Food Control 31 (2013) 353–358

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Evaluation of dispersive liquid–liquid microextraction for the determination of patulin in apple juices using micellar electrokinetic capillary chromatography

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ARTICLE INFO

Article history:
Received 13 February 2012
Received in revised form
23 October 2012
Accepted 3 November 2012

Keywords:
Dispersive liquid–liquid microextraction
Patulin
Apple juice
Micellar electrokinetic capillary chromatography

ABSTRACT

Patulin (PAT) is a mycotoxin naturally found in fruits, including apples. Its occurrence as a natural contaminant of fruit juices is indicative of fruit quality in production. The European Union has set the maximum content of patulin in 50 μg kg^{-1} for fruit juices and 10 μg kg^{-1} for infant fruit juices. In this paper, dispersive liquid–liquid microextraction (DLLME) has been proposed for the extraction and preconcentration of PAT in apple juice, followed by its determination by micellar electrokinetic chromatography (MEKC) with diode-array detection. PAT has been analyzed in the presence of 5-hydroxymethylfurfural (HMF), which is the main interference in this kind of matrix. Variables affecting DLLME efficiency were optimized and the calibration curve was established for PAT in analyte standard solutions, applying the DLLME–MEKC procedure. The limit of detection was 0.6 μg L^{-1} and recoveries obtained for spiked freshly-made apple juice samples at four different concentration levels (5, 20, 50 and 75 μg L^{-1}), were above 75% with RSD lower than 9%. This method can be classified as a green alternative, being successfully applied to the measurement of 19 apple juice samples obtained from different suppliers and supermarkets. The optimized DLLME–MEKC method is free from matrix effects and avoids the tedious matrix-matched or standard addition calibration method. Almost fifty percent of the samples were contaminated with a PAT content greater than the maximum content established by the European regulation.

1. Introduction

Mycotoxins are toxic secondary metabolites of fungi. Among them, patulin (PAT) is a naturally occurring mycotoxin produced by certain fungal species of *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruit. PAT has been mainly found in apple and apple products and occasionally in pears, grapes, apricots, strawberries, blueberries and peaches (González & Soriano, 2007, chap. 12). As PAT is very soluble in water and very stable in aqueous acid media, it reaches apple derivative products, such as juices.

Apple juice containing the potentially hazardous PAT continues to be a problem for human health, not only due to the effects of PAT but also due to the toxicity produced when PAT is combined with other mycotoxins. PAT is toxic for animals; it induces intestinal injuries, including epithelial cell degeneration, inflammation, ulceration, and hemorrhages. PAT has been classified by International Agency for Research on Cancer (IARC) as an agent whose evidence of carcinogenicity is inadequate in humans but sufficient in experimental animals (Group 3) (IARC, 1986). Thus, in the Directive (EC) 1881/2006 (European Regulation, 2006b), maximum contents of PAT for different fruit products have been considered: fruit juices, spirit drinks, cider and other fermented drinks derived from apples, solid apple products and baby foods (different to the processed cereal-based foods). The maximum permitted content is 10 μg kg^{-1} for infant apple juices and 50 μg kg^{-1} for non-infant apple juices. In this sense, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives established a provisional maximum tolerable daily PAT intake (PMTDI) at 0.4 μg kg^{-1} of body weight (European Commission, 1996; WHO, 1995). For all these reasons, the Association of Official Analytical Chemists (AOAC) recommends several mycotoxin quantification and determination methods in foods (AOAC International, 2002). As a consequence, very sensitive analytical methods for PAT detection, with performance characteristics fulfilling the established legislation are needed (European Regulation, 2006a).

Most of PAT determination methods have been based on thin layer chromatography (TLC) (AOAC, 1984), gas chromatography/mass spectrometry (GC–MS) (Cunha, Faria, & Fernandes, 2009; Sheu & Shyu, 1999; Roach, White, Trucksess, & Thomas, 2000; Rupp

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http://dx.doi.org/10.1016/j.foodcont.2012.11.003

& Turnipseed, 2000) and liquid chromatography (LC) (Boonzaaijer, Bobeldijk, & van Osenbruggen, 2005; Gaspar & Lucena, 2009; Gökmen, Acar, & Sarioglu, 2005; McDonald, Long, Gilbert, & Felgueiras, 2000; Tangni et al., 2003). The main disadvantage of TLC methods is that the detection limit is normally quite high. GC methods require derivatization due to the high polarity of PAT. Therefore, reverse-phase HPLC–UV has been the most frequently used technique.

Although satisfactory limits of quantification can be achieved by HPLC, capillary electrophoresis (CE) methods have some advantages such as being able to use a smaller volume of organic solvent and produce less waste volume (Vallejo-Córdoba & Vargas-Martínez, 2008 pp. 853-912). Micellar electrokinetic chromatography (MEKC) is the preferred electrophoretic mode to analyze PAT (Tsao & Zhou, 2000) and to separate it from 5-hydroxymethylfurfural (HMF) which is the main interference in apple juice (Murillo-Arbizu, González-Peña, & Amézqueta, 2008; Murillo-Arbizu, González-Peñas, Hansen, Amézqueta, & Ostergaard, 2008), being both of them quality indicators of this product. Recently the characteristics of HPLC and electrophoretic methods for monitoring PAT in infant apple juice have been discussed (Murillo-Arbizu, González-Peñas, & Amézqueta, 2010).

