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Determination of 5-Hydroxymethylfurfural Using Derivatization Combined with Polymer Monolith Microextraction by High-Performance Liquid Chromatography

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A simple and sensitive method for the determination of 5-hydroxymethylfurfural (HMF) in coffee, honey, beer, Coke, and urine by high-performance liquid chromatography (HPLC) is presented. This method is based on the formation of the 2,4-dinitrophenylhydrazone of HMF and subsequent polymer monolith microextraction (PMME) of this derivative. A poly(methacrylic acid-co-ethylene glycol dimethacrylate) (MAA-co-EGDMA) monolithic capillary column was selected as the extraction medium. Several parameters affecting the derivatization of HMF with 2,4-dinitrophenylhydrazine (DNPH) followed by extraction of the derivative were optimized. The procedure is simple and offers high sensitivity and specificity since the derivative of HMF is well preconcentrated by PMME with poly(MAA-co-EGDMA) monolith and well separated from the other components of the samples under examination. The recoveries in coffee, honey, beer, Coke, and urine samples were in the range of 83.9−110.8% spiked at different levels with HMF. The inter- and intraday precisions were less than 10%. The LOD (S/N = 3) and LOQ (S/N = 10) for HMF were 1.0 ng/mL and 3.4 ng/mL, respectively.

KEYWORDS: Polymer monolith microextraction; high-performance liquid chromatography; 5-hydroxymethylfurfural; 2,4-dinitrophenylhydrazine

INTRODUCTION

5-Hydroxymethylfurfural (HMF) exists in many carbohydrate-rich daily foodstuffs, such as balsamic vinegar, fruit juice, biscuits, bread, beer, coffee, honey, etc. Generally, it is formed either by acid catalyzed degradation of hexoses or via the Maillard reaction (*1*, *2*). Ulbricht et al. (*3*) estimated that humans may ingest up to 150 mg of HMF everyday. Thus, HMF is an important artificial compound in the human diet.

Since HMF and its metabolites, 5-sulfooxymethylfurfural (SMF) and 5-chloromethylfurfural (CMF), are cytotoxic (*4*), genotoxic (*5*), mutagenic, and carcinogenic (*6*−*8*), which may induce colrectal, hepatic, and skin cancers, the study of HMF in foodstuffs has received special attention recently. Moreover, because the concentration of HMF tends to rise during heating, it is a useful tool to evaluate the freshness and quality of foodstuffs. For instance, the Codex Alimentarius of the World Health Organization and the European Union have established the maximum HMF level consented in honey for 40 mg/kg as a deterioration and heat treatment indicator (*9*, *10*).

Various methods, including spectrophotometry (*11*), CE-UV (*12*, *13*), GC-FID/GC-MS (*14*, *15*), HPLC-UV (*16*−*23*), and LC-MS (*24*), have been reported for HMF assay in edible, potable, and other matrices. According to the derivatization of HMF, these methods can be divided into two categories of direct and indirect strategies. It has been demonstrated the indirect strategies are more selective and sensitive than the direct one. Currently, most of the derivatizations are based on the reaction of HMF with 2,4-dinitrophenylhydrazine (DNPH). For instance, Lo Coco et al. (*19*, *20*) used DNPH as the derivatization reagent for analyzing HMF in honey and beer by HPLC-UV. Wintersteiger et al. (*21*, *22*) analyzed HMF derived by DNPH or 2-nitrophenylhydrazine (NPH) in human plasma by HPLC-UV. Murkovic and Pichler (*23*) analyzed HMF derived by DNPH in human urine by HPLC-UV.

Nevertheless, no matter the use of direct or indirect methods, a common pretreatment process is often necessary to extract HMF and prevent matrix interference. To date, several methods, including liquid−liquid extraction (LLE) (*13*, *17*, *18*, *20*), solid-phase extraction (SPE) (*14*, *16*, *21*−*24*), and supercritical fluid extraction (SFE) (*25*), have been used to extract HMF from complex matrices. However, these traditional LLE, SPE, and SFE methods usually involved tedious extraction steps, low extraction uptakes, a prolonged elution time, and massive organic solvents as well as large volumes of samples.

However, the solid phase microextraction (SPME) method has become popular in sample preparation due to some advantages such as simplicity of procedures, solvent-free characteristics, convenience of automation, etc. (*26*). Recently, a novel microextraction setup named polymer monolith microextraction (PMME) was introduced by our group and has obtained great success in many applications (*27*−*29*). In our previous study, the poly(methacrylic acid-co-ethylene glycol dimethacrylate) (poly(MAA-co-EGDMA)) monolithic capillary column showed

