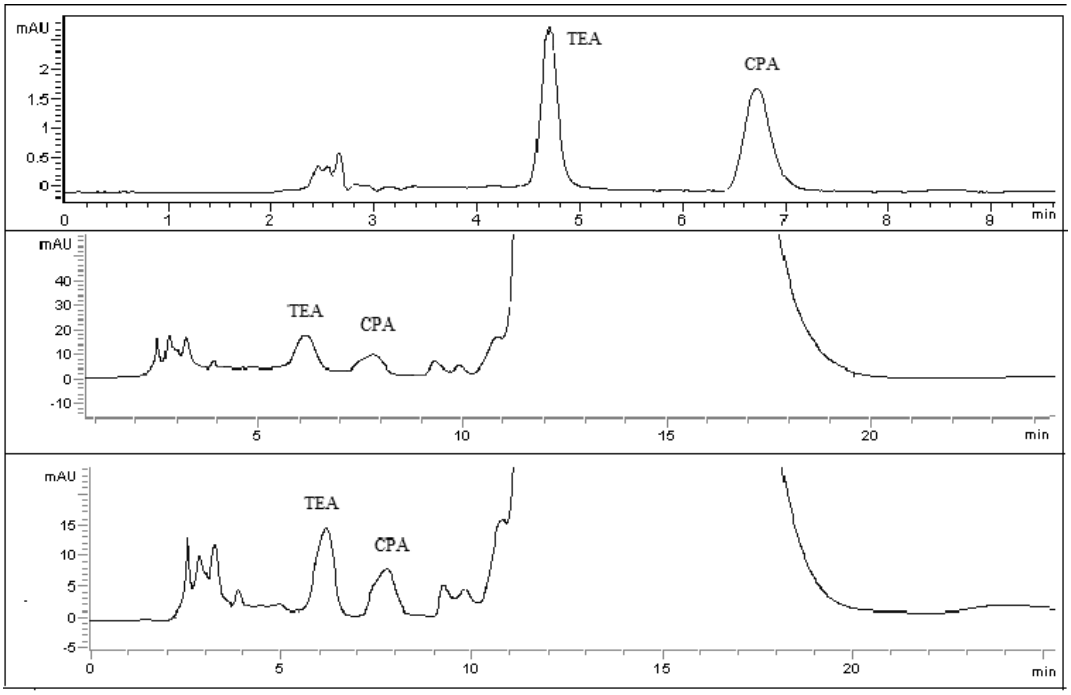

Figure 3.19 HPLC chromatogram of a) standard solution containing 0.05 ng CPA and TEA μL^{-1}, b) a tomato juice blank sample, c) fortified tomato juice sample spiked with 0.1 ng CPA and TEA μL^{-1}.

3.5 Amount of Some Major and Trace Elements in Wine and Beer Samples

The amounts of some major elements such as Na, K, Ca and Mg and trace elements such as Fe, Zn, Cu and Pb were analzed in all wine and beer samples. The levels of these elements were summarized in Table 3.7 and 3.8. The order of major elements in wine and beer samples was Na < Ca < Mg < K. The levels of trace elements were decreased in order Fe, Zn, Cu and Pb. In beer samples, the range area of elements are; K (424-244 $\mu g\ mL^{-1}$), Ca (54-24 $\mu g\ mL^{-1}$), Mg (49-30 $\mu g\ mL^{-1}$), Na (27-12 $\mu g\ mL^{-1}$), Fe (0.71-0.11 $\mu g\ mL^{-1}$), Zn (0.36-0.10 $\mu g\ mL^{-1}$), Pb (90-14 $ng\ mL^{-1}$) and Cu (37-14 $ng\ mL^{-1}$). In wine samples, the range area of elements are; K (1031-226 $\mu g\ mL^{-1}$), Mg (335-61 $\mu g\ mL^{-1}$), Ca (99-32 $\mu g\ mL^{-1}$), Na (88-10$\mu g\ mL^{-1}$), Fe (4.51-0.57 $\mu g\ mL^{-1}$), Zn (0.82-0.09 $\mu g\ mL^{-1}$), Pb (283-2 $ng\ mL^{-1}$) and Cu (277-54 $ng\ mL^{-1}$). The studied element contents in wine samples were found higher than in beer samples. The reason of this differentiation for each element in all wine samples could be their geographical origin and their production steps. For the accuracy of analysis,

wine and beer samples were spiked at two levels for each studied elements. The all recoveries were above 90% as tabulated in Table 3.9 and 3.10.

