Enzyme-assisted extraction and ionic liquid-based dispersive liquid–liquid microextraction followed by high-performance liquid chromatography for determination of patulin in apple juice and method optimization using central composite design

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HIGHLIGHTS
• A novel method was developed for extraction of patulin from apple juice.
• Enzyme-assisted extraction (EAE) was used to release of patulin from apple juice.
• IL-DLLME was used for quick pre-concentration of patulin from sample solution.
• Final separation and quantification was performed by HPLC-UV.
• The figures of merit for patulin in apple juice using this method were excellent.

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A simple and highly sensitive analytical methodology for isolation and determination of patulin in apple-juice samples, based on enzyme-assisted extraction (EAE) and ionic liquid-based dispersive liquid–liquid microextraction (IL-DLLME) was developed and optimized. Enzymes play essential roles in eliminating interference and increasing the extraction efficiency of patulin. Apple-juice samples were treated with pectinase and amylase. A mixture of 80 μL ionic liquid and 600 μL methanol (disperser solvent) was used for the IL-DLLME process. The sedimented phase was analyzed by high-performance liquid chromatography (HPLC). Experimental parameters controlling the performance of DLLME were optimized using response surface methodology (RSM) based on central composite design (CCD). Under optimum conditions, the calibration curves showed high levels of linearity (R² > 0.99) for patulin in the range of 1–200 ng g⁻¹. The relative standard deviation (RSD) for the seven analyses was 7.5%. The limits of detection (LOD) and limits of quantification (LOQ) were 0.15 ng g⁻¹ and 0.5 ng g⁻¹, respectively. The merit figures, compared with other methods, showed that new proposed method is an accurate, precise and reliable sample-pretreatment method that substantially reduces sample matrix interference and gives very good enrichment factors and detection limits for investigation trace amount of patulin in apple-juice samples.

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1. Introduction

Patulin ([4-hydroxy-4H-furul][3,2-c]pyran-2(6H)-one) is one of the several secondary metabolites produced by the fungi belonging to the genera Penicillium, Aspergillus and Byssoschlamys [1]. Penicillium expansum, the most common post-harvest invader of apples, causes blue mold rot during storage [2]. Although patulin was studied first as a potential new antibiotic, research revealed its toxicological properties. The occurrence of patulin as a natural contaminant of apple juice is a worldwide problem, and international recommendations and regulations have been made for maximum levels permitted in consumer products. Owing to its toxicity, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) has established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 μg kg⁻¹ body weight/day [3]. Recently, the European Commission (EC) proposed the following allowable levels for patulin: in fruit (apple) juices and apple-juice ingredients in other beverages 50 μg kg⁻¹; in solid products including dried compotes and apple puree, 25 μg kg⁻¹; in apple products intended for infants and young children and labeled as such, 10 μg kg⁻¹ [4].

Many methods have been applied for measuring patulin in apple-juice concentrate, including thin-layer chromatography (TLC) [5–7]; mass spectrometry [8]; colorimetry [9]; micellar electrokinetic chromatography (MEKC) [10]; gas chromatography mass spectrometry (GC–MS) [11,12]; liquid chromatography mass spectrometry (LC–MS) [13–16]; and high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [17–20]. Among these techniques, HPLC coupled with UV detection, is particularly well suited to determine the patulin, since it is a simple and accurate method that does not require derivatization of the analytes (in contrast to the GC method) [21]. Sample pretreatment is considered as the most critical step in the overall analytical process, since it plays a role in analyte extraction, preconcentration and clean-up from co-existing species. In most laboratories, a solution of ethyl acetate and sodium carbonate has been widely used for primary extraction and purification of patulin from apple-juice samples. This pretreatment is expensive and time-consuming, owing to the use of large amounts of organic solvents; moreover, clean-up procedures with sodium carbonate can degrade patulin, since it is more stable in an acidic medium. In recent years, interest in using enzymes in pretreatment to improve recovery has grown [22–24]. Enzymes are ideal catalysts to assist in the extraction of compounds from various sample matrices. Enzymes break down plant tissues complex polysaccharides into simpler molecules such as galacturonic acids. The synergistic activities of the enzymes enhance hydrolysis of the complex carbohydrates. Apple juice can be treated with enzymes such as pectinase and amylase to hydrolyze pectin and starch. The role of pectinases in reducing the cloudiness of fruit juices is well established. Results of enzyme treatment are to eliminate interference, patulin release from tissue samples and increase extraction efficiency [25].