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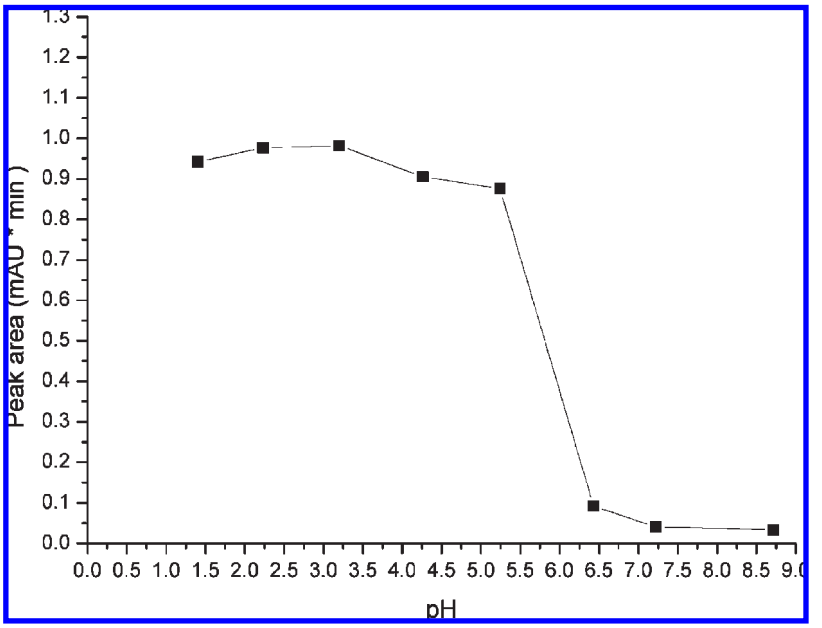

Figure 1. Influence of pH on the peak area of the HMF-DNPH derivative. The spiking level was 320 ng/mL, the phosphate buffer was 0.1 mol/L, and the molar ratio of DNPH to HMF was 40:1, direct injection after derivatization.

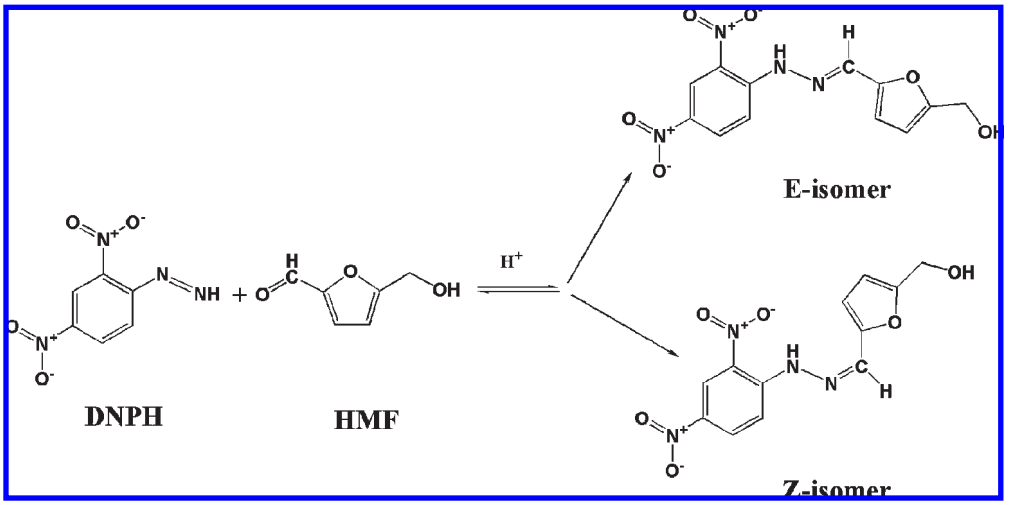

Figure 2. Scheme of the derivatization reaction.

strong extraction capability toward the derivatives of formaldehyde, acetaldehyde, acrolein, butyraldehyde, hexanal, and heptanal, which were labeled by DNPH (*30*, *31*). Low detection limits and high recoveries can be easily achieved by the combination of DNPH derivatization and PMME in the determination of trace aldehydes.

In this study, PMME using a poly(MAA-co-EGDMA) monolithic capillary column for the extraction of HMF derivatized by DNPH has been developed for HPLC determination of HMF in coffee, honey, beer, Coke, and urine.

MATERIALS AND METHODS

Reagents and Solutions. 5-Hydroxymethylfurfural (HMF) was purchased from Sigma (St. Louis, MO, USA). 2,4-Dinitrophenylhydrazine (DNPH) and other reagents (Analytical-grade) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Acetonitrile and methanol (HPLC-grade) were purchased from Fisher Company Inc. (Fairfield, OH, USA). Double distilled water was used for all experiments.

HMF stock solution (6.4 mg/mL) was prepared in water and stored at 4 °C. DNPH solution (2.0 mg/mL) was prepared by dissolving the reagent in 1 mol/L HCl and stored at 4 °C. The coffee, honey, beer, and Coke samples were purchased at a local market (Wuhan, China).

