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A SIMPLE AND SELECTIVE METHOD FOR SIMULTANEOUS DETERMINATION OF PATULIN AND 5- HMF IN HONEY AND APPLE JUICE BY HPLC-UV

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Abstract: A selective and sensitive method using a high-performance liquid chromatography-UV detector (HPLC-UV) for the simultaneous determination of Patulin (PAT) and 5-(hydroxymethyl)furfural (5-HMF) in honey and apple juice was developed. The sample preparation technique QuEChERS was applied to increase the sensitivity and selectivity. The linear ranges for PAT and

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5-HMF detected by the method were 2.0 ppb ÷ 200.0 ppb and 2.0 ppm ÷ 20 ppm. The results of method validation show that the recovery efficiency for PAT ranged from 92.8% ÷ 98.2% with % RSD_r from 0.56% ÷ 0.63% and from 97.9% ÷ 101.2% with %RSD 0.39% ÷ 0.67% for 5-HMF and LOD and LOQ values of the method for PAT and 5-HMF detection were respectively: 0.6 and 2.0 ppb; 0.6 and 2.0 ppm. The influence of the matrix effect on the accuracy, repeatability, and recovery of the method was insignificant. The proposed method was applied to quantify the content of PAT, and 5-HMF in actual samples, collected in Ho Chi Minh City, Vietnam. The result showed that PAT was not found in honey samples but was detected in 5 of 10 apple juice samples. In addition, 5-HMF was detected in 3 of 10 honey samples and 10 of 10 apple juice samples.

Keywords: HPLC-UV, Pantulin, 5-HMF, apple juice, honey.

Introduction

The organic compound 4-hydroxy-4H-furo (3,2-c) pyran - 2 (6H) - one, Patulin (PAT) (figure 1), a metabolite, is produced during the metabolism of many species of molds, such as *Aspergillus*, *Penicillium* including *A. clavatus, A. giganteus, A. terreus, P. urticae, P. expansum,* and *B. nivea.* Among them, *P. expansum* is the most important patulin-producing species.[2] PAT has been detected in many foods, including fruits, juices, vegetables, jams, cooked corn, meat, and honey. Patulin is commonly found in apples and apple products and is one of the important indicators of product quality related to apples.[3] PAT is a toxic metabolite, neurotoxic, mutagenic, and genotoxic. It can be toxic to many organisms, including microorganisms, higher plants, and animals.[4] The maximum allowable level of patulin in apple juice and cider is 50 µg/L, and in solid apple products is 25 µg/kg set by WHO. According to the EU, the permissible content of patulin in products for babies and children is 10 µg/kg.[5] In addition, JECFA has established a maximum allowable patulin intake of 0.4 µg/kg body weight/day. According to QCVN

8 - 1:2011/BYT, the limit of PAT in food for children under 36 months is 10 μg/kg.

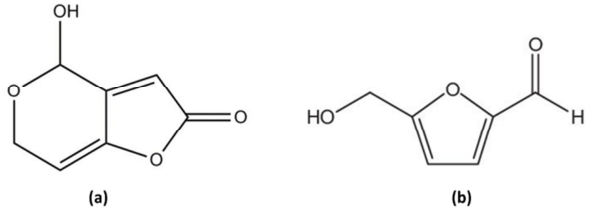

Figure 1. Chemical structures of PAT (a) and 5-HMF (b).

The compound 5-(hydroxymethyl) furfural (5-HMF), a cyclic aldehyde, is produced by the degradation of sugars in the Maillard reaction, the aldehyde group formed as a result of the dehydration of ketopentoses, especially in the acidic environment and under high temperatures. This compound is considered to be the most important intermediate formed in two reactions: (i) degradation of hexose under acid catalysis and (ii) degradation of 3-deoxyosone in the Maillard reaction.[6] 5-HMF is not present in fresh fruit but is produced from sugar-containing products during heat treatment such as drying or cooking.[7] Under acidic conditions, 5-HMF can form even at low temperatures, its concentration rises significantly with increasing temperature.[8] In addition to temperature, the rate of 5-HMF formation in foods also depends on pH, water activity,[8] the type of sugars,[9] and the type and concentration of cations.[10] The compound 5-HMF is used as a quality indicator for a wide range of fruits, coffee, honey, and milk to monitor the heating of cereal products.[11] Both 5-HMF and PAT compounds are important criteria for evaluating the quality of fruit juice and honey products. The presence of 5-HMF is considered a sign of deterioration in the quality of the product. 5-HMF can cause genetic mutations, DNA damage, cytotoxicity, nephrotoxicity, and cancer in studies at the cellular level.[12] The

Codex Alimentarius of the World Health Organisation[13] and the European Union[14] have established a maximum HMF quality level in honey (40 mg/kg) and apple juice (50 mg/kg) as deterioration and heat-treatment indicators. The International Federation of Fruit Juice Processors (IFFJP) recommends a maximum concentration of 5-10 mg/L of HMF in fruit juices and 25 mg/L in fruit concentrates.[15] There are several methods to determine PAT content in fruit samples that have been developed by researchers.[16,17] For the determination of 5-HMF, A.Serra-Cayuela et al. used LC-DAD-MS/MS and NMR spectrometry.[18] Angela Alcázar applied the HPLC method for the determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in alcoholic beverages.[19] Nail Altunay used the UV-VIS method with the sample preparation using magnetic ionic liquid ultrasound-assisted dispersive liquid-liquid microextraction.[20] Hassan et al. developed the method HPLC with Diode Array-Fluorimetric Detection for simultaneous quantitative assessment of Ochratoxin A, Patulin, and 5-Hydroxymethylfurfural.[21]

After investigating the equipment and methods of the previous researchers, it can be seen that using HPLC-UV equipment for PAT and 5-HMF quantitation combined with the sample preparation QuEChERS technique to increase sensitivity, and selectivity and shorten the time of procedure is meaningful and necessary.