After primary extraction of the patulin from apple juice to liquid phase, conventional liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have been widely used for pre-concentration and clean-up before analysis. LLE is time-consuming, expensive and hazardous to health due to the high volume of toxic solvents used [26–28]. SPE needs less solvent, but is still time-consuming, and often requires a concentration stage that presents disadvantages such as losses in the evaporation step, risk of contamination and loss of sensitivity due to the injection of only a small aliquot of the sample [29–31].

Recently, microextraction techniques have received a great deal of attention. They have several advantages, such as miniaturization (minimal volume of extraction solvent or even solvent-free extraction), automation, high-throughput performance and on-line coupling with analytical instruments. In-tube solid-phase microextraction (SPME) methods have been used to determine patulin in fruit juice and dried fruit samples [32]. In 2006, Assadi et al. introduced dispersive liquid–liquid microextraction (DLLME) as a simple and rapid pre-concentration and microextraction method [33]. In this method, a solvent system containing a mixture of extraction solvent (water-immiscible) and disperser solvent (water-miscible) is injected rapidly by syringe into an aqueous sample. The mixture is centrifuged and the extraction solvent separated and settled at the bottom of a conical tube. The short extraction time, simplicity of the operation, high enrichment factor and high recovery are some advantages of DLLME [34,35].

Ionic liquids (ILs) can be used as an extraction solvent in DLLME. ILs are ionic media resulting from combinations of organic cations and various anions that may be liquid at room temperature. The unique physicochemical properties of ILs, including air and moisture stability, non-volatility, good thermal stability, tunable viscosity and miscibility with water and organic solvents, have led to intense interest in these materials as alternatives to conventional organic solvents in a range of synthetic, catalytic, extraction and electrochemical applications [36]. The use of ILs as an extraction solvent in DLLME has been reported in a number of works [37–39]. Simplicity of operation, quickness, high enrichment factor and recovery, as well as environmental benignity, are advantages of ionic liquid-based dispersive liquid–liquid microextraction (IL-DLLME).

The objective of the present work was the development and optimization of a simple and fast method for determining patulin in apple juice. Enzyme-assisted extraction has been used to achieve high extraction yield and reduce matrix effects. After pretreatment of apple-juice samples, analysis of patulin in a sample solution was performed by IL-DLLME in combination with HPLC-UV for the first time. Several experimental parameters that influence the extraction performance of the proposed method were investigated and optimized by response surface methodology. The merit figures of the proposed method compare with other previous methods.

2. Materials and methods

2.1. Chemicals

Patulin was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) at purity higher than 99%. Methanol and water (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Other chemicals with purity higher than 99%, including acetic acid, potassium hexacyanoferrate, zinc acetate dehydrate, sodium hydroxide (NaOH), and sodium chloride (NaCl), were supplied by Merck. A liquid commercial pectinase and amylase were prepared from Unichem Company (Saveh, Iran), 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF6]), 1-Hexyl-3-methylimidazolium hexafluorophosphate ([HMM][PF6]) and 1-octyl-3-methylimidazolium hexafluorophosphate ([DOMIM][PF6]) were purchased from Acros Organics (NJ, USA). A stock solution of patulin was prepared at a concentration of 1500 mg L⁻¹ by dissolving 15 mg of patulin in 10 mL of water acidified with acetic acid (pH 4.0). Working standard solutions were prepared daily by appropriately diluting this stock solution with distilled water. Stock and working solutions were stored at 4°C in a refrigerator and protected from light. The pH of the solutions was adjusted by dissolving proper amount of sodium hydroxide in water (2 mol L⁻¹).

