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Determination of *Alternaria* mycotoxins in wine and juice using ionic liquid modified countercurrent chromatography as a pretreatment method followed by high-performance liquid chromatography☆

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ABSTRACT

Alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA) are some of the main *Alternaria* mycotoxins that can be found as contaminants in food materials. The objective of this study was to develop a pretreatment method with countercurrent chromatography (CCC) for enrichment and cleanup of trace *Alternaria* mycotoxins in food samples prior to high-performance liquid chromatography (HPLC) analysis. An Analytical CCC instrument with a column volume 22.5 mL was used, and a two-phase solvent system composed of ethyl acetate and water modified with 6% [HOOMIM][Cl] in mass to volume ratio was selected. Under the optimized CCC operation conditions, trace amounts of AOH, AME, and TeA in large volume of liquid sample were efficiently extracted and enriched in the stationary phase, and then eluted out just by reversing the stationary phase as mobile phase in the opposite flowing direction tail-to-head. The enrichment and elution strategies are unique and can be fulfilled online with high enrichment factors (87–114) and high recoveries (81.14–110.94%). The method has been successively applied to the determination of *Alternaria* mycotoxins in real apple juice and wine samples with the limits of detection (LOD) in the range of 0.03–0.14 $\mu g\,L^{-1}$. Totally 12 wine samples and 15 apple juice samples from the local market were analyzed. The detection rate of AOH and AME in both kinds of the samples were more than 50%, while TeA was found in relatively high level of 1.75–49.61 $\mu g\,L^{-1}$ in some of the apple juice samples. The proposed method is simple, rapid, and sensitive and could also be used for the analysis and monitoring of *Alternaria* mycotoxin in other food samples.

1. Introduction

Alternaria is a common genus of fungi, and contains numerous species that are both saprophytic on organic materials and pathogenic on many plants [1]. Due to their growth even at low temperatures, they are responsible for food spoilage during refrigerated transport and storage [2]. The *Alternaria* genus produces more than 70 known mycotoxins and the most important *Alternaria* mycotoxins are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins I, II, III (ATX-I, -II, -III) and tenuazonic acid (TeA) produced by *Alternaria alternata* posing a potential health hazard for the consumer if accumulated in food and feed [3]. AME and AOH are not very acutely toxic; however they are mutagenic and show remarkable cytotoxicity in cell culture [4–7]. In-vitro, AOH and AME were shown to induce DNA strand break in cell line systems [8]. TeA is considered to be of the highest toxicity among the *Alternaria* mycotoxins [9]. It inhibits protein biosynthesis at the ribosomal level in mammalian cells by suppression of the release of newly formed proteins from the ribosomes and is biologically active, exerting cytotoxic, phytotoxic, antitumor, antiviral and antibiotic effects [10,11]. Additionally, TeA has been made responsible for the outbreak of "onyalai", a human haematologic disorder disease occurring in Africa [12]. Natural occurrence of AOH, AME and in some cases other *Alternaria* toxins has been reported in grains and grain-based products, tomato and tomato products, sunflower seeds and sunflower oil, fruits and fruit products, beer, wine and so on, especially from several processed foodstuffs manufactured with damaged raw materials, e.g., juices, preserves, and sauces etc. TeA and AOH were most frequently analyzed and found in samples, while AME was not so frequently detected and occurred

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in lower concentrations [13]. AOH and AME were stable in apple juice at room temperature over 1–27 day periods and at 80 °C after 20 min. They were also found to be stable in white wine [14].

AOH, AME and other *Alternaria* mycotoxins have been determined by thin-layer chromatography, gas chromatography and liquid chromatography (LC) mainly with ultraviolet detection, although fluorescence and mass spectrometry and electrochemical detection have also been used, which have been reviewed by Scott [15] and Ostry [2], in which, previous preconcentration/cleaning steps such as solvent partition and solid phase extraction (SPE) or microextraction (SPME) were employed to selectively separate this mycotoxin from complex matrices in all of the cases. *Alternaria* mycotoxins are frequently found in variety of food at low level and have potential health risk for humans and animals. Therefore, monitoring of different foods and feeds as well as physiological samples using reliable and easily applicable methods, specifically which have lower limit of detection (LODs), is necessary in order to provide information on intake of these toxins by consumers, and will give impetus to further toxicological studies if occurrence of *Alternaria* toxins in foods becomes a concern [15].

Countercurrent chromatography (CCC) is a chromatographic separation technique based on the partition of solutes between two immiscible liquid phases as they interact in a thin tube under a centrifugal force field [16]. It inherits the advantages of traditional liquid–liquid extraction with high recovery, while its continuous elution system produces high partition efficiency in a short elution time. A few reported applications of CCC as a pretreatment method show that CCC can be successfully used in the environmental and food analysis [17–20]. CCC permits the analysis of crude and complex samples and can separate analytes from large volumes of such samples, therefore it has the potential to play an important role in food analysis [21]. Ionic liquids (ILs) are ionic, non-molecular solvents with unique physicochemical properties and good extractabilities. ILs have been successfully utilized as solvents in different extraction and microextraction schemes for sample preparation prior to analysis [22,23]. The employment of ionic liquids as a modifier of solvent system of conventional CCC in sample pretreatment can produce lower limits of detection and higher recoveries of analytes due to the unique physicochemical properties and more sufficient dispersion of ILs based on our study [24].