Sample Pretreatment. For instant coffee, 1.25 g was suspended in 5 mL of phosphate buffer (PBS, 0.1 mol/L, pH 2.2) in a 10 mL centrifuge tube. The tube was shaken vigorously for 1 min and centrifuged at 5000 rpm for 10 min. The supernatant was collected in a 10 mL volumetric flask, and two further extractions were performed using 2 mL of PBS. The supernatants were mixed, and the volume was made up to 10 mL with PBS. The honey was homogenized by a vortex mixer, and 1.25 g was diluted with PBS to 10 mL. Urine samples were collected from drug-free, healthy volunteers. Any precipitated material was removed by centrifuging the sample at 10000 rpm for 10 min and stored at −20 °C before use.

Derivatization Procedure. For HMF calibration graphs, the HMF standard solutions were diluted by phosphate buffer (0.1 mol/L, pH 2.2), and 40 times more concentrated DNPH solution was added, then additional PBS was added to prepare a series concentration of 5, 10, 20, 40, 80, 160, 320, and 640 ng/mL for HMF. Thereafter, the solutions were shaken for 1 min and allowed to react for 1 h at 50 °C in a water bath. For coffee, honey, Coke, and beer samples, 40 times more concentrated DNPH solution and PBS were added into 10 μL of the pretreatment sample solution (coffee and honey) and nontreatment sample (Coke and beer) to obtain a mixture of 1 mL. Then they were shaken for 1 min and allowed to react for 1 h at 50 °C in a water bath. For urine samples, 0.5 mL of urine sample was added to the standard HMF solution, 40 times more concentrated DNPH solution, and PBS to 1 mL to prepare a certain

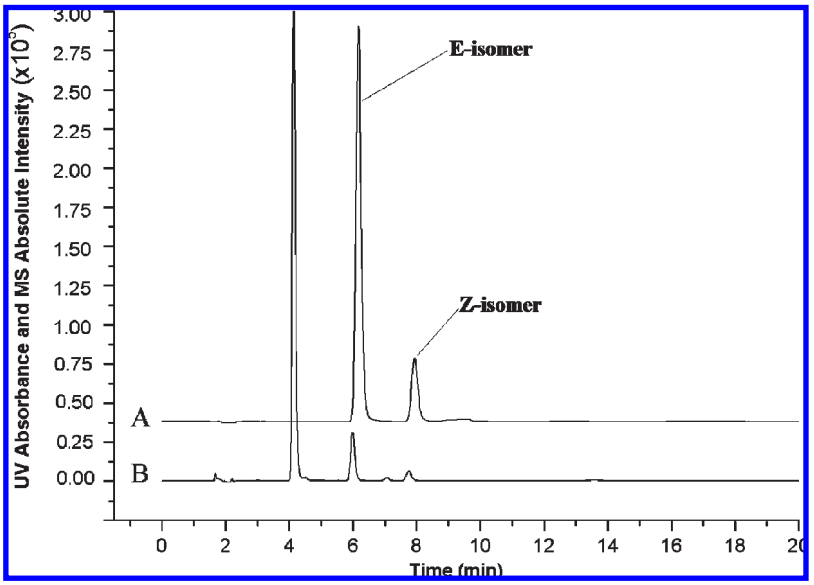

Figure 3. Chromatograms of HMF-DNPH derivative obtained by HPLC-UV-ESI-MS detection (A) (SIM, negative, $m/z = 305$) and PDA detection (B) (400 nm). The spiking level was 640 ng/mL, direct injection after derivatization.

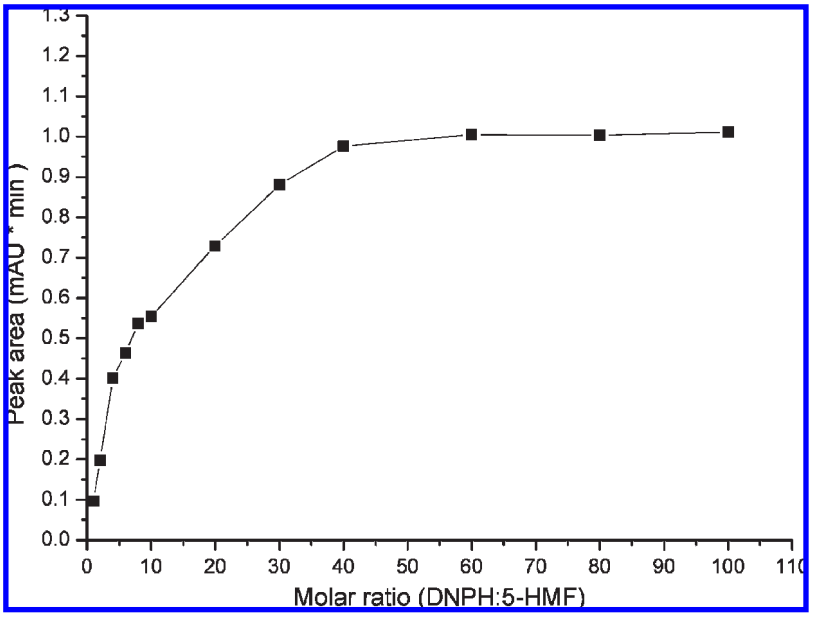

Figure 4. Influence of DNPH concentration on the peak area of the HMF-DNPH derivative. The spiking level was 320 ng/mL, and the phosphate buffer was 0.1 mol/L, pH 2.2, direct injection after derivatization.

concentration and then shaken for 1 min, and reacted for 1 h at 50 °C in a water bath.

