Cloud Point Extraction and Determination of Cyclopiazonic Acid and Tenuazonic Acid in Tomato Juice

Domates Suyundaki Siklopiazonik Asit ve Tenuazonik Asitin Bulutlanma Noktası Ekstraksiyonu ve Tayini

Research Article

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ABSTRACT

A cloud point extraction as a preconcentration technique was developed for the determination of a tenuazonic and a cyclopiazonic acid in tomato juices. These mycotoxins were extracted and preconcentrated using Triton-X 114 as surfactant and determined by high performance liquid chromatography with ultraviolet detection. The optimized extraction conditions were pH 2, 4% Triton X-114 (w/v), 1% KNO_3 (w/v), 50°C as the extraction temperature and 30 min for extraction time. The extraction recoveries were found as 40% and 95% for tenuazonic acid and cyclopiazonic acid, respectively. The linearity of the proposed method was in the concentration range of 10 to 2000 ng mL^{-1} for both mycotoxins.

Key Words
Cyclopiazonic acid, tenuazonic acid, cloud point extraction, tomato juice, HPLC

ÖZET

Domates suyunda tenuazonik asit ve siklopiazonik asit tayini için zenginleştirme yöntemi olarak bulutlanma noktası ekstraksiyonu geliştirildi. Mikotoksinler yüzey aktif madde olarak Triton X-114 ile zenginleştirildi ve ekstrakte edildi ve ultraviyole dedektörlü yüksek performanslı sıvı kromatografisi tayin edildi. Optimize edilen ekstraksiyon koşulları pH 2, %4 Triton X-114 (w/v), %1 KNO_3 (w/v), 50°C ekstraksiyon sıcaklığı ve ekstraksiyon süresi 30dk olarak belirlendi. Ekstraksiyon geri kazanımları tenuazonik asit ve siklopiazonik asit için sırasıyla %40 ve %95 olarak bulundu. Sunulan yöntemin derişim aralığı her iki mikotoksin için 10 ile 2000 ng mL^{-1}' dir.

Anahtar Kelimeler
Siklopiazonik asit, tenuazonik asit, bulutlanma noktası ekstraksiyonu, domates suyu, HPLC

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INTRODUCTION

Cyclopiazonic and tenuazonic acids are both toxic tetramic acids produced by the secondary metabolites of *Aspergillus, Penicillium* and *Alternaria spp*. Fungi [1, 2]. Cyclopiazonic acid (CPA) affects on the central nervous system in animals, also causes degeneration in liver, kidney, and skeletal muscle [3, 4]. Tenuazonic acid (TEA) that is the most toxic metabolite in *Alternia spp*. inhibits protein biosynthesis [5]. CPA has been found in a wide variety of foods as corn, wheat, peanuts, rice, poultry feed, milk, cheese, tomato products, and dried figs [6-9]. TEA has been commonly infesting a broad range of agricultural products, including cereals, wheat, barley, and their processed products such as beer, and also found in tomatoes [8, 10-14]. CPA has been detected at low levels in foods, but there is no regulatory limits published yet. But, the maximum limit of CPA has been only recommended in cheese is 4 μg g^{-1} and the average individual consumes 50 g of cheese daily for human exposure [15]. For TEA, the LD_{50} value is 162 and 115 mg kg^{-1} bodyweight for male and female mice, respectively [16]. The level of TEA has been reported as 49 μg/kg in cerelas and 25 μg/kg in a sample of cornflakes [13, 17].

Liquid liquid extraction (LLE), a traditional separation process, has been used for toxins effectively [18, 19]. However, this technique is time consuming, and the extraction efficiency depends on type of matrix and type of compounds being determined, and also spending a large amount of organic solvents is not an eco-friendly.