Currently, in Vietnam, the method to simultaneously determine the content of PAT and 5-HMF has yet to be published and has yet to set a standard. Therefore, we conducted this study intending to develop a quantitative method to analyze the content of these two substances for scientists to refer to and also help authorities have a perspective on the toxic substances in honey products and drinking beverages. Since then, this

research has warned about food safety and protected the community's health.

Materials and method

Chemicals and reagents

All reagents were of analytical grade, 100.0 µg/mL ± 0.1 µg/mL of PAT, and 5-HMF reference standards were purchased from Sigma-Aldrich (Germany). Methanol and Acetonitrile were of HPLC grade and acquired from J. T. Baker (Phillipsburg, USA). The SPE solid phase extraction powder $MgSO_4$, C18, PSA, and salt mixture (4.0 g of $MgSO_4$, 1.0 g of NaCl) were obtained from Waters (Milford, MA, USA).

Instrumentation

The method development and validation were performed on the ultrahigh-performance liquid chromatography (HPLC) system, including the column oven and thermostat autosampler (Agilent, Thermo Fisher Scientific, Bremen, Germany) combination with the UV detector and Cosmosil C18, 150 x 3.0 mm, 3 µm column.

Standard solutions

For preparing a stock solution of 1.0 mg/mL, 10 mg of pure crystal 5-HMF was accurately weighed into a 10 mL volumetric flask and dissolved in Acetonitrile (ACN). All stock solutions were kept in the freezer (-18^{o}C). The standard solution of 2 mg/L of PAT was prepared by diluting 0.2 mL of 100.0 µg/mL stock standard solution into 10 mL acetonitrile. A working solution mixture was prepared by diluting 0.2 mL of the stock standard solution 5-HMF 1 mg/mL and 0.1 mL of PAT standard solution 2 mg/L to 10 mL ACN to obtain a mixture solution containing 20 ppm for

5-HMF and 20 ppb for PAT. All standard solutions were kept refrigerated (2 - 8 °C) when not in use.

Sample Preparation

The technique QuEChERS was applied for sample preparation.[22] Firstly, 5.0 ± 0.1 g of the test sample was weighed and transferred directly into a 50-mL glass centrifuge tube. Next, 5.0 mL of water was precisely added and shaken in 3.0 min. Afterward, 10 mL of acetonitrile was added and shaken for 3.0 min at room temperature. Then, the salt mixture (4.0 g of $MgSO_4$ and 1.0 g of NaCl) was added and shaken vigorously. This mixture was centrifuged at 4000 rpm for 3.0 min). 2.0 mL of the upper layer was transferred into a 15 mL centrifuge tube containing clean-up powder (0.3 g of $MgSO_4$ and 0.1 g of C18), shaken for 2.0 min, and centrifuged at 4000 rpm for 3 min). Finally, 1.0 mL of the upper solution was diluted 5 times with deionized distilled water and filtered through a 0.45 μm membrane into the vial for analysis by HPLC-UV.

Method validation

Linear range

The stock solution (1 mg/mL) of PAT and 5-HMF reference standard was serially diluted with deionized water to obtain nine different calibration standards containing 0.4 ppb; 1.0 ppb; 2.0 ppb; 4.0 ppb; 10 ppb; 20 ppb; 40 ppb; 100 ppb, 200 ppb of PAT and 0.04 ppm; 0.1 ppm; 0.2 ppm; 0.4 ppm, 1.0 ppm; 2.0 ppm; 4.0 ppm, 10 ppm, 20 ppm of 5-HMF. Each sample was prepared in triplicate and analyzed in triplicate. Calibration graphs were generated by plotting peak areas versus PAT and 5-HMF concentrations and were used to determine the linear regression coefficient (R^2).

Matrix effect

Evaluation of the influence of the matrix on the accuracy of the method is necessary for assessing analytical procedures. If the effect of the matrix is negligible, it could use an external standard curve to quantify the analyte.[23] In contrast, when the influence of the matrix reaches serious on the accuracy, it could use a standard curve on the blank sample or standard addition, an internal isotopic standard could be used, or even change the sample processing procedure. In this study, to evaluate the influence of the sample matrix, a volume of 0.05 mL of 2 ppm PAT standard and 0.05 mL of 5-HMF 200 ppm standard was added to the sample solution after extraction, then filtered through a 0.45 µm filter into the vial and analyzed on HPLC – UV. Each sample was prepared in triplicate and analyzed in triplicate.

Recovery

Method recoveries were performed at three concentrations, low, medium, and high, to ensure that the analytical procedure achieves high accuracy at different concentrations of the analyte in the sample. In this work, the recovery of the method was determined by adding a standard to the apple juice and honey sample solution at three concentrations of 25 ppb, 50 ppb, 80 ppb for PAT, and 25 ppm, 50 ppm, and 100 ppm for PAT with 5-HMF. The analytical procedure was repeated seven times. In addition, the factors affecting the recovery, such as extraction solvent and extraction salt, were also investigated.