Several sample treatment methods have been developed for the extraction of PAT, mainly liquid–liquid extraction (LLE) using acetyl acetate as extraction solvent. This technique is expensive and involves long extraction time. Solid-phase extraction (SPE) has been proposed as a possible alternative because it is quicker, cheaper and cleaner than LLE (Jian-ke, Ri-na, Qiu-hui, & Jian-hua, 2007; Trucksess & Tang, 1999). Also, matrix solid-phase dispersion (MSPD), based on the simultaneous disruption and extraction of solid and semi-solid samples has been used for the extraction of PAT (Ri-Na, Ya-Li, Le, & Hong, 2008). Most of these methods are often complicated and their sample throughput is too low to meet the challenges of food analysis (Ri-Na, Feng-Lan, Jie, Hong, & Lei, 2009). In recent years, different strategies have been proposed for simplifying sample treatment and preconcentration in mycotoxins analysis, which include dispersive liquid–liquid microextraction (DLLME). This method was introduced by Rezaee et al. (2006) and shows simplicity of operation, low cost and high recovery. DLLME is based on a ternary component solvent system: the sample in an aqueous phase, an appropriate extraction solvent (i.e., a few microliters of an organic solvent such as chlorobenzene, chloroform or carbon disulfide) with high density, and a disperser solvent (such as methanol, acetonitrile, or acetone) with high miscibility in both extractant and aqueous phases. When the mixture of extractant phase and disperser is rapidly injected into the sample, a high turbulence is produced. This turbulent regimen gives rise to the formation of small droplets, which are dispersed throughout the aqueous sample. After the formation of the cloudy solution, the surface area between the extractant solvent and the aqueous sample becomes very large, so the equilibrium state is achieved quickly and, therefore, the extraction time is very short. In fact, this is the principal advantage of DLLME. After centrifugation of the cloudy solution, a sedimented phase is settled in the bottom of a conical tube and analyzed with a suitable analytical technique. The general aspects and applications of DLLME are compiled in some recent reviews (Bosch Ojeda & Sánchez Rojas, 2009; Herrera-Herrera, Asensio-Ramos, Hernández-Borges, & Rodriguez-Delgado, 2010; Rezaee, Yamini, & Faraji, 2010; Zang, Wu, Zhang, Xi, & Wang, 2009; Zgoła-Grzeskowiak & Grzeskowiak, 2011).

The combination of DLLME with a miniaturized technique such as MEKC is an environmentally friendly alternative to the determination of PAT, as the consumption of organic solvent is reduced in both steps (sample treatment and determination) of the method, being in agreement with the new trends of green analytical chemistry. To the best of our knowledge, this is the first time that DLLME has been applied to the extraction of PAT from apple juices.

2. Materials and methods

2.1. Chemicals and standard solutions

All the reagents were analytical reagent grade, solvents were HPLC-grade and PAT was analytical standard grade. Methanol, ethanol, acetonitrile, isobutanol, diethylether, dimethylformamide (DMF), sodium chloride, sodium hydroxide, SDS and sodium dihydrogen phosphate monohydrate were supplied by Panreac-Química (Madrid, Spain); acetic acid, dichloromethane, chloroform and tetrachloroethylene were purchased from VWR BDH Prolabo (West Chester, PA, USA); carbon disulfide by Carlo Erba (Rodano, MI, Italia), sodium tetraborate decahydrate, carbon tetrachloride and 2-propanol by Sigma–Aldrich (St. Louis, MO, EEUU), tetrahydrofurane (TFH) and ethyl acetate were supplied by Merck (Darmstadt, Germany).

Ultrapure water purified with a Milli-Q Plus system, (Millipore Bedford, MA, USA) was used throughout the work.

The separation buffer was prepared from sodium tetraborate. The pH was adjusted to 9.0 with 0.5 M hydrochloric acid obtained from Merck.

A 0.2 μm nylon membrane filter (Supelco, Bellefonte, PA, USA) was used for filtration of separation buffer, stock standard solutions and samples injected in the CE equipment.

PAT was provided by Sigma–Aldrich (St. Louis, MO, EEUU). A 1000 μg mL^{-1} stock standard solution was prepared by dissolving 2 mg PAT in 2 mL acetonitrile. 100, 50 and 10 μg mL^{-1} working standard solutions were prepared by diluting with acetonitrile. These solutions were stored in the dark at −20 °C.

HMF was provided by Sigma–Aldrich (St. Louis, MO, EEUU). A 1000 μg mL^{-1} stock standard solution was prepared by dissolving 5 mg HMF in 5 mL acetronitrile. 100 μg mL^{-1} working standard solutions were prepared by diluting with acetonitrile. These solutions were stored in the dark at −20 °C.

2.2. Instruments and equipments

CE experiments were carried out using an Agilent 7100 series CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Data were collected using the software provided with the HP ChemStation version B.02.01.

Separation was carried out in a 64.5 cm × 75 μm i.d. uncoated fused-silica capillary with an optical path length of 200 μm (bubble cell capillary from Agilent Technologies, Waldbron, Germany) and an effective length of 56 cm.

For pH measurements, a pH meter (Crison model pH 2000, Barcelona, Spain) was employed with a resolution of ±0.01 pH unit.

A centrifuge Model Universal 320R (Hettich, Tuttlingen, Germany), an evaporator with nitrogen (System EVA-EC, VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific industries, Bohemia, USA) were used for sample treatment.

2.3. Electrophoresis procedure

Before the first use, the new capillary was rinsed with 1 M NaOH at 60 °C for 20 min, then with deionized water for 10 min, and finally with the running buffer for 30 min. At the beginning of each session, the capillary was cleaned with deionized water for 3 min, then with 0.1 M NaOH for 7 min, deionized water for 1 min and finally with the running buffer for 20 min. Before each run, the capillary was pre-washed with 0.1 M NaOH for 2 min, deionized water for 1 min and finally with the running buffer for 2 min. In all