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Comparison between capillary electrophoresis and high performance liquid chromatography for the study of the occurrence of patulin in apple juice intended for infants

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ABSTRACT

Apple juice samples intended for infants purchased in Navarra (Spain) have been analyzed for PAT occurrence. Two capillary electrophoresis methods, based on a MEKC and a CEC system, and an HPLC method were evaluated for the aforementioned study. The CEC system gave less satisfying separations and several practical problems, so samples have been analyzed by MEKC and HPLC. Both methods have been comparable in terms of recovery, precision, limits of detection, volume of organic solvents used and adequate selectivity with regard to PAT and HMF. The analysis time in HPLC has been slightly lower than in the MEKC methodology. The PAT levels obtained in apple juice by both validated methods showed a strong correlation ($p < 0.001$). Therefore, both methodologies are useful for the accurate quantification of patulin in this matrix.

The PAT levels obtained in the 20 infant apple juices samples were in a range between <LOD and 29.6 μg L^{-1}, with a mean concentration of 8.0 μg L^{-1} which implies a dietary intake estimation of 104 ng kg^{-1} b.w. day^{-1} considering a body weight of 10 kg and an apple juice consumption of 130 mL day^{-1}, 26% of the PMTDI recommended by JECFA.

1. Introduction

Patulin (4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one) (PAT) is produced by filamentous fungi of the genera *Penicillium, Aspergillus* and *Byssochlamys* present in a wide variety of foodstuffs: mainly apples but also pears, grapes, apricots, strawberries, blueberries, peaches, vegetables, berries, bread, and meat products (Rychlik and Schieberle, 2001; Ritieni, 2003; Majerus and Kapp, 2002). PAT is very soluble in water, very stable in aqueous acid mediums and there are contradictory results concerning its stability after heat treatment (Kadakal and Nas, 2003); therefore, the use of unsound apples can result in the presence of patulin in derived products such as apple juice, puree, pies, jam and foodstuff intended for infants. Also, patulin can be used for quality control purposes of foods elaborated with apples, indicating the quality of raw materials employed, mainly the presence of rotten apples (Prieta et al., 1994).

The PAT toxic effects have been studied in different animal species (Mahfoud et al., 2002). PAT has mutagenic properties and can cause neurotoxic effects in rodents (Hopkins, 1993). The International Agency for Research on Cancer has classified patulin in group 3 (not classifiable regarding its carcinogenicity to humans and with no adequate evidence in experimental animals) (IARC, 1986).

In 1995, the Joint Expert Committee on Food Additives of the World Health Organization (JECFA, 1995), recommended that human exposure to PAT should be reduced to less than 0.4 μg kg^{-1} b.w. day^{-1}. Taking into consideration this recommendation and the data related to the occurrence of PAT in food, the European Commission approved the Directive (CE) No. 1881/2006 which established the PAT limits for different apple derivative commodities (Commission Regulation (EC) No 1881/2006 of 19 December, 2006). Taking into account that young children have a higher consumption of apple-based food compared to adults (Piemontese et al., 2005; Barreira et al., 2010) and in order to protect infants, this directive indicates a lower permitted level in the case of food for young children, infants, and baby foods (10 μg L^{-1}) than that permitted for apple juices not intended for infants (50 μg L^{-1}).

Abbreviations: CE, capillary electrophoresis; CEC, capillary electrochromatography; DAD, diode array detector; HMF, 5-hydroxymethylfurfural; HPLC, high performance liquid chromatography; LOD, limit of detection; MEKC, micellar electrokinetic chromatography; MES, 4-morpholino-ethyl-sulfonic-acid; PAT, patulin; PMTDI, provisional maximum tolerable daily intake; SDS, sodium dodecylsulphate; TLC, thin layer chromatography; TRIS, trihydroxymethylamoniomethane; TFA, trifluoroacetic acid; RSD, relative standard deviation.