Repeatability

The repeatability of the method is used to control for reliability and evaluate the random effects on the analysis results. The repeatability limit

parameter r^{24} (repeatability limit) is determined by the evaluation of the method's repeatability limit. Repeatability must be less than or equal to that value with a 95% confidence level: r= 2.8 x SDr (where: SDr is the standard deviation). For conducting experiments, we added standards at 45 ppb with PAT and 150 ppm with 5-HMF to two samples: a juice sample and a honey one, which was positive for these two substances. The analytical procedure was repeated 7 times to calculate the SDr value.

LOD and LOQ

To estimate the LOD and LOQ of the method, the experiment was carried out as follows: (i) the estimated minimum concentration (C_{min}) of the PAT and 5-HMF standards was 2 ppb, and 2 ppm was added to the apple juice and honey sample matrix; (ii) After sample processing, the analytical procedure was repeated 11 times to calculate the standard deviation (SD); (iii) LOD and LOQ were determined by the expression LOD = 3SD and LOQ = 3LOD, in which, LOD must match the condition: $LOD < C_{min} < 10LOD$ and $\frac{S}{N} = \frac{\overline{X}}{SD}$ in the range 3÷10, where: S: Signal, N: Noise, and $\overline{X}$ is the average concentration.[23]

Results and discussion

Chromatographic conditions

Selection of detection optimum wavelength

For UV detectors, the selection of the wavelength at which absorbance reaches the maximum is very important. The absorption spectra of PAT and 5-HMF were scanned in the wavelength range of 200 - 300 nm, the results showed that both the UV spectrum of PAT and 5-HMF has a

fairly wide absorption band with a maximum at 276 nm for PAT and 284 nm for 5-HMF. Therefore, wavelengths at 276 nm and 284 nm were chosen for the analysis of PAT and 5-HMF.

Mobile phase

The mobile phase composition is the most important factor in high-performance liquid chromatography and directly affects the ability separating of the analyte from those in the matrix sample. In this study, the used stationary phase is a silica compound with a C18 alkyl chain, which is consistent with the PAT analysis and 5-HMF of medium polarity (both compounds contain the –OH group). Therefore, the mobile phase is suitable for this study and selected from strong polarity (water) to medium (ACN) and the acetic acid is chosen for the changing pH values.

Compound 5-HMF contains -CHO group, capable of Aldol addition reaction in an acidic medium, so the solvent system including 1.0 % acetic acid - ACN (95: 5) was selected to compare with mixed solvent water - ACN (95: 5). The chromatograms in Figures 2 and 3 show that with the mobile phase water and ACN, a noise signal appears at the retention time of PAT in the honey sample matrix is 4.4 min, and retention time of 5-HMF is over 8 min. While with the 1.0% acetic acid - ACN mobile phase, the noise signal around the PAT peak is negligible and the retention time of 5-HMF is about 7.3 min (Figure 4 and Figure 5). Figure 6 reveals that with the increasing concentration of acetic acid from 0.2 % to 1.0 %, the retention time of 5-HMF decreases. the growing concentration of acetic acid in the mobile phase causes the decrease in pH, increasing the Aldol addition reaction rate of the 5-HMF compound. Then, the 5-HMF molecules were more positively charged and reduced the interaction with the stationary

phase. Therefore, a mobile phase solvent containing 1.0 % acetic acid was chosen for this analytical procedure.

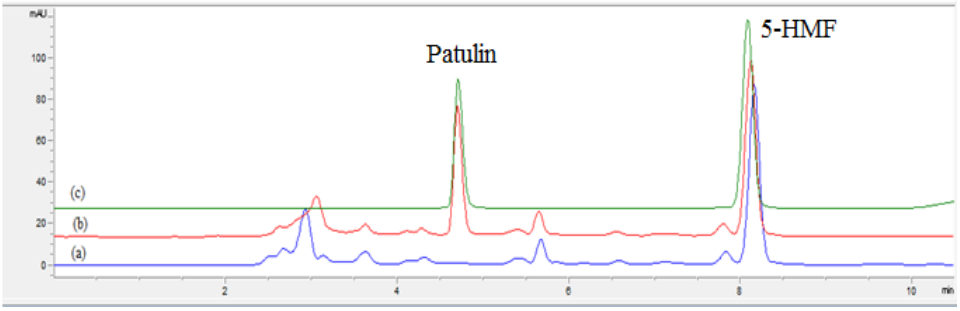

Figure 2. Typical chromatogram of (a) apple juice extract, (b) apple juice extract + 10 ppb of PAT, (c) 10 ppb of PAT + 5 ppm of 5-HMF in ACN, with mobile phase water: ACN (95:5), Column C18, 150 x 3.0 mm, 3 µm, 0.8 mL/min flow rate, 20 µL injection volume.