2.2. Instrumentation

The chromatographic HPLC system (Agilent 1260 series, USA) was equipped with a G1311C quaternary pump, vacuum degasser,
six-port valve (Rhodyne model 7125, USA), G1314B UV–vis detector and ODS column (250 mm × 4 mm I.D., 5 μm). The injection volume was 20 μL and the column temperature was 25 °C (ambient temperature). Agilent Chemstation G2170AA for LC software was used for data processing. Separation of the patulin was achieved with a water–methanol (90:10, v/v) as the mobile phase at a flow rate of 0.8 mL min⁻¹. The effluent was monitored at 276 nm for patulin.

2.3. Sample preparation

2.3.1. Enzyme-assisted extraction

Enzyme-assisted extraction of patulin from apple juice was carried out as detailed below. Portions of solid parts of apple (10 g) were mixed with 45 mL of distilled water and mixed with a blender to obtain a very homogeneous sample solution for 2 min. Then 25 and 100 μL from amylase and pectinase enzymes, respectively, was added to 50 mL of apple juice solution in conical flask, and the mixture incubated in the oven for 120 min at 45 °C. The sample solution was centrifuged at 6000 rpm for 5 min and the supernatant separated and filtered, 1 mL potassium hexaferrocyanide (Carrez solution I) and 1 mL zinc acetate (Carrez solution II) were added to 10 mL of the filtered sample solution to precipitate the protein and soluble carbohydrates. This mixture was agitated using a flat shaker (2 min), and then it’s centrifuged at 6000 rpm for 5 min. The obtained supernatant was immediately used for the DLLME process. The spiked apple juice solution was used for the optimization of the effective parameters in the DLLME process.

2.3.2. Ionic liquid-based dispersive liquid–liquid microextraction

Under optimum conditions, 10 mL of depectinized sample was placed in a 15 mL centrifuge tube and the pH was adjusted to 6.5; then 2.8 g NaCl was added to the sample. Thereafter, a solution consisting of 80 μL [HMIM][PF₆] (as extraction solvent) and 600 μL methanol (as disperser solvent) was injected rapidly by syringe into the extraction device containing 10 mL of sample solution. The mixture was centrifuged for 5 min at 6000 rpm. The dispersed fine particles of extraction solvent separated and settled at the bottom of the conical tube. Finally, 20 μL of the sedimented phase was injected directly into the HPLC using a microsyringe.

2.4. Experimental design

The most effective parameters on DLLME process performance, including pH (A), volume of extraction solvent (B), volume of dispersive solvent (C) and ionic strength (salt amount) (D) were chosen based on the literature and preliminary experiments. For each of the four studied variables, high and low set points were selected to construct an orthogonal design (Table 1). Central composite design (CCD) was used to optimize the values of these factors and reach the best response. The employed CCD included 30 treatments in five levels for four factors, and consisted of six center points. The peak area of patulin was used as the HPLC response to evaluate the extraction efficiency.

A quadratic polynomial model (Eq. (1)) was obtained to predict the response of dependent variable for the extraction of patulin:

$$Y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{6} b_{ij} x_i x_j + \sum_{i=1}^{4} b_{ii} x_i^2$$

where Y is the dependent variable, $x_i$ the independent variable, $b_0$ the constant coefficient, $b_i$ the coefficient of linear effect, $b_{ij}$ the coefficient of interaction effect and $b_{ii}$ the coefficient of squared effect. The software package Design-Expert 8.0.5 (Minneapolis, USA) was employed to analyze the data and experimental design.