PMME Apparatus and Procedures. The self-designing PMME apparatus as previously reported (*28*) was used in this work. The poly (MAA-co-EDGMA) monolithic capillary column (3 cm × 530 μm i.d.) from Micromole Separation and Testing Technology (Beijing, China) was selected as the extraction medium for PMME.

A laboratorial syringe pump (TS2-60, Baoding Longer Precision Pump Co., Ltd., Hebei, China) was employed for the delivery of solutions in the whole extraction process including precondition, sorption, washing, and desorption. For preconditioning, 0.5 mL of methanol and 0.5 mL of PBS (0.1 mol/L, pH 2.2) were ejected through the monolithic capillary at 0.05 mL/min. For the sorption, 0.8 mL of sample solution was pushed through the capillary at 0.1 mL/min, and then 0.5 mL of PBS (0.1 mol/L, pH 2.2) was driven through at the same velocity to eliminate the residual matrix for avoiding the interference of separation and detection. Thereafter, the residual PBS was expelled from the monolithic capillary by air via a clean syringe. For the desorption, 0.05 mL of methanol was injected into the monolithic capillary at 0.05 mL/min, and the eluate was collected into a vial for the subsequent analysis by HPLC.

Chromatographic Conditions. The HPLC profiles were recorded using a Dionex HPLC system (Dionex Co., CA, USA) equipped with a Dionex P680 four-unit pump, a ASI-100 Autosampler, TCC-100 Thermostatted Column Compartment, and a PDA-100 Photodiode Array Detector. The analytical column was a Diamonsil (TM) C18 column (250 × 4.6 mm, 5 μm, Dikma Technologies). The optimized mobile phase was acetonitrile/water (45/55; v/v), and the flow rate was kept at 1.0 mL/min. The detection wavelength was 400 nm, and the column temperature was kept at 25 °C. The injection volume was 10 μL. Data collection and processing were performed on Chromeleon software (Dionex Co., CA, USA).

A HPLC-UV-ESI-MS system (Shimadzu LCMS-2010EV, Tokyo, Japan) was used for identification of the derivatives of geometrical isomers. The column was Shim-pack VP-ODS (Shimadzu, 150 × 4.6 mm, 5 μm). Isocratical elution was carried out with a mobile phase of acetonitrile (45%) and water (55%) at a flow rate of 0.2 mL/min.

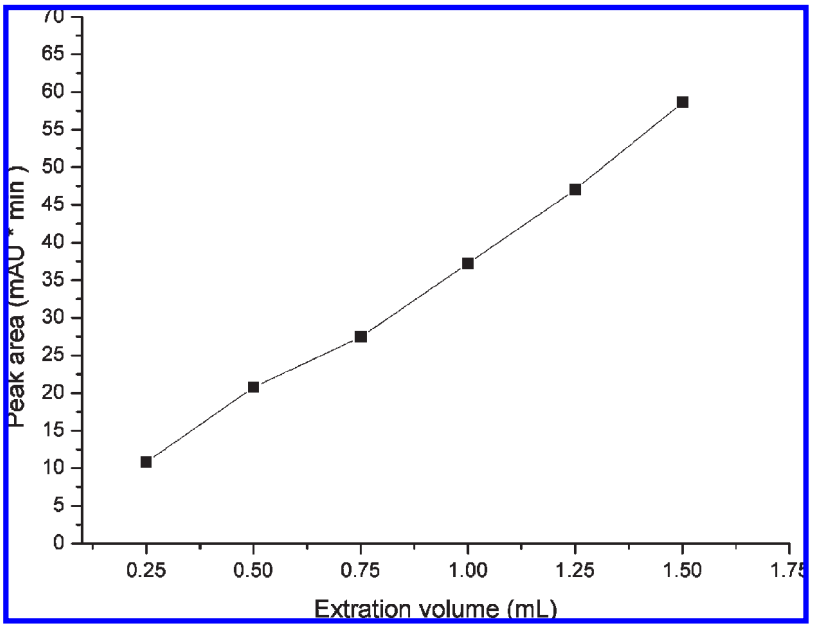

Figure 5. Extracted sample volume profile for the HMF-DNPH derivative using PMME. The spiking level was 320 ng/mL. The optimal derivatization condition was used.

