Optimization of solid phase extraction based on molecularly imprinted polymer for patulin determination

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Abstract: The modified Stöber silica particles were used as support for the development of molecular imprinted polymer matrix for extraction of patulin. γ-MPTS was covalently attached to the surface of Stöber silica particles by means of condensation reaction between surface silanol groups of silica and methoxy groups of γ-MPTS. The prepared silica SiO_2-γ-MPTS was polymerized through a non-covalent approach using patulin (PAT) as a template, maleic acid (MA) as a functional monomer, ethylene glycol dimethacrylate (EGDMA) as a cross linker, 2,2-azobis- (2- methylpropionitrile) (AIBN) as a precursor and acetonitrile (MeCN) as a porogen solvent to prepare SiO_2MA@MIP. The non-imprinted polymer SiO_2MA@NIP was prepared following the same synthetic scheme, but in the absence of the template. Molecular imprinted material was used to selective solid phase extraction (SPE) of patulin in apple matrix. The best conditions for PAT extraction using the novel MIP@SPE were: 50 mg mass of SiO_2MA@MIP packing in solid phase extraction cartridges, solution of sodium bicarbonate with (1%) acetic acid as washing solvent and 5 mL MeCN as eluting solvent. The developed MIP@SPE has the advantages of MIPs and SPE and could have potential applications for high selective enrichment and determination of PAT in apple juices with a major impact on quality control in food processing, improving product quality and safety with minimal investment.

Keywords: Patulin, molecularly imprinted polymer, Solid phase extraction, MIP@SPE cartridge.

INTRODUCTION

Patulin (PAT) (figure 1) is a polyketide-derived mycotoxin produced by several species of filamentous fungi belonging to the genera *Penicillium*, *Aspergillus* and *Byssochlamys*. Of these, *Penicillium* is the mainly prolific with 14 species identified as patulin producers [2,3]. Fruit products in general and apples in particular, are one of the sectors most affected by this pathogen, and are considered by far the main route of entry of patulin into the food chain [4,5]. However, the major sources of contamination are apples and apple products, which are also the most important source of PAT in the human food [5-8]. Indeed, PAT is recognized to be immunotoxic, mutagenic, neurotoxic, and it can cause undesirable effects on the gastrointestinal tract; it also has adverse effects on the developing foaetus [9,10]. Given the health risks posed by PAT, governments have created regulatory guidelines for maximal levels permitted in apple and apple products. In the United States, the maximum acceptable level of PAT was set at 50 ppb [11]. the European commission regulations 1425/2003 and 1881/2006 established the

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maximum recommended concentration of PAT equal to 50 mg/kg in apple fruit juice and apple juice, 25 mg/kg for solid apple products such as apple puree and 10 mg/kg for solid apple products intended for babies and young infants [12,13]. To meet such requirements, many methods were developed to analyze PAT in apple juice matrices [14-17] such as thin layer chromatography (TLC), mass spectrometry, colorimetry, gas chromatography/mass spectrometry (GC-MS) and high performance liquid chromatography-mass spectrometry (LC-MS). At the moment, the high performance liquid chromatography with ultra-violet light detection (HPLC-UV) is the most frequently used method for PAT determination and has been validated as an AOAC international official method [5, 6, 18]. The deficit of UV detection however, take place when poor resolution is observed between PAT and other interfering substances especially 5-(hydroxy-methyl)furfural (HMF) (see Scheme 1). Therefore, the pretreatment of the sample for HPLC analysis prior to the analysis is necessary and crucial in the analytical procedures. Recently solid phase extraction (SPE) have been used as alternative clean-up method substituting the traditional liquid-liquid extraction (LLE) which is time consuming and uses organic solvents [19]. The most common SPE for routine determination of PAT are based on C18 or C8 modified silica are applied for PAT extraction and clean-up as solid phase but these phases exhibit a poor selectivity for the separation of patulin from complex matrices [20,21]. Molecularly Imprinted Polymers (MIPs) have been used as SPE sorbents (MIP@SPE) and seem to become the promising development to circumvent the draw backs of traditional SPE sorbents owing to the recognition ability of MIPs for extraction of mycotoxins from various food matrices [19,22]. Generally, the imprinting process involves prearrangement of the functional monomers around a template molecule, then polymerization in the presence of a crosslinking monomer and finally removing the template in order to leave a cavity specific for template molecule. They are cross linked functional polymers synthesized in the presence of template, which after removal leads to formation of specific cavity complementary to the template molecule [23]. As a consequence, the objectives of the present study were to develop a novel extraction procedure using a molecularly imprinted polymer MIP@SPE for selective clean-up and quantification of PAT from apple juice or its related products samples prior to analysis.