Cloud point extraction (CPE) is one of the alternative extraction techniques which was developed by Watanabe and Tanaka in 1978 [20]. In CPE, phase separation is achieved by formation of micellar phase using surfactant after changing temperature or adding salt to an aqueous solution [21]. The aggregation of micelles depends on the minimum concentration of surfactant which is called as the critical micelle concentration (CMC). Below the CMC, the surfactant can be degraded due to its low concentration in aqueous solution and it is mostly in nonassociated monomer form. However above the CMC, these monomers associate to form micelles spontaneously because of its decreased solubility in aqueous medium [22]. CPE is largely applied for determination of metals in various analytical matrix using Triton X-100, Triton X-114 and PONPE 7.5 [23-25]. Additionally, CPE has been used in the determination of some organic and biological compounds such as adrenalines in milk, carbamate pesticides and phthalates in vegetables and allura dye in food samples [26-28]. CPE has rarely been reported for the extraction and preconcentration of mycotoxins from food samples. Garcia-Fonseca and coworkers have reported the determination of ochratoxin A in wine using decanoic acid as a surfactant in CPE following HPLC with fluorescence detection [29]. The preconcentration of ergotamine in pharmaceutical and biological samples by CPE with fluorimetic detection has been studied by Wang and coworkers using a non-ionic surfactant as PONPE 7.5 [30]. This technique is preferred due to its cheapness, usage of less amount of surfactant, less laboratory residues and eco-friendliness. The used surfactants are generally nontoxic, nonvolatile, and less flammable.

The aim of this study was to develop a new, simple and quick extraction methodology for the analysis of CPA and TEA in tomato juice samples. For this, we developed the CPE method before HPLC analysis.

MATERIALS & METHODS

Reagents, Solvents, Samples and Preparation of Standard Solutions

The standards of CPA and TEA were purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored in a freezer at -20°C. Acetonitrile (ACN) and methanol (MeOH) were supplied from Merck (Darmstadt, 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.).

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) were prepared by diluting with MeOH. Also, each working standard solution was prepared prior to analysis.

Tomato juice samples were purchased from local stores in Izmir and stored their original bottles at 4°C before analysis.

Extraction method

CPA and TEA were extracted from tomato juice samples using liquid-liquid extraction method described by Da Motta, & Soares [8]. Briefly, a 5 g of tomato juice sample was put into a reaction flask and mixed with 15 mL of methanol. 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 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. Then, the recent methanolic extract was shaked with 4 mL of chloroform two times after removing the hexane phase. The chloroform extract was washed with 3 mL of water after separation methanolic phase. The obtained chloroform extract was evaporated under N_2 stream and then redissolved with 3 mL of pH 2 phosphate buffer for getting ready cloud point extraction.

CPE method

In cloud point extraction, the tomato juice sample extract was mixed in turn with 2 mL of 4% Triton X-114 (w/v) solution and 2 mL of 1% KNO_3 (w/v) solution and its final volume was completed to 10 mL with distilled water. The final mixture was heated at 50°C for 30 min, and then centrifuged at 4000 rpm for 15 min. The upper aqueous phase was discarded using a long-needled syringe. An obtained 1.4 mL surfactant-rich phase was diluted to 2 mL with MeOH to reduce the viscosity and then analyzed by HPLC.

Analysis of CPA and TEA by HPLC

The Agilent 1100 model HPLC system (Waldbronn, Germany) consisting of an online vacuum degasser (G1322A), a column oven (G1316A), a quaternary pump (G1311A) and diode array detector (G1315B) with manuel injection was used. The separation was carried out using analytical column ODS-2 hypersyl C18 (Thermo, 250 x 4.6 mm, 5 μm). A Hamilton stainless steel manual injector as 100 μL was used. Each sample was injected 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 was MeOH: water (75:25, v/v) containing 300 mg/L $ZnSO_4$. H_2O. The wavelength of determination was set at 280 nm, and the mobile phase at isocratic elution was pumped at a flow rate of 1.0 mL/min. The column temperature was controlled at 30°C. The retention times were 4.8 and 6.9 min for CPA and TEA, respectively [8].

RESULTS & DISCUSSION

Effect of Surfactants

The interaction of an analyte and surfactant can take places differently based on the 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 partitioning 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 separation and preconcentration of TEA and CPA. The surfactant rich phase was only observed with Triton X-114.

Effect of pH

The effect of pH on the extraction was studied by using the phosphate buffer solutions as pH 2, pH 3.5 and pH 6. The extraction yield decreased with the increase of pH from 2 to 6. So, the value of 2 was chosen as the optimum pH. The reason of decrease could be due to the deprotonation of CPA and TEA (pK_a of CPA and TEA is 2.97 and 3.5, respectively).

Effect of Surfactant Concentration

To examine the effect of Triton X-114 concentration on the extraction of CPA and TEA, the nine different concentration of Triton X-114 solution varing from 0.20 to 10% (w/v) were used. The highest recoveries were detected as 79% for CPA and 25% for TEA at 4% (w/v) surfactant solution and then diminished especially for CPA. Hence, Triton X-114 as 4% (w/v) was used for further studies. This surfactant concentration was above critical micelle concentration ($0.28x10^{-3}$ M, 24°C) [30,31].