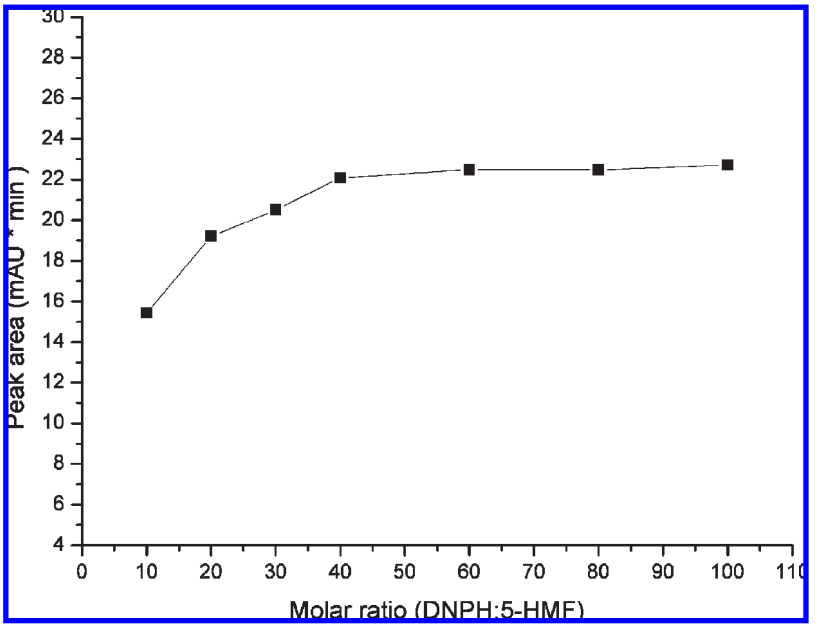

Figure 6. Influence of DNPH concentration on the peak area of the HMF-DNPH derivative for the PMME. The spiking level was 320 ng/mL. The optimal derivatization condition was used.

The sample injection volume was 10 μL. The wavelength of 400 nm was selected for UV detection. Selected ion monitoring (SIM) was conducted to monitor ions at m/z 305, which corresponded to the deprotonated molecular ions of the HMF-DNPH derivative. Capillary voltage was 4.5 kV. Curved desolvation line (CDL) and heat block temperatures for the analysis were set at 250 and 200 °C, respectively. Drying and nebulizer gases of nitrogen were set at 1.5 L/min with a pressure of 0.02 MPa. The detector voltage was set at 1.4 eV.

RESULTS AND DISCUSSION

Optimization of Derivatization. As a typical derivatizing reagent for carbonyl compounds, the derivatization reaction of DNPH with aldehydes has been studied in detail (*32*). Lo Coco and colleagues (*19*, *20*) reported a method of precolumn derivatization with DNPH for the analysis of HMF in beer and honey. The reaction was affected by various parameters including the pH of the reaction solution, the concentration of the reagent, and the reaction temperature. In this study, the derivatization conditions were optimized in terms of both high derivatization yield and high extraction efficiency.

The pH plays a vital role in the derivatization reaction of carbonyl compounds with DNPH because an acidic medium is required to gain the corresponding hydrazones (*19*). Optimization was performed in the pH range of 1.4−8.7, and the molar ratio of DNPH to HMF was 40:1. As shown in **Figure 1**, the derivatization yield did not change significantly over the pH range from 1.4 to 5.5 and had a maximum at about pH 2.2. In the case of the pH value being over 5.5, the yield of the HMF derivative sharply decreased. Therefore, pH 2.2 of PBS was suitably used as the reaction pH value for the derivatization.

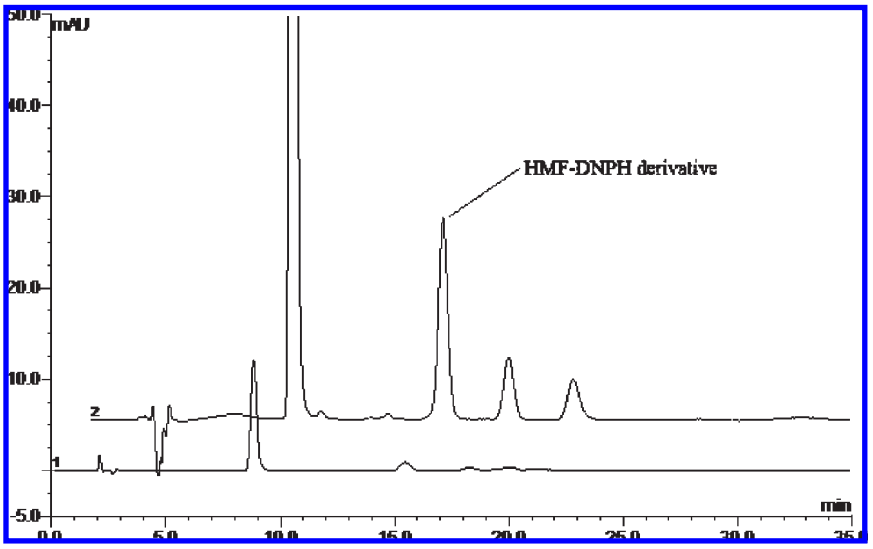

Figure 7. Chromatograms of the HMF-DNPH derivative obtained by direct injection (1) and PMME (2). The spiking level was 160 ng/mL. The optimal derivatization condition was used.