Table 3.7 Some major and trace element levels in wine samples

Sample	Ca (II) ($\mu g\ mL^{-1}$)	Mg (II) ($\mu g\ mL^{-1}$)	Na (I) ($\mu g\ mL^{-1}$)	K (I) ($\mu g\ mL^{-1}$)	Fe (II) ($\mu g\ mL^{-1}$)	Zn (II) ($\mu g\ mL^{-1}$)	Cu (II) ($ng\ mL^{-1}$)	Pb (II) ($ng\ mL^{-1}$)
Wine 8	99 ± 1	120 ± 2	50 ± 6	1031 ± 13	1.78 ± 0.05	0.48 ± 0.01	188 ± 2	185 ± 1
Wine 9	40 ± 1	71 ± 2	88 ± 11	576 ± 12	1.27 ± 0.05	0.34 ± 0.03	58 ± 6	42 ± 1
Wine 10	35 ± 6	80 ± 6	21 ± 1	868 ± 72	1.95 ± 0.00	0.81 ± 0.01	149 ± 6	159 ± 8
Wine 11	44 ± 1	71 ± 1	78 ± 8	397 ± 21	1.52 ± 0.24	0.62 ± 0.03	65 ± 1	41 ± 2
Wine 12	71 ± 2	84 ± 4	47 ± 1	674 ± 12	2.98 ± 0.05	0.20 ± 0.07	91 ± 8	283 ± 7
Wine 13	78 ± 1	335 ± 2	24 ±2	1030 ± 45	4.48 ± 0.03	0.39 ± 0.02	156 ± 1	238 ± 6
Wine 14	80± 5	98 ± 1	14 ± 1	988 ± 4	2.09± 0.05	0.48 ± 0.01	143 ± 3	167 ± 2
Wine 15	43 ± 9	120± 9	19 ± 2	541 ± 28	1.40 ± 0.04	0.34 ± 0.03	132 ± 3	39 ± 2
Wine 16	41 ± 9	73 ± 2	28 ± 1	517 ± 4	1.54 ± 0.03	0.45 ± 0.06	61 ± 11	85 ± 4
Wine 17	32 ± 3	68 ± 4	19 ± 2	476 ± 2	0.83 ± 0.17	0.42 ± 0.07	74 ± 11	27 ± 5
Wine 18	50 ± 3	66 ± 8	28 ± 2	313 ± 35	4.51 ± 0.00	0.82 ± 0.06	54 ± 2	50 ± 3
Wine 19	46 ± 3	74± 4	41 ± 4	475 ± 6	0.74 ± 0.00	0.12 ± 0.00	119 ± 1	52 ± 3
Wine 20	69 ± 1	79 ± 1	16 ± 1	240 ± 31	0.57 ± 0.27	0.09 ± 0.02	110 ± 1	77 ± 4
Wine 21	45 ± 1	61 ± 1	11 ± 3	373 ± 3	2.16 ± 0.43	0.42 ± 0.04	277 ± 8	28 ± 1
Wine 22	40 ± 2	64 ± 5	13 ± 1	459 ± 3	0.64 ± 0.00	0.80 ± 0.01	90 ± 1	80 ± 10
Wine 23	53 ± 2	64 ± 1	10 ± 2	226 ± 22	1.13 ± 0.23	0.61 ± 0.06	174 ± 3	33 ± 2