3. Results and discussion

Pectin, the most abundant carbohydrate in apple, is thought to interfere with the extractability and chromatographic separation of patulin. The pectinization is being reduced the extraction efficiency of Patulin. Hydrolysis of pectins (depectinization) after pressing is necessary when making a clear apple juice. In the extraction of concentrated apple juice it is necessary to prevent jellification during the concentration. Cloudy juices and solid apple products are usually subjected to depectinization by enzyme to remove co-extraction interferences [40]. Cell-wall-degrading enzymes (i.e. glucanases and pectinases) can weaken or break down the cell wall, rendering the intracellular materials more accessible for extraction. Depectinization is employed specifically for cloudy apple juice, not for clear. Depectinization by pectinase and amylase demulsifies and clarifies cloudy apple juice and enhances the release of patulin from the apple-juice matrix. DLLME was used as a novel sample preparation method. Extraction and disperser solvents are two important variables that can affect the extraction yield in the DLLME procedure. In the selection of extraction solvent, several factors should be considered: (i) efficient extraction of the target compound; (ii) the ability to formation a stable cloudy solution; (iii) low solubility in water; and (iv) good chromatographic behavior. Miscibility of disperser solvent in extraction solvent and aqueous phase (sample solution) is the most important point for selecting a disperser solvent. Among three ionic liquids—1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF₆]), 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIM][PF₆]) and 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIM][PF₆])—[HMIM][PF₆] was selected as a suitable extraction solvent. The capacity of acetone, ethanol and methanol as disperser solvent was evaluated; the results indicated that methanol gave the maximum recovery.

Between 40 and 120 μL of 1-hexyl-3-methylimidazolium hexafluorophosphate and between 300 and 1000 μL of methanol were used. The solubilities of the target analyte and organic extraction solvent in aqueous phase usually decrease with an increase of ionic strength, which is favorable for reaching high recovery. Based on preliminary study, the range 0–3 g of NaCl was selected for this study. Changes in pH can change the solubility of patulin in the aqueous sample and affect the extraction efficiency; therefore pH adjustment was necessary. The range 3–10 of pH was selected to optimize this parameter in the experimental design, and achieved the best response.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter</th>
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<td>+1</td>
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<td></td>
<td></td>
<td>+3 (high)</td>
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</table>

| B | Volume of extraction solvent (μL) | 40 | 60 | 80 | 100 | 120 |
| C | Volume of disperser solvent (μL) | 300 | 475 | 650 | 825 | 1000 |
| D | Salt (g) | 0 | 0.75 | 1.5 | 2.25 | 3 |
Table 2
Analysis of variance (ANOVA) for response surface quadratic model.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>d.f.$^c$</th>
<th>Mean square</th>
<th>F-value$^b$</th>
<th>p-value$^a$</th>
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</table>

$^c$ Degrees of freedom.
$^b$ Test for comparing model variance with residual (error) variance.
$^a$ Probability of seeing the observed F-value if the null hypothesis is true.

3.1. Optimization of the IL-DLLME method

The extraction efficiency by IL-DLLME for the target analyte is influenced by many factors, such as the volume of extraction and disperser solvents, pH and salt effect. In order to obtain the optimized extraction conditions, and thus the best extraction efficiency, we used the peak area of patulin as the HPLC response to evaluate the extraction efficiency under different conditions. Three replicate extractions and determinations were performed for each level. The optimization of significant parameters on the efficiency of the DLLME process was done using CCD, one of the most common response surface designs. It is constructed from several supernumeral designs and comprises a factorial design (2$^f$) augmented with (2$^f$) star points, where $f$ is the number of factors to be optimized, and with (n) central points [41].