In this paper, a method based on unique CCC separation strategy and modified solvent system with ionic liquids was developed for the enrichment and clean-up of *Alternaria* mycotoxins from juice and wine samples followed by HPLC determination. The effects of several experimental parameters were studied and the optimized method was successfully applied to the analysis of minor *Alternaria* mycotoxins in real wine and apple juice samples.

2. Material and methods

2.1. Apparatus

The present study employed DE Spectrum HPCCC (Dynamic Extraction, UK). The coil column was prepared by winding polytetrafluoroethylene (PTFE) tubing (0.8 mm i.d.) in multilayer on a holder hub with a total capacity of 22.5 mL. The maximum rotational speed is 1600 rmp. A SH150-1500 constant temperature regulator (Lab Tech, Beijing, China) was used to maintain the temperature at 30 °C. The HPCCC system was equipped with a KNAUER Smartline HPLC system (Berlin, Germany), which contained two P-1000 pumps, a UV-2500 detector and a EuroChrom workstation.

The HPLC equipment used was Agilent 1260HPLC system (Agilent Technologies, Waldbronn, Germany), including G1311A QuatPump, G1315B UV–vis photodiode array detector, G1329B autosampler with a 20 μL loop, G1332 degasser and Agilent HPLC workstation.

2.2. Chemicals and reagents

The standards including AOH, AME and TeA, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ionic liquids including [BMIM][Cl], [BMIM][BF_4], [BMIM][PF_6], [BMIM][NTF_2] (where [BMIM], 1-butyl-3-methylimidazolium, [NTF_2], bis(trifluoromethylsulfonyl) amide), [HOOCMIM][NTF_2], [HOOCMIM][Cl] (where [HOOCMIM], 1-carboxymethyl-3-methylimidazolium) were obtained from Chengjie Chemical Co., Ltd. (Shanghai, China). Chromatographic grade acetonitrile was from Fisher Scientific Company (UK). All other reagents were of analytical-reagent grade and from Beijing Chemical Factory (Beijing, China). Ultrapure water was obtained with a Milli-Q water purification system (Millipore Co., USA). The wine and apple juice samples were purchased from local markets and stored in the dark at 4 °C.

2.3. Sample preparation

The mixture stock solutions of AOH, AME and TeA were prepared by dissolving in acetonitrile, resulting in a concentration of 200 μg mL^{-1} of each compound. The spiked water sample was prepared by dissolving 0.01 mL of the stock solution in 200 mL ultrapure water to make a concentration of 10 μg L^{-1} of each compound. Before pretreatment with CCC, each wine and apple juice sample was filtered through a 0.22 μm membrane to remove the suspended particulates, and acidified by 0.2% H_3PO_4 to pH 1.91 as discussed below.

2.4. Selection of the two-phase solvent systems

The composition of the two-phase solvent system was selected by measuring the partition coefficients (K) of target *Alternaria* mycotoxins. The K-values for the target compounds were measured by HPLC as follows: a suitable amount of standard *Alternaria* mycotoxins was dissolved in 1 mL of aqueous phase of the thoroughly equilibrated two-phase solvent system. The solution was determined by HPLC and the peak area was recorded as A_1. Then 1 mL of the organic phase was added to the solution and mixed for 40 s. After the distribution equilibrium was established, the aqueous phase was measured by HPLC again at the same injection volume and the peak area was recorded as A_2. The partition coefficient was defined as $K_1 = (A_1 - A_2)/A_2$ [16].

2.5. CCC pretreatment procedures

A two-phase solvent system composed of ethyl acetate and water (modified with 6% [HOOMIM][Cl], w/v) was used in this study. In CCC operation, the solvent system ethyl acetate: water (1:1, v/v) was pre-equilibrated in a separatory funnel before use. The ethyl acetate upper phase was used as a stationary phase for the enrichment of *Alternaria* mycotoxins from the liquid samples. Firstly, the multilayer coil column was entirely filled with upper ethyl acetate phase, and then the column was rotated at 1400 rpm while the lower aqueous phase was introduced into the column as mobile phase in head-to-tail direction at a flow rate of 3.0 mL min^{-1} to establish a dynamic equilibrium until the water emerged from the end of column. (The retention factor S_f (%) was calculated as a ratio of the stationary phase volume V_s to the total column volume V_c.) Secondly, 50 mL spiked sample or real sample modified with 6% [HOOMIM][Cl] (w/v) was pumped through the column at the same flow rate of 3.0 mL min^{-1}, and the *Alternaria* mycotoxins were extracted into the stationary ethyl acetate phase continuously.