Table 3.8 Some major and trace element levels in beer samples

Sample	Ca (II) ($\mu g\ mL^{-1}$)	Mg (II) ($\mu g\ mL^{-1}$)	Na (I) ($\mu g\ mL^{-1}$)	K (I) ($\mu g\ mL^{-1}$)	Fe (II) ($\mu g\ mL^{-1}$)	Zn (II) ($\mu g\ mL^{-1}$)	Cu (II) ($ng\ mL^{-1}$)	Pb (II) ($ng\ mL^{-1}$)
Beer 1	54 ± 1	31 ± 1	14 ± 1	247 ± 9	0.34 ± 0.01	0.25 ± 0.02	18 ± 1	18 ± 2
Beer 2	22 ± 1	38 ± 2	13 ± 1	244 ± 5	0.37 ± 0.04	0.21 ± 0.00	26 ± 1	90 ± 5
Beer 3	33 ± 2	37 ± 1	20 ± 1	3318 ± 8	0.11 ± 0.00	0.10 ± 0.00	36 ± 1	41 ± 3
Beer 4	29 ± 1	37 ± 1	14 ± 1	314 ± 5	0.51 ± 0.07	0.36 ± 0.01	35 ± 1	31 ± 1
Beer 5	41 ± 1	30 ± 1	16 ± 1	424 ± 1	0.71 ± 0.13	0.24 ± 0.00	37 ± 2	48 ± 8
Beer 6	14 ± 1	49 ± 1	20 ± 2	283 ± 2	0.38 ± 0.02	0.12 ± 0.02	23 ± 4	31 ± 2
Beer 7	33 ± 1	37 ± 2	22 ± 1	331 ± 8	0.18 ± 0.02	0.15 ± 0.04	14 ± 2	22 ± 4
Beer 8	35 ± 1	42 ± 1	23 ± 1	354 ± 4	0.30 ± 0.01	0.21 ± 0.07	29 ± 1	45 ± 2
Beer 9	34 ± 1	45 ± 1	27 ± 1	347 ± 2	0.16 ± 0.01	0.17 ± 0.01	18 ± 4	14 ± 2
Beer 10	38 ± 1	40 ± 2	22 ± 1	394 ± 4	0.30 ± 0.02	0.20 ± 0.03	19 ± 1	31 ± 6
Beer 11	32 ± 1	45 ± 1	12 ± 1	302 ± 5	0.32 ± 0.05	0.22 ± 0.06	17 ± 1	44 ± 1
Beer 12	31 ± 1	38 ± 2	12 ± 1	337 ± 4	0.45 ± 0.03	0.29 ± 0.02	19 ± 2	23 ± 5
Beer 13	32 ± 1	42 ± 1	17 ± 2	389 ± 10	0.28 ± 0.07	0.19 ± 0.03	21 ± 1	31 ± 7

Table 3.9 Average recoveries of wine samples spiked at two levels (n = 2)

Sample	Added standard concentrations ($\mu g\ mL^{-1}$)												Added standard concentrations ($mg\ mL^{-1}$)			
	Ca (II)		**Mg (II)**		**Na (I)**		**K (I)**		**Fe (II)**		**Zn (II)**		**Cu (II)**		**Pb (II)**	
	0.5	2	0.5	2	0.25	1	0.5	2	0.125	0.5	0.0125	0.05	10	40	2.5	10
	Recovery (%)															
Wine 8	99	99	96	95	100	110	98	99	97	97	100	103	98	100	99	105
Wine 9	97	100	102	98	94	97	99	97	98	104	103	100	96	102	93	101
Wine 10	105	103	101	99	103	105	100	98	102	95	110	109	99	103	109	105
Wine 11	103	100	97	96	97	108	104	108	99	97	109	97	99	95	95	98
Wine 12	104	108	98	99	103	108	105	100	107	102	100	99	95	94	101	102
Wine 13	106	107	103	101	99	101	96	97	99	101	99	95	96	98	97	99
Wine 14	104	100	104	101	99	97	97	96	102	96	97	104	99	99	98	104
Wine 15	104	97	99	98	88	106	96	101	97	99	95	93	119	107	101	102
Wine 16	103	99	98	100	101	103	97	100	101	97	109	102	102	102	105	94
Wine 17	103	98	106	100	101	98	107	105	104	103	104	102	96	99	104	104
Wine 18	98	98	96	98	116	103	99	107	97	108	103	110	95	97	97	101
Wine 19	104	109	101	104	100	97	103	107	103	99	104	100	98	101	103	101