A quadratic model was fitted to the obtained data. This model is used to predict response at any point, even those not contained in the design. The RSM model equation is shown in Eq. (2) in terms of coded value:

$$R = +16.86 - 0.89A + 0.69B + 0.22C + 1.59D - 0.12AB + 0.15AC + 0.0004AD + 0.075BC + 0.075BD - 0.075CD - 8.80A^2 - 5.40B^2 - 2.60C^2 + 5.20D^2$$

where $R$ is the sum of relative peak areas for patulin as a function of $A$ (pH), $B$ (volume of ionic liquid), $C$ (volume of disperser solvent) and $D$ (salt).

In addition to describing the linear effects of factors on the response, CCD explains the interaction and quadratic effects of the variables. In this study, analysis of variance (ANOVA) was used to evaluate the significance of each factor and interaction term (Table 2). The quality of the polynomial model was expressed by the coefficient of determination ($R^2$ and adjusted-$R^2$). An $R^2$ of 0.9923 and an adjusted-$R^2$ of 0.9852 showed a good relationship between the experimental data and the fitted model, and a high potential for the model to predict response. From the ANOVA summary, the model was found to be significant, with a $p$-value less than 0.0001 and an $F$-value of 138.46. The lack-of-fit (LOF) $F$-value of 0.2960 implies that the LOF was not significant relative to the pure error. The parameters of pH ($A$), volume of extraction solvent ($B$), volume of disperser solvent ($C$) and salt ($D$) had significant linear effects on response. The interactive effect of pH and volume of extraction solvent (the $AB$ term in the equation) and the interactive effect of volume of extraction solvent and volume of disperser solvent (the $BC$ term) on response were also significant. Fig. 1 indicates the predicted versus actual responses. Most plots were scattered monotonously around the line; this indicates a good correlation between predicted and actual responses, and thus a good fit for the proposed quadratic model. Fig. 2 shows the residuals versus the predicted responses. The residual plots were scattered randomly, indicating that the variance of the experimental measurements was constant for all values of Y. The next step was to find the optimum value of each factor to achieve the maximum response. To evaluate the interactive effect of two variables on response, the use of three-dimensional graphs of the model is suggested. In the proposed model, the coefficient AB (coefficient of interaction for pH value and volume of ionic liquid) is large. Their interaction was significant and simultaneous changes in both variables (increasing the pH value to 6.5 and decreasing the ionic-liquid volume to 80 µL) led to enhanced extraction performance. Thus the volume of ionic liquid and pH had positive effect on the extraction performance, and increasing pH from 3 to 6.5 increased response. But the extraction efficiency was decreased in pH over 6.5. The ionic-liquid volume had a large effect on the enrichment factor.

Fig. 1. The predicted response vs. the observed response.
With increased ionic-liquid volume, the final sediments phase obtained by centrifugation increased, resulting in a decrease of the concentration of the target analyte in the sediments phase. Although the extraction recovery remained almost constant, the enrichment factor decreased, leading to a decrease of the sensitivity of the determination for the target compounds. Therefore, the optimal extraction-solvent volume should ensure both a high enrichment factor and the enough volume for subsequent determination after centrifuging (Fig. 3a). The combined effect of salt and volume of disperser solvent is shown in Fig. 3b. The maximum response was obtained at 600 μL disperser solvent and 2.8 g of salt. Fig. 3c shows the response surface obtained by plotting pH versus salt. The extraction efficiency improves with increasing pH and salt. The addition of salt to the sample solution due to the salting-out effect often has a positive influence on extraction efficiency, and it is expected that with increasing salt, the amount of extracted analyte increases [42]. This can be explained by the participation of polar molecules of solution surrounding the target analytes in electrostatic interaction with the salt ions in solution, which releases the analyte and, thus, the extraction efficiency increased. Fig. 3d shows the response surface obtained by plotting pH versus volume of disperser solvent, with the center points of the volume of ionic liquid 80 μL and salt 1.5 g held fixed. The extraction efficiency increased with increasing pH and disperser solvent volume. Therefore, the maximum response was obtained at a pH of 6.5 and 600 μL of disperser solvent. According to the results from the optimization study, the optimal experimental conditions were: salt 2.8 g, pH 6.5, volume of ionic liquid 80 μL and volume of disperser solvent 600 μL.