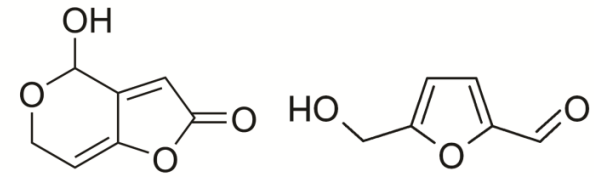
MATERIALS AND METHODS

1. Chemicals and reagents

Patulin standard (purity ≥ 98%) was provided from AG Scientific (AG Scientific Inc, San Diego, CA, USA) dissolved in water/acetonitrile 90:10 and stored at 4 °C. 5-(hydroxymethyl) furfural (HMF) (purity ≥ 95%) was obtained from TCI (Tokyo Chemical Industry CO. LTD). Its molecular structure is shown in Scheme 1. All reagents (acetic acid, hydrochloric acid and sodium carbonate) were purchased from Sigma-Aldrich (France). γ-Methacryloxy-propyltrimethoxysilane (γ-MPTS) and tetraethyl orthosilicate (TEOS, 98%) were purchased from Sigma-Aldrich. Polysorbate 20 used as stabilizer was purchased from Aldrich under the trade name Tween®20. 2,2-azobisisobutyronitrile (AIBN), maleic acid (MA), ethylene glycoldimethacrylate (EGDMA) were purchased from Sigma-Aldrich All solvents (acetonitrile (MeCN), ethyl acetate (EtOAc), hexane (HA), acetone (Ac) of HPLC grade were obtained from Fisher Scientific (Illkirch-Graffenstaden, France). Water was doubly-deionized and filtered with 0.45-µm filter membrane before use.

2. Synthesis of molecular imprinted polymer

The synthesis of SiO_2MA@MIP comprised two steps as described elsewhere [24]. Step i): At first, SiO_2-γ-MPTS was prepared by adding tetraethoxysilane (TEOS) to γ-methacryloxy-propyltrimethoxysilane (γ-MPTS) solution. The molar ratio of γ-MPTS to TEOS in the final solution was 1:4. The mixture was kept at 80°C over night. In the second step, the obtained silica SiO_2-γ-MPTS and 0.01 mmol of patulin (template)



4,6-dihydro-4-hydroxy-2H-furo [3,2-c]pyranne-2-one (PAT)

5-(hydroxymethyl)furfural (HMF)

Figure 1. Molecular structure of PAT and HMF.

were introduced in 10 mL of acetonitrile containing 20 mmol maleic acid (MA), the cross-linker EGDMA (20 mmol) and AIBN (0.2 mmol) as initiator. The mixture was polymerized by heating at 60°C for 6h. The obtained powder denoted SiO_2MA@PAT was separated and stored under vacuum for subsequent uses. To remove the template, repeated extractions of SiO_2MA@PAT with ethyl acetate for 8h and methanol-acetic acid (90:10, v/v) in a Soxhlet apparatus were established. For control, non-imprinted polymer (SiO_2MA@NIP) was also synthesised by using the same manner and conditions but without PAT.

3. Chromatographic conditions

A liquid chromatography apparatus Agilent Technologies) equipped with two pumps, DAD detector, automatic injector with a six-port injection valve (Agilent 110) was used for the identification and quantification of patulin in apple juice. Chemstation workstation for LC software was used for data processing. The HPLC operation conditions included reversed-phase C18 column (4 x 250 mm, 5 mm, EC Nucleosil, Macherey-Nagel) and a mixture of solvent as mobile phase (water/Acetonitrile, v/v = 90:10). Flow rate: 1mL.min^{-1}; injection: 10µL and chromatograms were recorded at 276 nm.

4. Standard solutions and sample preparation

Stock standard solution of PAT was prepared by dissolving 5 mg of pure crystalline patulin in 50 mL of water/acetonitrile (90:10 v/v) and working solutions were obtained by successive dilutions in water/acetonitrile. HMF (figure 1) stock standard solution (500 µg.L^{-1}, dissolved in deionized water). Working standard solutions were prepared by appropriate dilution of this solution with water. Stock standard and working standard solutions were stored at 4°C until used. Apple juice samples provided from local market were centrifuged at 5000 tr/min for 5 min. The supernatant was then diluted with water and stored at 4°C before analysis.