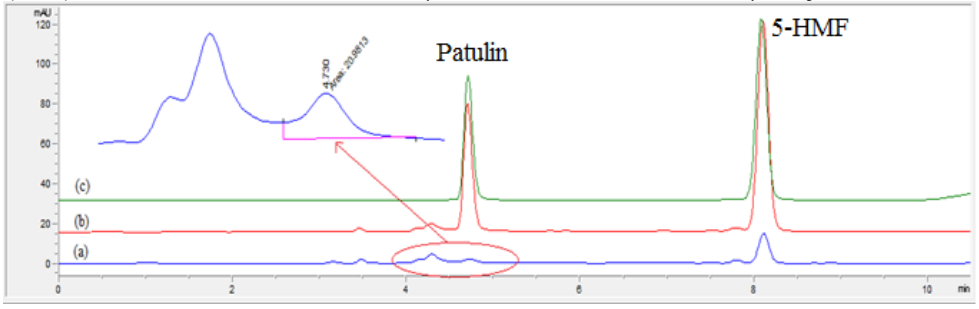

Figure 3. Typical chromatogram of (a) honey extract, (b) honey extract + 10 ppb of PAT+5ppm of 5-HMF, (c) 10 ppb of PAT + 5 ppm of 5-HMF in ACN, with mobile phase water: ACN (95:5), Column C18, 150 x 3.0 mm, 3 µm, 0.8 mL/min flow rate, 20 µL injection volume.

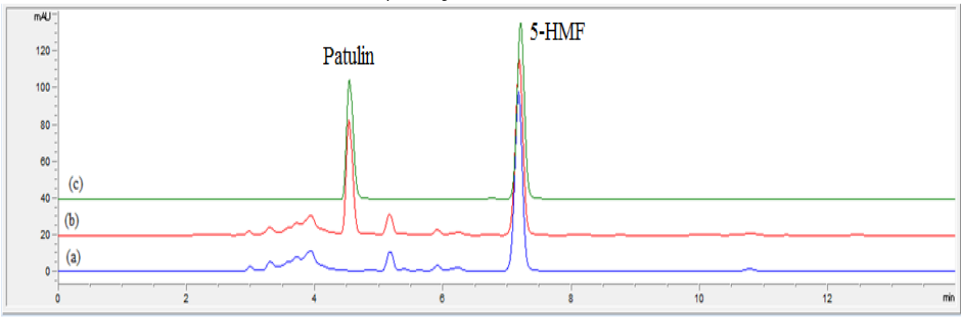

Figure 4. Typical chromatogram of (a) apple juice extract, (b) apple juice extract + 10 ppb of PAT + 5 ppm of 5-HMF, (c) 10 ppb of PAT + 5 ppm of 5-HMF in ACN, with mobile phase 1% of CH_3COOH: ACN (96:4), Column C18, 150 x 3.0 mm, 3µm, 0.8 mL/min flow rate, 20 µL injection volume.

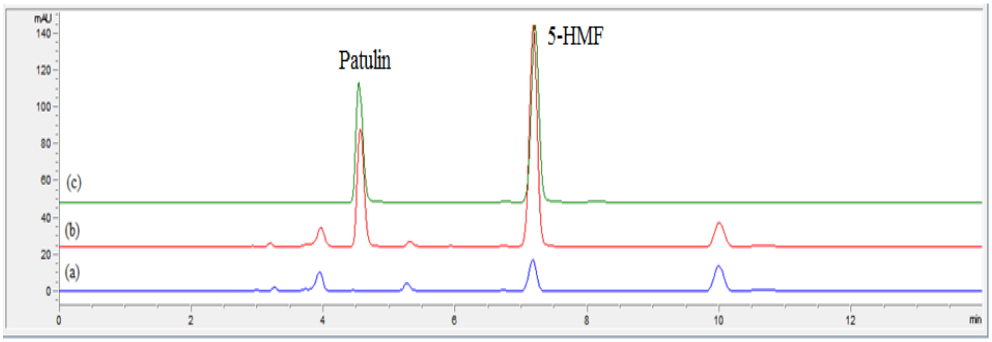

Figure 5. Typical chromatogram of (a) honey extract, (b) honey extract + 10 ppb of PAT+5 ppm of 5-HMF, (c) 10 ppb of PAT + 5 ppm of 5-HMF in ACN, with mobile phase 1% of CH_3COOH: ACN (96:4), Column C18, 150 x 3.0 mm, 3µm, 0.8 mL/min flow rate, 20 µL injection volume.

In reverse phase chromatography, water has the weakest elution, so as the proportion of organic solvents increases, the analytes tend to elute faster because of the strong interaction of the mobile phase with the analyte than the interaction force of the analyte with the stationary phase. Therefore, when analyzing many compounds of different polarities in the same analytical sample, the mobile phase gradient mode is often applied for faster analysis time and sharper and more symmetrical chromatographic peaks. However, increasing the organic solvent ratio too quickly will lead to peak overlap.

Since PAT and 5-HMF are compounds with moderate polarity, the interaction force with the stationary phase is not too strong; so for the analytes not to be eluted too quickly and to avoid peaking with the background interference, the selection of a solvent with a high ratio of water and a solvent isocratic program is necessary. Therefore, in this experiment, we have investigated the percentage of solvent in the mobile phase (1.0 % of CH_3COOH: ACN) as follows: (97:3), (96:4), (95:5), (94:6), and (92:8), with sample processing as mentioned above.