After these optimization experiments, a comparison between enzymes and non-enzymatic treatment regarding their performances of patulin extraction from apple juice sample from mottled apples was performed (Fig. 4). The extraction yield of patulin without enzyme treatment was very low (Fig. 4a) while, the highest extraction yields was obtained with enzyme treatment (Fig. 4b). It is also evident in Fig. 4 that EAE stage before microextraction analysis is capable of removing co-extraction interference considerably, and a clear chromatogram was obtained.

### 3.2. Quantitative analysis of patulin

To evaluate the performance of the proposed method, linearity, repeatability, recovery, limit of detection (LOD) and limit of quantification (LOQ) were investigated under optimized experimental conditions. The results are shown in Table 3. Nine-point calibration curves of patulin were constructed by peak area of patulin versus their concentrations. Good linearity was obtained ($R^2 > 0.99$) in the
concentration range of 1–200 ng mL$^{-1}$ for patulin. The comparative peak area, calculated from seven replicates extraction from one apple juice, was employed to estimate the repeatability and is shown as relative standard deviation percentage (RSD %). As the Table 3 shows, the RSD % was 7.5 for patulin using proposed method. The enrichment factor expressed as the ratio of the final concentration of the analyte in the sedimented phase to its concentration in the original solution was 162. The recovery for patulin was determined by comparing the amount of analyte added to an apple-juice sample with the concentration found after the procedure. The recovery value of the extraction of patulin from the apple-juice sample was 89%. The limit of detection (LOD) and limit of quantification (LOQ) (based on signal-to-noise ratios of 3 and 10, respectively) for patulin when using the optimized conditions and HPLC were 0.15 ng mL$^{-1}$ and 0.5 ng mL$^{-1}$, respectively (Table 3).

A comparison of the results for extraction and determination of patulin using this optimized novel method with literature data using other methods followed by HPLC-UV [29,30] shows that the proposed method is comparable or better for analysis of patulin (Table 4).

3.3. Application to real sample

The reliability of the proposed method was evaluated to analyze of patulin from apple juice samples. These results showed that HPLC analysis after enzyme treatment and IL-DLLME is a powerful method for monitoring patulin at very low concentrations in different apple-juice samples (Table 5). Fig. 5 shows the chromatograms obtained by EAE-IL-DLLME-HPLC-UV for an apple-juice sample. A clean separation and a good chromatogram are readily achieved without the presence of sample matrix interference.

4. Conclusion

In the present work, we successfully developed the EAE-IL-DLLME procedure followed by HPLC-UV for rapid extraction and quantification of patulin at very low levels in apple juice for the

![Fig. 4. The chromatogram obtained by IL-DLLME-HPLC for a real sample under optimum conditions. (a) without enzyme treatment and (b) with enzyme treatment.](image)

![Fig. 5. The chromatogram obtained by EAE-IL-DLLME-HPLC for an apple juice under optimum conditions. (a) Non-spiked and (b) spiked with 50 ng mL$^{-1}$ of patulin (peak 1).](image)
first time. Enzyme-assisted extraction (EAE) was employed in the pretreatment stage to break down the complex polysaccharides in plant tissues into simpler molecules, such as galacturonic acids, and to enhance the extraction efficiency for patulin. The results also show that the use of carréz solvent to sediment proteins and carbohydrates can greatly decrease the interference of the real sample matrix. RSM based on CCD was used to determine the interaction and quadratic effects of variables, and also optimize the parameters for microextraction. Low consumption of solvent, simple experimental setup, a high enrichment factor, good precision and no matrix interference are clear advantages of this method. The comparison of the proposed method with others methods for patulin analysis showed comparable or better results and proved the applicability of EAE-IL-DLLME followed by HPLC-UV for extraction and determination of patulin in apple juice.

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