RESULTS AND DISCUSSION

1. Method development of MIP@SPE

A novel procedure for the development of MIP@SPE that ensures an efficient extraction of PAT was optimized. To this end, the ability of the developed SiO_2MA@MIP to selectively extract PAT was assessed at different mass of adsorbent

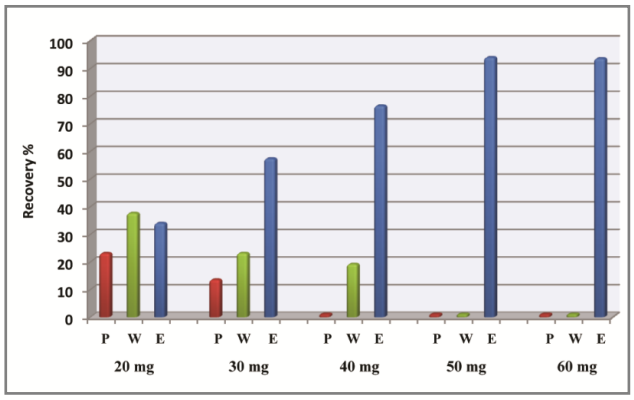


Figure 2. Extraction profiles of patulin using different masses of SiO_2MA@MIP.

(20, 30, 50 and 60 mg). The effects of washing and eluting solvents on the recovery of PAT were also evaluated.

1.1. Mass of adsorbent and binding capacity

As shown in figure 2, the lower recovery (38%) was observed with 20 mg adsorbent (SiO_2MA@MIP), suggesting a rapid saturation of the recognition sites and that the free (non attached) molecules of PAT were loosed during the percolation and washing steps. Increasing the mass of adsorbent remarkably improved the retention of PAT, with a maximum recovery (94%) were found at 50 mg or above (60 mg) of adsorbent, which indicates that the increased number of binding sites leads to better fixation of PAT molecules under the experimental conditions used. Additionally, the

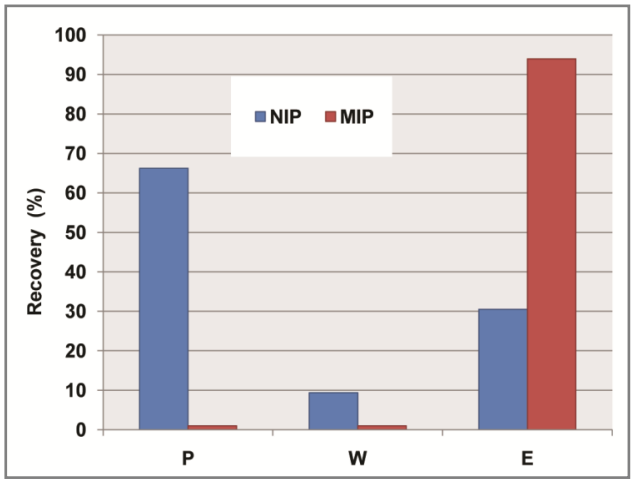


Figure 3. Extraction profiles obtained with MIP and NIP after percolation of 1mL apple juice spiked with PAT (0.5 µg.mL^{-1}).

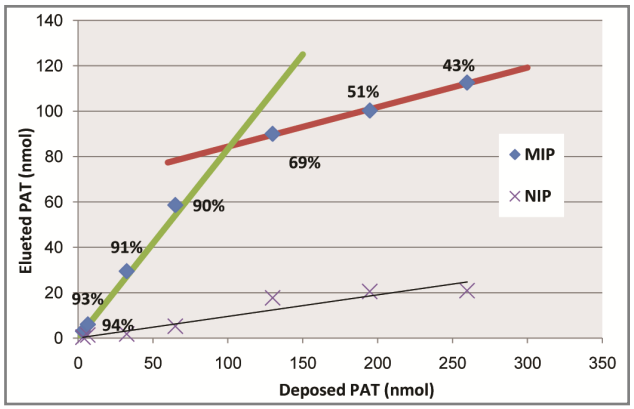


Figure 4. Retain capacities of PAT using SiO_2MA@MIP and SiO_2MA@NIP materials.

high recovery confirms that this elaborated SiO_2MA@MIP could be useful in extracting PAT.