Table 3.9 Continue

Sample	Added standard concentrations															
	(μg mL^{-1})												(mg mL^{-1})			
	Ca (II)		**Mg (II)**		**Na (I)**		**K (I)**		**Fe (II)**		**Zn (II)**		**Cu (II)**		**Pb (II)**	
	0.5	2	0.5	2	0.25	1	0.5	2	0.125	0.5	0.0125	0.05	10	40	2.5	10
	Recovery (%)															
Wine 20	95	107	101	104	98	107	101	102	98	103	110	95	97	104	101	107
Wine 21	105	106	103	102	98	107	105	105	108	98	97	96	105	100	108	98
Wine 22	101	99	101	97	102	104	109	100	97	95	104	98	101	97	100	105
Wine 23	101	102	95	99	98	107	103	102	97	102	100	98	95	98	99	97

Table 3.10 Average recoveries of beer samples spiked at two levels (n =2)

Sample	Added standard concentrations ($\mu g\ mL^{-1}$)												Added standard concentrations ($mg\ mL^{-1}$)			
	Ca (II)		**Mg (II)**		**Na (I)**		**K (I)**		**Fe (II)**		**Zn (II)**		**Cu (II)**		**Pb (II)**	
	0.5	2	0.5	2	0.25	1	0.5	2	0.125	0.5	0.02	0.08	2	8	2	8
	Recovery (%)															
Beer 1	100	104	98	101	106	100	101	99	107	102	103	98	115	99	104	107
Beer 2	110	100	95	96	105	105	98	98	104	100	96	96	101	99	103	97
Beer 3	101	98	106	104	98	99	100	99	100	101	116	112	99	100	104	100
Beer 4	96	99	98	99	99	103	103	100	101	96	93	94	97	99	94	102
Beer 5	97	104	103	100	103	102	101	101	97	101	102	104	104	101	98	99
Beer 6	110	103	97	99	103	98	96	98	109	97	102	106	98	98	98	98
Beer 7	95	102	101	101	108	110	97	96	104	108	102	100	112	104	108	109
Beer 8	102	102	110	110	104	99	98	99	98	100	95	102	98	104	84	98
Beer 9	102	102	99	98	99	100	99	100	108	107	96	99	101	98	102	98
Beer 10	110	105	108	104	98	99	96	99	101	101	98	105	95	108	102	107
Beer 11	100	101	98	103	108	101	98	101	103	96	95	99	100	104	110	92
Beer 12	96	97	101	103	96	100	96	101	100	100	109	106	94	96	113	106
Beer 13	109	99	99	96	100	107	101	103	98	96	103	109	106	106	96	110

CHAPTER FOUR
CONCLUSIONS

In this study, HMF contents in vinegar and fruit wine samples were determined by liquid-liqiud extraction combined with TLC-densitometry. All samples were extracted simply without spending more labour and very less amount of organic solvent was used. The HMF levels were within the acceptable limit established by European Union for apple juice.

The ZEN and OTA contents in beer and wine samples were studied using dispersive liquid-liquid microextraction following TLC-densitometry or HPLC. Developed DLLME-HPLC or HPTLC methods for ZEN and OTA have good sensitivity and effectivity in different kinds of wine and beer samples. These DLLME methods are alternative to the mostly used extraction techniques such as solid phase extraction and immunoaffinity columns. These methods have some properties such as simplicity, rapidity, low-cost, eco-friendly and high recovery and sensitivity.

It can be concluded that HPTLC technique can be successfully used for optimization and quantification. The progressed HPTLC methods for HMF, OTA and ZEN are accurate, linear, simple, fast and rugged.

Newly cloud point extraction method combined with HPLC for CPA and TEA in tomato juice samples was developed. The high extraction recovery and preconcentration was achieved using Triton X-114. Besides commonly used liquid extraction techniques, CPE has versatile, simplicity, safety, use of less chemicals and provides good enrichment factors and efficient separation.

Besides mycotoxins, four major and four trace elements in wine and beer samples were determined by atomic emission/absorption spectrometry techniques. The amounts of studied elements were in good agreement with European Union established permissible limits. The element contents in wine samples were found

higher than beer samples, but the order of elements was same in both of them. The reasons of diversity in the amount of elements in all wine and beer samples are soil, processing equipment, and production steps.

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