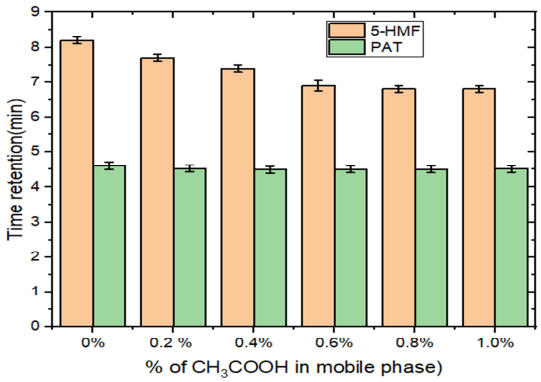

Figure 6. The dependence of retention time of PAT and 5-HMF in real samples on acetic acid concentration in the mobile phase.

The results show that when the proportion of organic solvents was increased, the analytes are eluted faster, and at the same time, the spreading of the peaks reduces. However, when the organic solvent content was increased to the ratio of 6 % and 8 %, patulin was eluted quickly, and peaking occurred at the peak foot, so a mobile phase ratio of 1.0 % acetic acid: ACN (96:4) was chosen for this analytical procedure.

In conclusion, after the composition and mobile phase ratio were optimized on the HPLC - UV instrument, the optimal parameters were obtained as follows: chromatographic column: Comosil C18, 150 x 3.0 mm, 3µm, mobile phase composition including 1.0 % acetic acid: ACN (96:4), flow rate: 0.8 mL/min, sample injection volume: 20 µL, and retention times of PAT and 5-HMF were respectively 4.74 and 7.17 minutes in the sample backgrounds.

Sample extraction

Effect of extraction solvent

In QuEChERS extraction, ACN is the commonly used solvent because of its good extraction ability with organic compounds.[22,26] Depending on the properties of the compounds to be analyzed, the ACN

solvent can be added with acids or bases to create a suitable pH environment for the extraction substances. It can be seen in the sample preparation section, the apple juice and honey samples were added to 20 ppb for PAT and 8.0 ppm for 5-HMF. Next, the extractions were carried out to determine the recovery efficiency by using the following solvents: ACN, ACN 1% acetic acid, ACN 5% acetic acid, ACN 2% NH_3, ACN 4% NH_3. Each sample was prepared in triplicate and analyzed in triplicate. The results showed that the acidic and neutral solvent extraction presented almost similar recovery efficiency. However, this value of 5-HMF in the honey sample matrix decreased with the ACN 2% NH_3 and 4% NH_3 solvent (76.1% and 67.2%), and the recovery efficiency of 5-HMF in the apple juice sample also decreased slightly from 97.0% to 93.8%. Hence, the solvent mixture ACN+1% CH_3COOH is the optimal extraction solvent selected for the analytical procedure.

Effect of extraction aid salt on the recovery efficiency

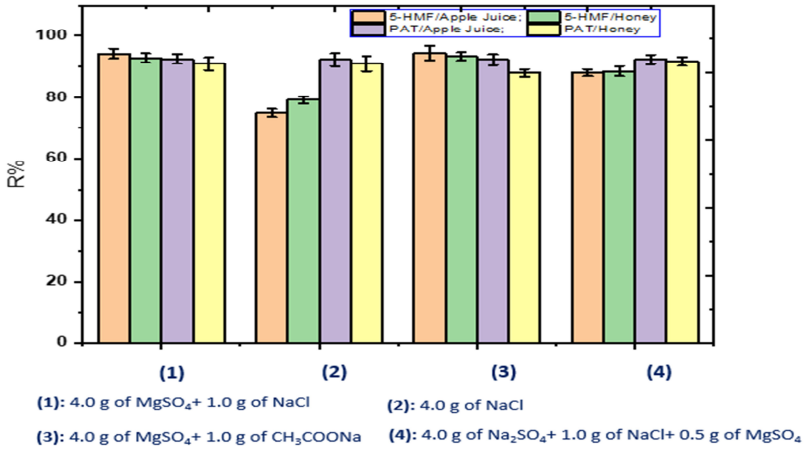

Figure 7. Effect of aiding extraction salt on the recovery of the method.

For the QuEChERS extraction technique, $MgSO_4$ is used as the extraction aid salt.[22, 27–29] The salt dissolved in the solution causes to

generate strong heat that makes the separation of the two phases of water and ACN more favorable. We firstly experimented with the procedure in the sample preparation section and turn, changed the salts to support the extraction of samples to be investigated as follows: 4.0 g of NaCl, 4.0 g of $MgSO_4$ + 1.0 g of NaCl, 6.0 g of $MgSO_4$ + 1.5 g of CH_3COONa, 4.0 g of Na_2SO_4 +1.0 g of NaCl + 0.5 g of $MgSO_4$. Each sample was prepared in triplicate and analyzed in triplicate. Then, the extraction efficiency at standard addition concentrations at 40 ppb for PAT and 8.0 ppm for 5-HMF was determined.

Figure 7 shows that with the extraction aid salt of 4.0 g NaCl and 4.0 g Na_2SO_4 +1.0 g NaCl + 0.5 g $MgSO_4$, the 5-HMF extraction efficiency was lower than that of other ratios. Meanwhile, the extraction efficiency of the mixture of 4.0 g $MgSO_4$ + 1.0 g NaCl and 6.0 g $MgSO_4$ + 1.5 g CH_3COONa is almost equivalent. Therefore, the mixture of 4.0 g $MgSO_4$ + 1.0 g NaCl was the extraction aid salt mixture of choice for the analytical procedure.