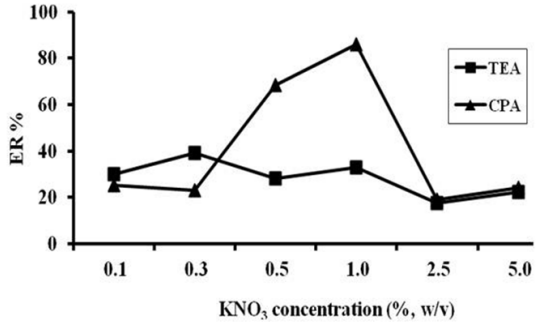

Figure 1. Effect of KNO_3 on CPE and TEA extraction. Extraction conditions: volume of synthetic solution: 2 mL; pH: 2; volume of 4%(w/v) Triton X-114: 2 mL; Teq: 60°C.

Salting Effect

Salt addition assists the solubilization or partitioning of solute into organic phase. For this purpose, the effect of salts using KNO_3, $NaNO_3$, NaCl, KCl, Na_2SO_4 and K_2SO_4 was studied at a concentration of 1% (w/v) at 60°C of cloud point temperature. As shown in Figure 1, the high extraction of CPA was obtained by KNO_3. For TEA, the extraction yields obtained by sulphate salts were good. But, to extract both mytoxins together, KNO_3 was selected. Later on, the amount of KNO_3 ranging from 0.1 to 5 % (w/v) was tested at six different concentrations. Extraction efficiency of TEA was not noticeably influenced by addition of different concentration of KNO_3. The highest extraction efficiency of CPA (85%) and TEA (40%) were obtained at 1 % (w/v) KNO_3.

Effect of Temperature

Cloud point temperature is very important to form a cloudy state. In order to obtain cloudy formation, the equilibrium temperature was controlled in the range between 20 and 80°C (Figure). While 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 especially CPA. However, the extraction yield of TEA was not influenced so much. So, the optimum lowest extraction possible temperature was preferred as 50°C for CPA and TEA.

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 (Figure 3). 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.

Performance Characteristics of CPE Method Using HPLC

The instrumental calibration curves of these studied mycotoxins were established by injecting at least five standard solutions. The linear working ranges were between 0.01-20 μg mL^{-1} and 0.02-10 μg mL^{-1} for CPA and TEA, respectively. Additionally, the regression coefficients were calculated as 0.9984 for CPA and 0.9981 for TEA.

Under the optimum conditions of CPE-HPLC method, the analytical performance of the proposed method was evaluated in terms of the dynamic linearity, the limit of detection and quantitation, the precision and the extraction recovery. The matrix-matched calibration curves

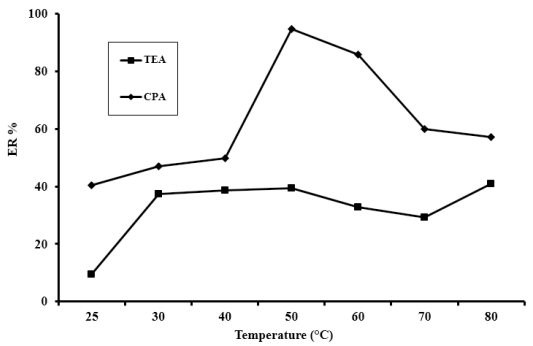

Figure 2. Effect of equilibrium temperature on CPA and TEA extraction. Extraction conditions: volume of synthetic solution: 2 mL; pH: 2; volume of 4%(w/v) Triton X-114: 2 mL; volume of 1%(w/v) KNO_3: 2 mL.

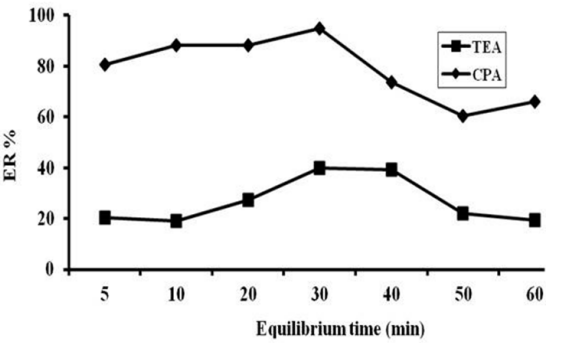

Figure 3. Effect of equilibration time on CPA and TEA extraction. Extraction conditions: volume of synthetic solution: 2 mL; pH: 2; volume of 4% (w/v) Triton X-114: 2 mL; volume of 1% (w/v) KNO_3: 2 mL; T_{eq}: 50°C.