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In order to evaluate the exposure to patulin, different studies have been conducted (Beretta et al., 2000; Ritieni 2003; Piemontese et al., 2005; Legarda and Burdaspal, 2005; Cano-Sancho et al., 2009; Murillo-Arbizu et al., 2009). A Scientific Cooperation Study (Majerus and Kapp, 2002) showed that apple juice and apple nectar are the main sources of patulin intake in most countries, particularly for young children; patulin can be considered a typical mycotoxin, with baby and children as the target (Ritieni, 2003). Some studies on the occurrence of patulin in baby food products have been reported (Prieta et al., 1994; Cano-Sancho et al., 2009; Mhadhbi et al., 2007; Legarda and Burdaspal, 2005; Plessi et al., 1998; Ritieni, 2003), but there is less data for baby food products than for adults products (Cano-Sancho et al., 2009).

Several studies have been performed to develop sensitive and selective methods for the determination of patulin in food. At first, patulin was semi-quantitatively analyzed using TLC methods (Scott and Kennedy, 1973; Stinson et al., 1977). Currently, the analysis of patulin is generally carried out using HPLC with diode array detection (Gökmen et al., 2005; Spadaro et al., 2007; González-Osnaya et al., 2007; Valle-Algarra et al., 2009). In comparison with HPLC methods, capillary electrophoresis (CE) methods have some advantages, such as being able to use a smaller volume of organic solvents, highly efficient separation and producing less waste volumes. Indeed, micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) methods have become powerful separation techniques for both neutral and charged compounds. Few studies on the use of capillary electrophoresis for the analysis of patulin have been conducted (Tsao and Zhou, 2000; Murillo et al., 2008; Murillo-Arbizu et al., 2008). To date, there have been no reported attempts to compare the CE methodology with the HPLC one in the determination of this mycotoxin.

The objectives of this study have been: (a) to report the results of the analysis of patulin in apple juice intended for infants purchased in Spain, in view of the adverse effects caused by this mycotoxin, and the continuous need of exposure data for risk assessment. (b) To compare the HPLC and CE techniques for PAT analysis in this matrix. In order to do that, samples have been analyzed by two validated methods, one of them being a previously reported method using MEKC (Murillo et al., 2008) and the second one being an HPLC method that has been developed and validated for PAT quantification in this type of matrix. Linearity, recovery, LOD, selectivity, time of analysis, cost and their applicability in real apple juice samples intended for infants have been compared.

2. Material and methods

2.1. Reagents

Pure crystalline patulin (CAS No. 149-29-1, >98%), 5-hydroxymethylfurfural, acetic acid, trihydroxymethyl-amoniomethane (TRIS), 4-morpholino-ethyl-sulfonic-acid (MES), trifluoroacetic acid, anhydrous sodium bisulphite and anhydrous sodium carbonate were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Sodium tetraborate 100 mM, sodium tetraborate 50 mM/sodium dodecylsulphate (SDS) 100 mM solution were obtained from Agilent Technologies (Waldbronn, Germany). Ethylacetate, acetonitrile, methanol and ethanol HPLC grade were purchased from Riedel-de Haen (Seelze, Germany). Millipore type I water was used to prepare all of the aqueous solutions and it was obtained daily from a Milli-Q water-purifying system.

2.2. Apparatus

The electrophoretic separations were performed in an Agilent Technologies capillary electrophoresis system (model G1602A) controlled by Chemstation 3D software. DAD detection was used at a wavelength of 276 nm.

The MEKC method used in this study was previously described (Murillo et al., 2008). Briefly, an extended light path capillary of 56 and 64.5 cm for effective and total length, respectively, and 75 μm I.D was used; the electrolyte composition was sodium tetraborate 33.3/66.6 mM of SDS/5% acetonitrile, analysis temperature 35 °C, run voltage + 15 kV, and hydrodynamic injection (50 mbar for 15 s).

In previous studies, we have obtained CE methods (using MEKC and MEEKC) useful in the determination of PAT levels in apple juices, and so CEC has been proved in this study as a possible alternative technique to the analysis of patulin. However, several technical problems arose. The excessive time and labor required to obtain stable current/baseline and reproducible retention times, the long time necessary for conditioning the column between runs, the lack of reliably and reproducibility, the fragility of the column affecting the method robustness, the bubble formation during analysis found during the optimization steps as well as the overall analysis price, and the fact that no improvements were found with regard to the previously MEKC method developed, led us to discard the CEC method for further investigation, and MEKC was chosen as the CE method to be used for the comparison with the HPLC system.