Effect of SPE clean-up powder

The apple juice and honey sample matrix contain a variety of organic acids and colored compounds. Therefore, the use of cleaning powders is necessary to reduce the influence of these substances on the accuracy of the method as well as to avoid contamination of the chromatographic system.

In this study, the used cleaning powder was C18 powder to remove the color of the extract solution and PSA powder to remove organic acids. In addition, we also used $MgSO_4$ to anhydrous the sample extract. As in the procedure in the sample preparation section, the standard of 40 ppb for PAT and 8.0 ppm for 5-HMF was added to two samples of apple juice and honey,

the sample solution was then cleaned as the following systems: 0.3 g $MgSO_4$ + 0.1 g C18, 0.6 g $MgSO_4$ + 0.1 g C18, 0.3 g $MgSO_4$ + 0.1 g PSA, 0.6 g $MgSO_4$ + 0.1 g C18 + 0.1 g PSA. Each sample was prepared in triplicate and analyzed in triplicate.

The recovery efficiency was determined to evaluate the cleaning ability of the selected powder systems. As can be seen in Figure 8, the results showed that the extraction efficiency of PAT was not affected by cleaning powder containing PSA, but the extraction efficiency of 5-HMF in both apple juice and honey samples were severely affected (extraction efficiency < 10%). The influence of PSA on the extraction capacity of 5-HMF can be explained that the aldehyde functional group (-CHO) in the 5-HMF compound reacted with the primary amine group of PSA powder, so 5-HMF was kept in the extract powder. The mixture of 0.3 g $MgSO_4$ + 0.1 g C18 and 0.6 g $MgSO_4$ + 0.1 g C18 gave a similar performance; therefore, a cleaning powder mixture consisting of 0.3 g $MgSO_4$ + 0.1 g C18 was selected in the following experiments.

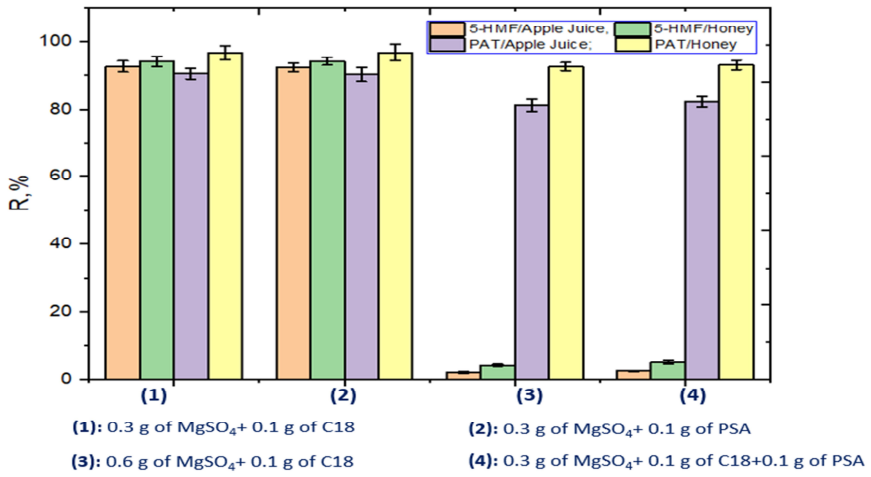

Figure 8. Effect of cleaning up powder on the recovery of the method.

Method validation

Linearity ranges

The linear range of PAT was 2.0 ppb to 200 ppb and the regression equation was y = 21.72x - 4.49, R^2 = 0.9999, but 5-HMF showed a linear range from 2.0 ppm to 20 ppm and the regression equation was y = 164.34x - 9.43, R^2 = 0.9999. In addition, the concentration range selected meets all of the standard curve requirements for quantitative analysis according to AOAC.[30]

Matrix effect

The influence of the matrix sample on the method's selectivity is an essential factor in the evaluation of the analytical procedure, so the study carried out this procedure. The obtained results showed that the influence of both honey and apple juice matrix on the measurement's selectivity with analytes was insignificant because the effect of the matrix samples on the recovery was performed at the level of 20 ppb for PAT and 2.0 ppm for 5-HMF. At the above concentrations, recoveries ranged from 99.5% to 100.4%, and RSD varied from 0.8% to 1.9%.

Recovery

The results of method validation show that the recovery efficiency for PAT ranged from 92.8 % ÷ 98.2 % with $\%RSD_r$ from 0.56% ÷ 0.63 % and from 97.9 % ÷ 101.2 % with % RSD 0.39 % ÷ 0.67 % for 5-HMF as in Table 1. These data also show that this analytical procedure has a high recovery.

Repeatability

The repeatability and reproducibility were checked and expressed as the relative standard deviation (RSD) for a series of evaluations. The

observed values in all samples in Table 1 show that this method is adequately precise for the analysis. The recovery and precision obtained in this research are related to or better than those in several published works because these results indicated adequate repeatability and reproducibility of the proposed method.