Table 1. 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 (%)
Tomato juice 1	-	10.2 ± 0.02	-	ND	-
	50	58.4 ± 0.03	97	43.29 ± 0.32	86
	100	94.6 ± 0.05	86	83.65 ± 0.26	83
Tomato juice 2	-	ND	-	ND	-
	50	47.6 ± 0.05	94	83.4 ± 0.02	93
	100	98.4 ± 0.07	98	97.6 ± 0.04	97
Tomato juice 3	-	12.3 ± 0.05	-	13.8 ± 0.04	-
	50	60. 1 ± 0.06	96	54.8 ± 0.02	86
	100	94.7 ± 0.03	84	102.5 ± 0.08	90

[nd] not detected. It means below LOQ.

of CPA and TEA after CPE method were linear in the range of 10 - 2000 ng mL^{-1} containing eigth concentrations with the regression coefficients as 0.9969 and 0.9956 for CPA and TEA, respectively. The limit of detection (3xS/N) and the limit of quantitation (10xS/N) were 0.6 ng mL^{-1} for CPA and 0.7 ng mL^{-1} for TEA and 7.6 ng mL^{-1} for CPA 8 ng mL^{-1} for TEA. The precision as percentage of relative standard deviation 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 μg mL^{-1} synthetic solution. The extraction recovery (ER) was calculated as the ratio between the amount of analyte determined after CPE and the amount of anayte before CPE. The percentage of ER of the proposed CPE method was 95% for CPA and 40% for TEA. The preconcentration factor was calculated as the ratio of the volume of original sample solution to the volume of obtained surfactant-rich phase [32]. Under the optimized conditions, the preconcentration factor for CPA and TEA was found as around 7 wherefore the volume of rich phase as 1.4 mL.

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 1. The typical HPLC chromatograms of tomato juice and the fortified tomato juice samples after the CPE method were given in Figure 4. To check the accuracy of the proposed method, tomato juice samples were fortified at two levels of standard CPA and TEA (50 and 100 ng mL^{-1}). The calculated recoveries of each level were also given in Table 1. They show high recoveries and they were in acceptable limit according to current legislation in European Commission [33].

CONCLUSION

In this study, a newly cloud point extraction method combined with HPLC for CPA and TEA in tomato juice samples was developed. The good extraction recovery and preconcentration was achieved using a non-ionic surfactant as Triton X-114. Unlike commonly used liquid extraction

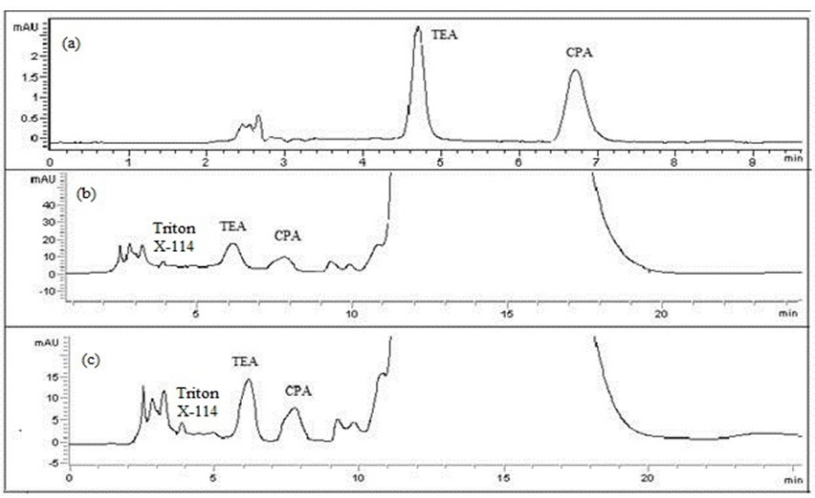

Figure 4. HPLC chromatogram of a) a standard solution containing 0.05 ng CPA and TEA μL^{-1}, b) a tomato juice sample, c) a tomato juice sample spiked with 0.1 ng CPA and TEA μL^{-1}.

techniques, CPE has versatile, simplicity, safety, usage of less amount chemicals and provides good enrichment factors and efficient separation.

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