Table 1. Linearity Characteristics of HMF by Derivatization Combined with the PMME and HPLC-UV Detection Method

		regression line				
compound	concentration range (ng/mL)	slope	intercept	R^2	LOD (ng/mL)	LOQ (ng/mL)
HMF	5−640	0.06856	−0.5403	0.9996	1.0	3.4

Table 2. Results Obtained from the Analysis of Real Samples by Derivatization Combined with the PMME and HPLC-UV Detection Method[a]

		recovery (%, n = 3)		
sample	concentration	20 ng/mL	160 ng/mL	640 ng/mL
coffee	36.2 μg/g	110.8	103.1	93.8
honey	129.3 μg/g	108.3	83.9	87.0
beer	1.8 μg/mL	110.2	92.0	84.8
Coke	2.4 μg/mL	101.8	86.5	96.2
urine	N.D.	98.5	92.4	85.6

[a] N.D.: Not detected.

Table 3. Intraday and Interday Precision of Peak Areas at Three Different Concentrations for PMME of HMF Derivatized by DNPH from Coffee, Honey, Beer, Coke, and Urine Samples

	intraday (RSD, %, n = 5)			interday (RSD, %, n = 5)		
sample	low (20 ng/mL)	medium (160 ng/mL)	high (640 ng/mL)	low (20 ng/mL)	medium (160 ng/mL)	high (640 ng/mL)
coffee	7.7	5.3	3.2	8.4	6.4	4.2
honey	5.4	5.1	2.1	6.3	5.1	4.4
beer	5.6	4.3	2.3	6.7	5.4	3.7
Coke	5.6	4.9	2.2	6.4	5.2	2.9
urine	9.7	5.3	1.2	4.2	6.4	3.1

The influence of the buffer concentration on the reaction was also investigated. It can be found that the yields of the HMF derivative remained almost stable in the concentration range of 0.05−2.0 mol/L. Since the derivative can be salted out at high buffer concentrations, the buffer concentration of 0.1 mol/L was chosen in our studies.

The concentration of DNPH is also critical for the reaction and subsequent extraction. The molar ratios of DNPH to HMF were investigated in the range from 1:1 to 100:1. As shown in **Figure 2**, it can be found that higher concentration of reagents could give higher reaction yield. However, when the molar ratios of DNPH to HMF reaches 40-fold, the derivatization yield almost stayed constant. In view of the possible interference of excess DNPH to PMME and subsequent HPLC separation, the 40-fold molar ratio was chosen for the derivatization.

Under the above optimal conditions, the effect of the reaction temperature was also tested in PBS. From 25 to 80 °C, the peak area of the HMF derivative from the chromatograms reaches the greatest value at 50 °C. Therefore, 50 °C was selected as the optimum heating temperature for the derivatization reaction.

In order to obtain the optimum heating time for the derivatization, the reaction was performed in a water bath at 50 °C for 0.5 to 2.5 h. It can be found that the average peak area of the HMF derivative was the greatest after 1 h of reaction. Therefore, 1 h was selected as the optimum heating time. The calculated yields of the HMF derivative was generally around 90% under the optimized derivatization conditions employed. The HMF derivative can be stable at room temperature for 12 h according to the experiment results.

It has been well known that imines generally exist as stereoisomers, which may influence the analysis of such samples. In our experiments, we found that, under optimized conditions, a quite constant isomeric ratio of the E-HMF-DNPH derivate to Z-HMF-DNPH derivate of about 4.0 was obtained. The scheme of the derivatization reaction is described in **Figure 3**. **Figure 4** shows the chromatogram for the HMF-DNPH derivatives. Obviously, two stereoisomers were well separated. However, in our studies, only the first peak (E-isomer) was adopted for quantification not only because of the higher concentration of this isomer but also because of no other interference.

Optimization of the PMME Conditions. To achieve the best extraction efficiency of the poly(MAA-co-EGDMA) monolithic capillary toward the HMF derivative, various parameters affecting the extraction efficiency such as pH, extraction equilibrium profile, extraction flow rate, desorption flow rate, and the concentration of DNPH for PMME were investigated. These experiments were performed with the optimized derivatization conditions and water samples spiked with 320 ng/mL of HMF.