Support to these results was provided by figure 3, where the potential to extract PAT from apple juice was compared between SiO_2MA@MIP and SiO_2MA@NIP. Results indicated that most of loaded PAT was lost in the loading fraction, and that SiO_2MA@NIP was not able to retain PAT. In contrast, when using SiO_2MA@MIP, a sufficient retention of PAT was underscored in the elution. It is worthy to note that SiO_2MA@NIP was also able to retain a residual amount of PAT possibly via non-specific interactions.

Support to this assumption is given by figure 4, where the capacities of MIP and NIP polymers to retain PAT are presented. Results show that 50 mg SiO_2MA@MIP exhibited high capacity for PAT than that of the SiO_2MA@NIP. The maximum binding capacity of SiO_2MA@MIP was found to be 261.8 $\mu g.g^{-1}$ versus 30 $\mu g.g^{-1}$ of SiO_2MA@NIP. Collectively, these results indicate that the novel material showed great level of adsorption and good affinity towards PAT.

1.2. Effects of washing and eluting solvent on PAT recovery

In order to remove PAT contaminant HMF, different solvents have been considered for the washing and eluting steps during MIP@SPE analysis. To this end, apple juices spiked with HMF and PAT were loaded into the developed MIP@SPE at a flow rate of $1mL.min^{-1}$ and washed with hexane, petroleum ether, diethyl ether, mixture of ethyl acetate/hexane at different proportions (10:90, 20:80 and 30:70) and sodium bicarbonate (1%, w / v) followed by acetic acid (1%). Among these solvents, the best recovery (75%) was observed with sodium bicarbonate solution followed by acetic acid, presumably through its high capacity to break the non specific interactions of HMF on SiO_2MA@MIP surface and to achieve selective preconcentration of PAT (figure 5).

Another point to be considered is that cartridge acidification allowed better stability to PAT [25], leading hence to better recovery. These results were similar to those reported by Khorrami and

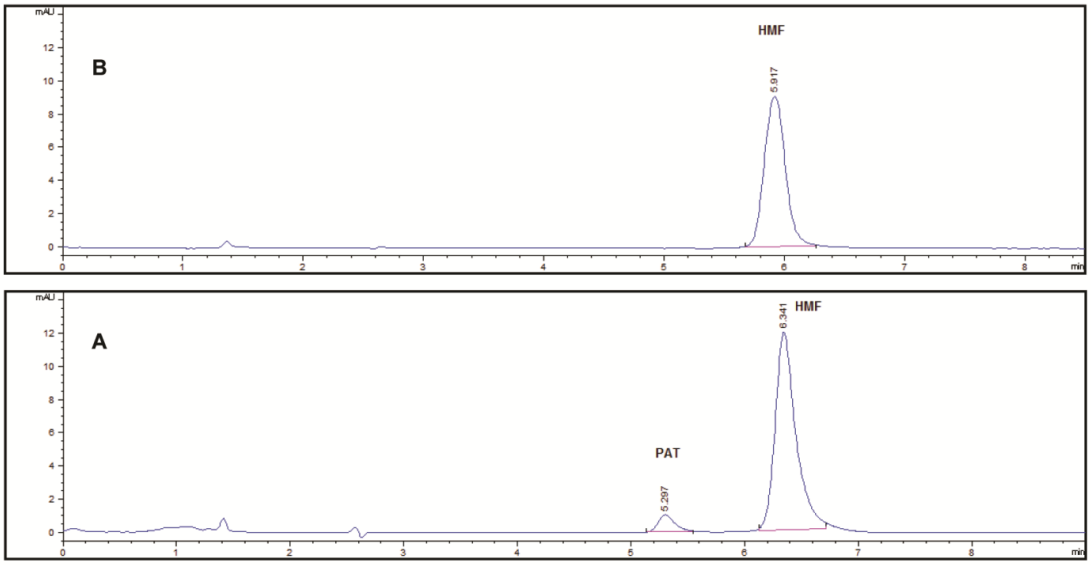


Figure 5. (A) Chromatogram of apple juice spiked with PAT ($0.1 \mu g.mL^{-1}$) and HMF ($0.1 \mu g.mL^{-1}$), (B) Chromatogram of washing fraction obtained after loading spiked apple juice through MIP@SPE cartridge.