The HPLC instrument used was an Agilent Technologies 1100 liquid chromatographic system equipped with a diode array detector, controlled by Chemstation 3D software. Separations were obtained by using a 5 μm (15 × 0.46 cm) Zorbax Eclipse XDB-C18 column with a Tracer Extrasil ODS-2 precolumn, from Agilent Technologies (Waldbronn, Germany) and Teknokroma (Barcelona, Spain), respectively. The injection volume was 50 μl. Chromatography was performed at 40 °C, and a detection wavelength of 276 nm was used. The composition and proportion (aqueous and organic percentage) of the mobile phase were studied in order to obtain a good resolution between PAT and HMF.

2.3. Samples

Twenty apple juice samples, labeled as intended for infants older than four months were obtained from different supermarkets within Navarra (Spain) during 2008. Different brand names were selected in order to have a market-representative sampling. The volume of the samples was between 130 mL and 260 mL. They were stored in their original packets (glass bottles) at room temperature (18–25 °C) until analysis was carried out (no longer than 6 months), always before the sample expiration dates indicated on the containers.

2.4. Standard sample preparation

Stock solutions of patulin and HMF standards were prepared in ethyl acetate (10 mg in 50 mL and 5 mg in 25 mL, respectively). Aliquots of the PAT and HMF solutions were stored at −20 °C due to the fact that PAT is stable in ethyl acetate for several months under these storage conditions (MacDonald and Felguieras, 1997).

A working solution of PAT (10 μg mL^{-1} approximately) was prepared after evaporating 1 mL of the corresponding stock solution to dryness under a stream of nitrogen at 40 °C and redissolving the residue in 20 mL of ethanol. The accurate concentration of PAT was determined by UV spectrophotometry at 276 nm (MW = 154.12; ε = 14600 L mol^{-1} cm^{-1}) (MacDonald and Felguieras, 1997). A working solution of HMF was prepared by evaporating 7 mL of its stock solution to dryness under a stream of nitrogen at 40 °C and then by dissolving the residue in 20 mL of ethanol, obtaining an HMF solution of 70 μg mL^{-1}. These solutions were stored at 0–4 °C. They were stable at this temperature for at least 6 months (data not shown). Calibration samples were prepared by evaporating the adequate volumes of PAT and HMF working solutions, after having been temperated at room temperature for 30 min, under a nitrogen stream at 40 °C, followed by dissolution in 200 μl of water at pH = 4.0 acidified with 0.1% acetic acid and filtering through a 0.45 μm filter (Syringe Driven Filter, Millipore Corporation, MA Bedford USA).

2.5. Sample preparation

The extraction of PAT for MEKC analysis was the same as that previously described (Murillo et al., 2008). Briefly, 20 mL of apple juice were extracted with 20 mL of ethylacetate. Ten milliliters of the organic phase were evaporated under a nitrogen stream in a water bath at 40 °C. The residue was then dissolved in 200 μl of water acidified at pH = 4.0 with a 0.1% solution of acetic acid; afterwards, it was filtered through a 0.45 μm filter before analysis.

The method used in PAT extraction from apple juice samples for the HPLC analysis was based on the method developed by Yurdun et al. (2001), with some modifications: PAT was extracted from 20 mL of apple juice samples with 20 mL of ethylacetate in a vertical shaker model ABT4 (SBS®) for 10 min. After letting the mixture stand for 10 min, the two phases were segregated and the organic phase was mixed with anhydrous sodium carbonate at 1.5% and shaken for 30 s. Then, it was left standing for 1 min. The organic phase was filtered through a filter containing 10 g of anhydrous sodium bisulphite. Eight milliliters of the filtrate was evaporated to dryness under a weak stream of nitrogen at 40 °C. The residue was redissolved in 0.2 mL of water acidified at pH = 4.0 with a 0.1% solution of acetic acid, and then filtered through a 0.45 μm filter.

2.6. Quantification and method evaluation

The PAT levels in the samples were determined by using a calibration graph of concentration versus response achieved by injection of patulin standard solutions and subsequent extrapolation. In the HPLC method, the response was measured