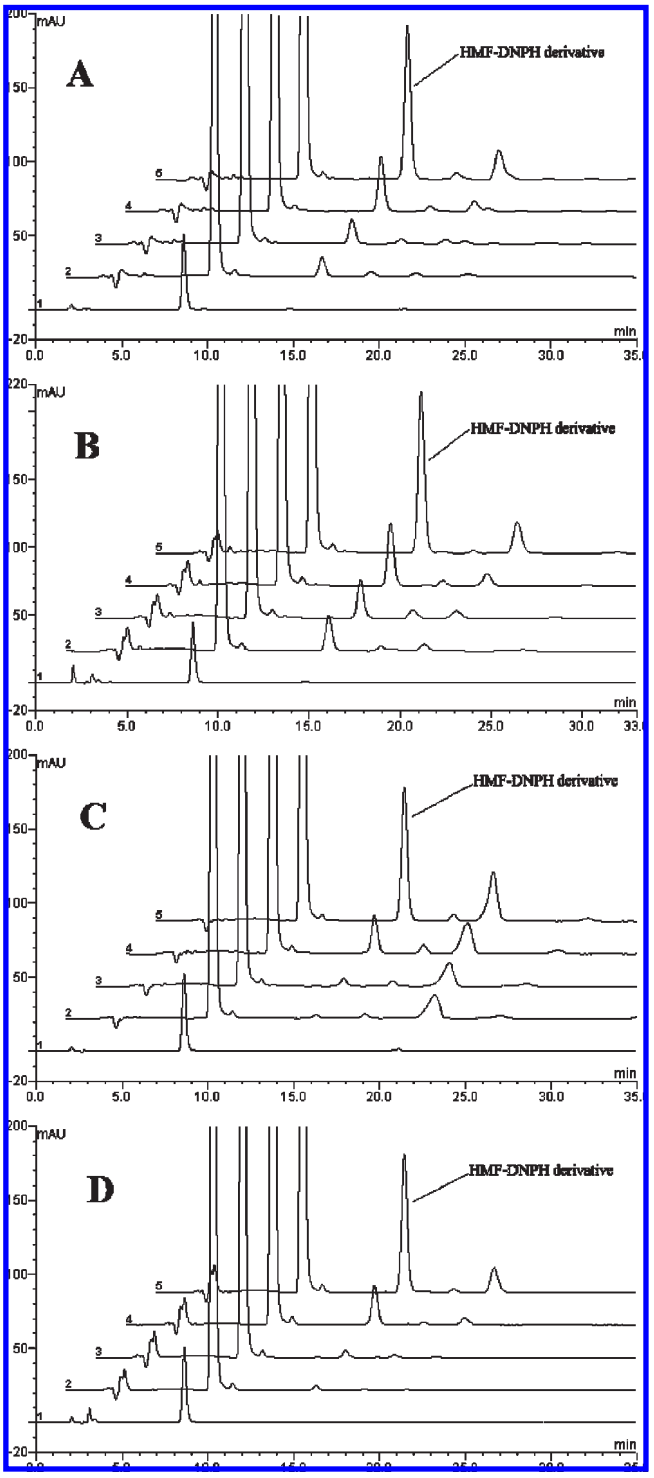

Figure 8. Typical derivatization-PMME-HPLC chromatograms for coffee (**A**), honey (**B**), beer (**C**), and Coke (**D**) samples and these samples spiked with HMF (1, derivatized samples by direct injection; 2, derivatized samples by PMME; 3, derivatized samples, spiked with 20 ng/mL HMF by PMME; 4, derivatized samples, spiked with 160 ng/mL HMF by PMME; 5, derivatized samples, spiked with 640 ng/mL HMF by PMME). Optimal derivatization and microextraction conditions were used.

The pH of the sample solution is an important parameter affecting the PMME process. The results showed that the extraction efficiency was high and constant in the pH range of 1.4−5.2. It would probably be ascribed to the fact that the interaction between the analyte and the monolithic capillary was mainly based on the hydrophobic interaction. Considering the derivatization process and operational convenience, the microextraction was carried out with a sample solution of pH 2.2.

The extraction equilibrium profile was obtained by increasing the sample volume from 0.25 to 1.5 mL at a constant flow rate. As shown in **Figure 5**, the peak area of the HMF derivative increased rapidly, and the extraction equilibrium was not reached even after 1.5 mL of sample solution was fed. The sharp slope of the profile indicated that the monolithic capillary exhibited remarkable extraction capacity for the HMF derivative. To achieve sufficient sensitivity within a short time, a sample volume of 0.8 mL was selected for subsequent analysis.