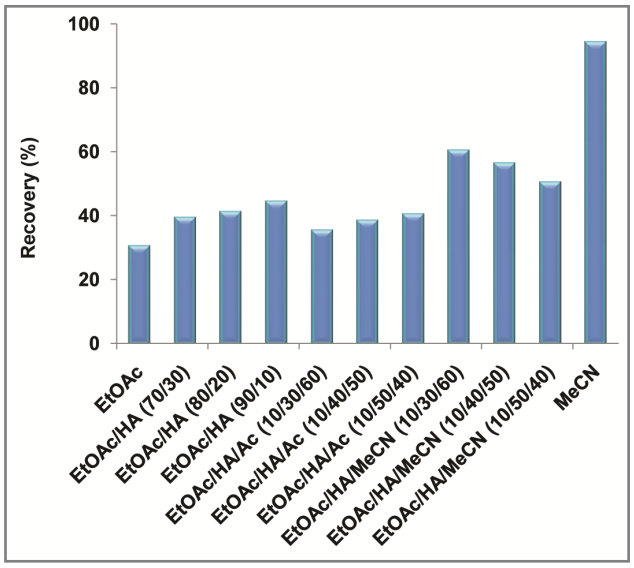


Figure 6. Recoveries of PAT (0.5 µg.mL^{-1}) with different eluting solvents.

Taherkhani [26] who found that the use of 1.5% sodium bicarbonate solution at a flow rate of 1mL.min^{-1} overcomes the problem of interfering phenolic compounds and improve the recovery of PAT in a polymethacrylic MIP@SPE.

In order to increase PAT recovery, an appropriate choice of eluting solvent is required. In this study, eleven eluting solvents including ethyl acetate, acetonitrile, binary mixture (ethyl acetate/hexane in different proportions) and ternary mixture (ethyl acetate/hexane/acetone and ethyl acetate/hexane/ acetonitrile in different proportions) were screened for their PAT recovery. Results showed that the average recoveries ranged from 30% (using ethyl acetate) to 94% with acetonitrile (Figure 6). The remaining eluting solvents showed intermediate recovery values (50-60%). The potential of acetonitrile to desorbs PAT was at least in part attributed to a decrease in the number of hydrogen-bond donating groups of PAT due to its relatively high dielectric constant (ε= 37.5). Earlier studies focused on PAT analysis in different apple-based commercial products also mentioned the potential of polar aprotic solvents like acetonitrile to desorb PAT from SPE-C18 [27].

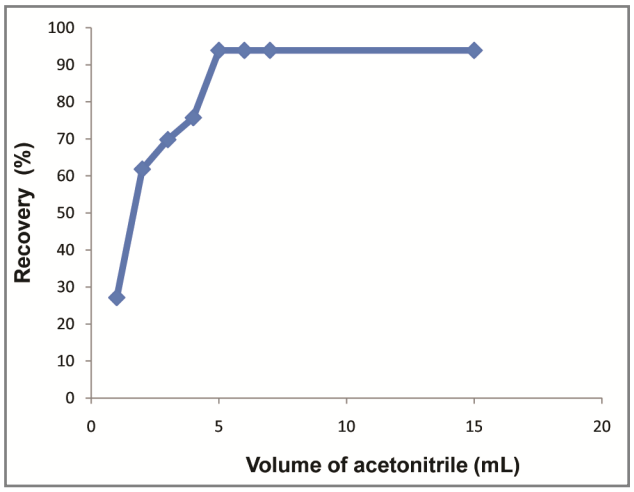


Figure 7. Recoveries of PAT from the MIP@SPE cartridge as function as volume of acetonitrile.

It is worthy to note that 5 mL of acetonitrile provided the highest efficiency for PAT extraction (Figure 7). Consequently, on the basis of the elution efficiency and minimum solvent consumption, 5 mL of acetonitrile was adopted for the elution of PAT from the MIP@SPE cartridge.

CONCLUSION

Synthesis of a molecular imprinted polymer through polymerization of maleic acid using PAT as template molecule was achieved. Polymerisation was conducted from surface attached groups in the presence or absence of Patulin. The imprinted material was applied as a sorbent of the MIP@SPE method. Various parameters including mass of adsorbent, washing solvent, eluting solvent and its amount have been optimized for the selective extraction of PAT. The selectivity of the MIP@SPE was established using non-imprinted polymer solid phase extraction (NIP@SPE). Best results were attained using 50 mg of adsorbent, sodium bicarbonate followed with (1%) acetic acid as washing solvent and 5 mL acetonitrile as eluting solvent. The recoveries of PAT from spiked apple juices are in the range of 82-98%.

Sensitivity, simplicity, precision and rapidity of the developed MIP@SPE method were validated for SPE extraction of Patulin. This study is ongoing and will be published in a separate paper.

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