LOD and LOQ

The method's limit of detection (LOD) for PAT was 0.6 ppb and 0.6 ppm for 5-HMF. The limit of quantification (LOQ) for PAT was 2.0 ppb, and 2.0 ppm for 5-HMF in both sample matrices. For confirming these LOQ values, PAT and 5-HMF standards were added to the blank background at these LOQ levels of the method (PAT 2 µg/kg and 5-HMF 2 mg/kg), repeated 7 times for calculating the recoveries. The results show that the recovery efficiency of the measurement for PAT is 93.5% with 2.1% of RSD and 95.2% and 2.5% of RSD. The recovery efficiency and repeatability are good, meeting the requirements for method validation of AOAC; therefore, the LOQ values of this method were acceptable.[30]

Application to real samples

This method was applied to determine the content of PAT and 5-HMF in 10 samples of apple juice and 10 samples of honey. The results are shown in Table 2. The obtained data showed that up to 5 of 10 apple juice samples were contaminated with PAT at concentrations higher than the acceptable limit (50 µg/L). These infected products have a different color than products on the market, of which 3 of 5 samples have expired. Besides, 5-HMF was detected in all samples, but the 5-HMF content was still within the allowable range (no more than 25 mg/L). Meanwhile, PAT was not detected in the honey samples but 5-HMF was found in 3 of 10 honey samples with concentrations greater than 40 mg/kg.

Table 1. Results of method validation.

Analytes	**PAT**		**5-HMF**	
Sample matrices	Apple juice	Honey	Apple juice	Honey
LOD	0.6 ppb	0.6 ppb	0.6 ppm	0.6 ppm
LOQ	2.0 ppb	2.0 ppb	2.0 ppm	2.0 ppm
Recovery, %	92.8 – 98.2	92.2 – 93.1	98.1 – 101.2	97.9 – 100.3
Repeatability, $\%RSD_r$	0.63	0.56	0.67	0.39
Repeatability limit, %r	0.79	0.68	2.9	1.9
Reproducibility, $\%RSD_R$	0.92	0.76	1.1	1.0
Reproducibility limit, %R	1.1	0.92	4.8	5.2

Table 2. The results of analyzing PAT and 5-HMF in real samples.

Sample No	**Apple Juice**		**Honey**	
	PAT, ppb	**5-HMF, ppm**	**PAT, ppb**	**5-HMF, ppm**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
1	137.35 ± 0.50	11.4 ± 0.13	< LOD (0.6)	76.1 ± 1.1
2	116.59 ± 0.29	15.22 ± 0.20	< LOD (0.6)	13.09 ± 0.45
3	165.99 ± 0.50	6.33 ± 0.18	< LOD (0.6)	36.04 ± 0.85
4	116.25 ± 0.50	11.73 ± 0.13	< LOD (0.6)	13.32 ± 0.16
5	75.22 ± 0.29	20.60 ± 0.22	< LOD (0.6)	13.74 ± 0.24
6	< LOD (0.6)	10.55 ± 0.16	< LOD (0.6)	99.0 ± 1.4
7	< LOD (0.6)	24.87 ± 0.16	< LOD (0.6)	10.33 ± 0.21
8	< LOD (0.6)	10.80 ± 0.16	< LOD (0.6)	14.34 ± 0.18
9	< LOD (0.6)	15.85 ± 0.24	< LOD (0.6)	13.05 ± 0.20
10	< LOD (0.6)	13.66 ± 0.19	< LOD (0.6)	95.1 ± 1.4

Conclusion

The study described a simple, precise, and sensitive HPLC-UV method for determining furfurals 5-HMF and patulin. With data collection, the proposed method showed effectiveness and straightforwardness. Especially it is allowed the simultaneous determination of PAT and 5-HMF contained in food matrices. Furthermore, the content of PAT and 5-HMF can be attributed as an indicator that reveals the stability and quality of carbohydrate-containing foods. These values are helpful for routine quality control of food products.

Conflicts of interest: There are no conflicts to declare.

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References

1. Wright, S. A. Patulin in food. *Curr. Opin. Food Sci.*, **2015**, *5*, 105-109.
2. Hawar, S.; Vevers, W.; Karieb, S.; Ali, B. K.; Billington, R. and Beal, J. Biotransformation of patulin to hydroascladiol by Lactobacillus plantarum. *Food Control* **2013**, *34(2)*, 502–508.
3. Fernández-Cruz, M. L.; Mansilla, M. L. and Tadeo, J. L. Mycotoxins in fruits and their processed products: Analysis, occurrence, and health implications. *J. Adv. Res.* 2**010**, *1(2)*, 113–122.
4. Pal, S.; Singh, N. and Ansari, K. M. Toxicological effects of patulin mycotoxin on the mammalian system: an overview. *Toxicol. Res.* **2017**, *6(6)*, 764–771.
5. Mbundi, L.; Gallar-Ayala, H.; Khan, M. R.; Barber, J. L.; Losada, S. and Busquets, R. Chapter two - advances in the analysis of challenging food contaminants: nanoparticles, bisphenols, mycotoxins, and brominated flame retardants. *Adv. Mol. Toxicol.* **2014**, *8*, 35–105.
6. Shapla, U. M.; Solayman, Md.; Alam, N.; Khalil, Md. I. and Gan, S. H. 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health. *Chem. Cent. J.* **2018**, *12(1)*, 1–18.