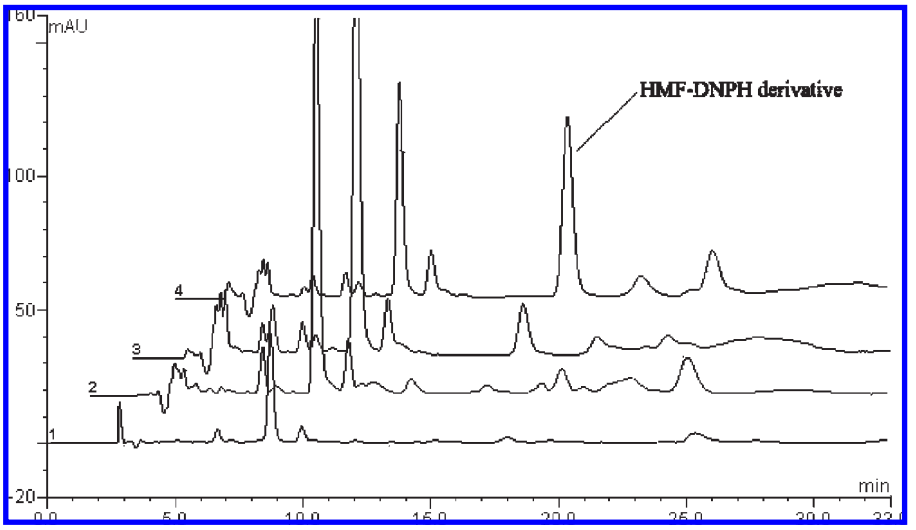

Figure 9. Typical derivatization−PMME-HPLC chromatograms for urine sample (1, blank urine spiked with 20 ng/mL HMF by direct injection after derivatization; 2, blank urine spiked with 20 ng/mL HMF by PMME after derivatization; 3, blank urine spiked with 160 ng/mL HMF by PMME after derivatization; 4, blank urine spiked with 640 ng/mL HMF by PMME after derivatization). Optimal derivatization and microextraction conditions were used.

The flow rate of the sample solution was optimized in the range of 0.05−0.4 mL/min with a total loading of 0.8 mL of sample solution. It was found that the flow rate had no obvious influence on the extraction efficiency. Therefore, the flow rate of 0.1 mL/min was selected considering the extraction time as well as the pressure on the monolithic capillary.

The monolithic capillary can also extract the excess DNPH in the sample matrix, which may be detrimental to the extraction efficiency of the HMF derivative. Therefore, the influence of DNPH concentration on PMME should be investigated. It can be found from **Figure 6** that the concentration of DNPH had no influence on the extraction yield of HMF derivative, which would be due to the high extraction efficiency of the poly(MAA-co-EGDMA) monolith to the HMF derivative under optimal derivatization conditions.

The desorption was optimized to achieve accurate quantification of the analytes. After sample extraction, 0.05 mL of methanol was used to elute the analyte. The same procedure was repeated twice. Each of the 0.05 mL eluates was collected for HPLC analysis. The result indicated that the first 0.05 mL of methanol could elute more than 90% extracted HMF-DNPH derivative from the monolithic capillary. Moreover, the flow rate of the desorption was optimized in the range of 0.025−0.1 mL/min. In view of the capillary pressure and the desorption time, a flow rate of 0.05 mL/min was suitable.

The chromatograms obtained after PMME and direct injection are shown in **Figure 7**. In comparison with the chromatogram of direct injection, a dramatic enhancement of the peak height was observed, indicating the remarkable preconcentration capability of the monolithic capillary to the HMF-DNPH derivative. On the basis of these optimal experiment conditions, the calculated enrichment factor and extraction yield of the HMF derivative were 14.6 and 93.2%, respectively.

Validation of the Method. To assess the linearity range of this method, various concentrations of HMF ranging from 5 ng/mL to 640 ng/mL were derivatized and extracted for analysis. The calibration curve was constructed by comparing the peak areas against the analyte concentrations. As shown in **Table 1**, a good linearity for HMF is obtained with a correlation coefficient (R^2) value of 0.9996. The LOD and LOQ were determined with low concentration (1.0 and 3.4 ng/mL) and calculated with the S/Ns of 3 and 10, respectively under HPLC-UV conditions.

The recoveries of the HMF-derivative spiked at low, medium, and high concentrations in coffee, honey, beer, Coke, and urine samples were studied. As shown in **Table 2**, satisfactory recoveries of the spiked analyte from different samples were achieved, yielding recoveries in the range of 83.9−110.8% (n = 3).

The method reproducibility was assessed by the intra- and interday precisions that were expressed as the relative standard deviation (RSD). Five extractions over a day gave the intraday precisions, which were based on the analysis of coffee, honey, beer, Coke, and urine samples spiked at three levels of concentration. The interday precisions were determined by extracting the samples that were independently prepared continuously for 5 days. Results showed that the intra- and interday precisions of the peak areas were both less than 10%. The detailed data are listed in **Table 3**.

Quantitative Analysis of HMF in Real Samples. The derivatization combined with the PMME and HPLC-UV detection method was applied to analyze the HMF in coffee, honey, beer, Coke, and urine samples. The results are listed in **Table 2**. All of the real samples were spiked with the analyte standard at different concentration levels to assess the matrix effects. The coexistence of some carbonyl compounds and the matrix did not influence the separation under the optimized conditions. The chromatogram obtained by HPLC-UV of coffee, honey, beer, Coke, and urine samples spiked with the target compound at low, medium, and high concentrations of HMF after the developed method is shown in **Figures 8** and **9**.

In conclusion, the derivatization coupled with the PMME method was successfully applied to extract HMF in coffee, honey, beer, Coke, and urine. In comparison to the previously reported pretreatment and analysis methods, the proposed method has some advantages such as being environmentally friendly, providing rapid sample handling, and being inexpensive. The high sensitivity makes this method attractive for the trace determination of HMF in different biologic matrices such as plasmaa and urine. Additionally, in comparison to traditional LLE or other techniques, the good permeability of the monolithic capillary can realize the extraction process very quickly, indicating its wide usage in high-throughput applications.

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