7. Zhang, L.-L.; Sun, Y.; Zhang, Y.-Y.; Sun, B.-G. and Chen, H.-T. Determination and quantification of 5-hydroxymethylfurfural in vinegar and soy sauces. *J. Food Qual.* **2017**, 8314354.
8. Lee, H. S. and Nagi, S. Relative reactivities of sugars in the formation of 5-hydroxymethylfurfural in sugar-catalyst model systems. *J. Food Process. Preserv.* **1990**, *14(3)*, 171–178.
9. Capuano, E. and Fogliano, V. Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. *LWT - Food Sci. Technol*. **2011**, *44(4)*, 793–810.
10. Gökmen, V. and Şenyuva, H. Z. Effects of some cations on the formation of acrylamide and furfurals in glucose–asparagine model system. *Eur. Food Res. Technol.* **2007**, *225(5–6)*, 815–820.
11. Yang, W.; Zhang, C.; Li, C.; Huang, Z. Y. and Miao, X. Pathway of 5-hydroxymethyl-2-furaldehyde formation in honey. *J. Food. Sci. Technol*. **2019**, *56(5)*, 2417–2425.
12. Severin, I.; Dumont, C.; Jondeau-Cabaton, A.; Graillot, V. and Chagnon, M.-C. Genotoxic activities of the food contaminant 5-hydroxymethylfurfural using different in vitro bioassays. *Toxicol. Lett.* **2010**, *192(2)*, 189–194.
13. Thrasyvoulou, A.; Tananaki, C.; Goras, G.; Karazafiris, E.; Dimou, M.; Liolios, V.; Kanelis D. and Gounari, S. Legislation of honey criteria and standards. *J. Apic. Res.* **2018**, *57(1)*, 88–96.
14. Directive 2001/110/EC of 20 December 2001. *Official Journal of the European Communities (OJEU)* **2001**, 47–52.
15. Gaspar, E. M. S. M. and Lucena, A. F. F. Improved HPLC methodology for food control – furfurals and patulin as markers of quality. *Food Chem.* **2009**, *114(4)*, 1576–1582.
16. Gökmen, V. and Acar, J. Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography. *J. Chromatogr. A.* **1999**, *847(1–2)*, 69–74.
17. Sargenti, S. R. and Almeida, C. A. A. Determination of patulin in apple juice by HPLC using a simple and fast sample preparation method. *Eclet. Quim.* **2010**, *35(2)*, 15–21.
18. Serra-Cayuela, A.; Castellari, M.; Bosch-Fusté, J.; Riu-Aumatell, M.; Buxaderas, S. and López-Tamames, E. Identification of

5-hydroxymethyl-2-furfural (5-HMF) in Cava sparkling wines by LC-DAD-MS/MS and NMR spectrometry. *Food Chem.* **2013**, *141(4)*, 3373–3380.

19. Alcázar, A.; Jurado, Pablos, J. M. F.; González, A. G. and Martín, M. J. HPLC determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in alcoholic beverages. *Microchem. J.* **2006**, *82(1)*, 22–28.
20. Altunay, N. Experimental design of magnetic ionic liquid ultrasound-assisted dispersive liquid-liquid microextraction for the determination of 5-HMF in honey samples. *J. Food. Compost. Anal.* **2022**, *114*, 104817.
21. Hassan, N. H., Al Othman, H. I. A.; Malek, N. R. A.; Zulkurnain, M.; Saad, B. and Wong, Y. F. Simultaneous quantitative assessment of Ochratoxin A, Patulin, 5-Hydroxymethylfurfural, and Bisphenol A in fruit drinks using HPLC with Diode Array-Fluorimetric Detection. *Foods*, **2020**, *9(11)*, 1–14.
22. Rejczak, T. and Tuzimski, T. A review of recent developments and trends in the QuEChERS sample preparation approach, *Open Chem.* **2015**, *13(1)*, 880-1010.
23. Hieu, T. Q.; Pham, P.; Vi, H.; Phuong, P. K.; Thi, L. and Bien, K. Validation of the method for determination of phenolphthalein and sibutramine in weight-loss functional foods from Vietnam by UPLC-MS/MS. *Vietnam J. Chem.* **2021**, *59(4)*, 467–474.
24. Association of Analytical Communities International. Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals, AOAC International, **2013**, 1–38.
25. Tran, Q. H.; Nguyen, T. T. and Pham, K. P. Development of the high sensitivity and selectivity method for the determination of histamine in fish and fish sauce from Vietnam by UPLC-MS/MS. *Int. J. Anal. Chem.* **2020**, 2187646.
26. Both, E. B.; Moreno-González, D.; García-Reyes, J. F. and Dernovics, M. Monitoring the degradation of atropine and scopolamine in soil after spiking with naturally contaminated organic millet. *Sci. Total Environ.* **2018**, *625*, 1088–1092.
27. Annunziata, L.; Stramenga, A.; Visciano, P.; Schirone, M.; De Colli, L.; Colagrande, M. N.; Campana, G. and Scortichini, G.

Simultaneous determination of aflatoxins, T-2 and HT-2 toxins, and fumonisins in cereal-derived products by QuEChERS extraction coupled with LC-MS/MS. *Anal. Bioanal. Chem.* **2017**, *409(21)*, 5143–5155.

28. Hieu-Tran, Q. Validation of the method for determination of melamine and investigation its trace in milk from Vietnam by LC-MS/MS. *Asian J. Appl. Chem.* **2021**, *8(1)*, 13–19.
29. Marsol-Vall, A.; Delpino-Rius, A.; Eras, J.; Balcells, M. and Canela-Garayoa, R. A fast and reliable UHPLC-PDA method for determination of patulin in apple food products using QuEChERS extraction. *Food Anal. Methods* **2014**, *7(2)*, 465–471.
30. AOAC-appendix F., Official Methods of Analysis of AOAC International, Association of Official Analytical Chemists International, **2016**.