

Determination of Hydroxymethylfurfural in Honey and Biomass

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Introduction

Hydroxymethylfurfural (HMF), or 5-hydroxymethyl-2-furaldehyde, is a water-soluble heterocyclic organic compound derived from sugars. It is a derivative of furan and has both aldehyde and alcohol functional groups (Figure 1). Very low amounts of this compound are naturally found in fresh sugar-containing foods including milk, honey, fruit juices, spirits, and bread. Additionally, HMF is produced during food pasteurization and cooking as a result of dehydration of sugars such as glucose and fructose¹ and in the initial stages of the Maillard reaction,² a reaction between sugars and proteins responsible for changes in color and flavor of food. HMF is also formed during extended food storage under acidic conditions that favor its generation. Therefore, it is an indicator of excessive heat-treatment, spoilage, and of possible adulteration with other sugars or syrups.

Although HMF is not yet considered a harmful substance, the National Institute of Environmental Health Sciences nominated HMF for toxicity testing³ based on the potential for widespread exposure through consumed foods, and evidence for carcinogenic potential of other members of this class. As a result, many countries impose restrictions on maximum levels of HMF in food and beverages.⁴

Beyond being an indicator of food quality, HMF is a biomass platform chemical because it can be used to synthesize a number of compounds that are currently derived from crude oil, including solvents, fuels, and monomers for polymer production (Figure 1).^{5,6} HMF is readily derived from cellulose, either directly or through a two-step process involving hydrolysis and the formation of simple sugars.⁷ Thus, it is important to measure HMF in matrices ranging from foods to treated cellulosic biomass.

There are spectrophotometric and HPLC methods available for HMF determination. One commonly used method is based on spectral absorbance at 284 nm.^{8,9} This direct-absorbance measurement could have interferences from other compounds present in the complex matrices. In the HPLC method, HMF is separated on a reversed-phase column, with water and methanol as the mobile phase, and then detected by UV absorbance.¹⁰

This work describes a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)-based method for the determination of HMF in samples ranging from food (honey and pancake syrup) to treated biomass (corn stover and wood hydrolysate). A Thermo Scientific™ Dionex™ ICS-3000 system with a Thermo Scientific™ Dionex™ CarboPac™ PA1 column, electrolytically generated hydroxide eluent, and electrochemical detection with disposable Au-on-polytetrafluoroethylene (PTFE) working electrodes are used. The Dionex CarboPac PA1 is a high-capacity, rugged column suitable for determining mono- and disaccharides, and has high resolution for HMF in a wide variety of matrices.

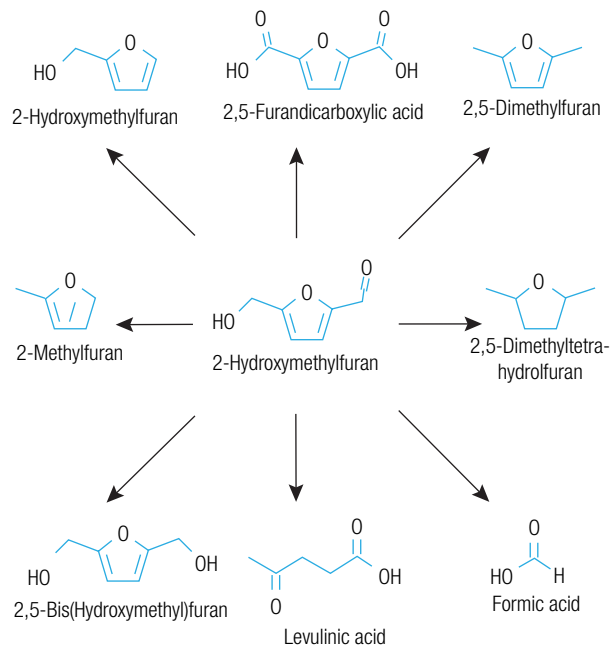


Figure 1. HMF as precursor for a number of commercial chemicals.

The testing here demonstrates the linearity, limit of quantitation, limit of detection, precision, and recovery of HMF in diverse matrices ranging from honey to corn stover. It shows that PAD is an appropriate detection technique for HMF, with a broad linear range and low detection limit. Disposable electrodes provide short equilibration time and greater electrode-to-electrode reproducibility, compared to conventional electrodes. Compared to other disposable Au electrodes, the Au-on-PTFE electrodes have longer lifetime and can operate at higher hydroxide concentrations. The described method provides good sensitivity, consistent response, and can be routinely used for HMF analysis in foods and biomass applications, demonstrating the capability of HPAE-PAD for HMF determination in varied matrices.

Equipment

- Dionex ICS-3000 or ICS-5000 system including:
 - Gradient or Isocratic Pump, with the vacuum degas option installed
 - EG Vacuum Degas Conversion Kit (P/N 063353)
 - DC Detector/Chromatography Module
 - 10 μ L Injection loop
 - Electrochemical Detector (P/N 079830)
 - Carbohydrate PTFE Disposable Au Working Electrodes (P/N 066480, package of 6)
 - Ag/AgCl Reference Electrode (P/N 061879)
 - 3 mil PTFE gaskets (P/N 63537)
- AS Autosampler
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software
- Eluent Organizer, including 2 L plastic bottles and pressure regulator
- Polypropylene injection vials with caps (0.3 mL vial kit, P/N 055428)
- Nalgene® 125 mL HDPE narrow mouth bottles (VWR P/N 16057-062)
- Nalgene 250 mL HDPE narrow mouth bottles (VWR P/N 16057-109)
- Nalgene 250 mL 0.2 μ m nylon filter units (VWR P/N 28199-371)
- Nalgene 1000 mL 0.2 μ m nylon filter units (VWR P/N 28198-514)

Reagents and Standards

Reagents

- Deionized (DI) water, Type I reagent grade, 18 M Ω -cm resistivity or better, filtered through a 0.2 μ m filter immediately before use

Standards

- HMF (Sigma Aldrich Cat # W501808)
- Fructose (Baker Analyzed® Cat # M556-05)
- Xylose (Aldrich Chemical Company Cat # X-107-5)
- Sucrose (Sigma Cat # S-9378)
- Glucose (Sigma Cat # G-5250)
- Glycerol (J.T. Baker Cat # M778-07)
- Arabinose (Sigma Cat # A3131)

Conditions

Method

Columns:	Dionex CarboPac PA1 Analytical, 4 × 250 mm (P/N 035391) Dionex CarboPac PA1 Guard, 4 × 50 mm (P/N 43096)
Flow Rate:	1.0 mL/min
Inj. Volume:	10 μ L (full loop)
Temperature:	30 °C
Back Pressure:	2400 psi
Eluent:	50 mM KOH
Eluent Source:	Thermo Scientific™ Dionex™ EGC II KOH cartridge with Thermo Scientific™ Dionex™ CR-ATC Continuously Regenerated Anion Trap Column
Detection:	PAD
Background:	30–70 nC
Working Electrode:	Carbohydrate PTFE Disposable Au Working Electrodes
Reference Electrode:	
Mode:	Ag/AgCl mode
Noise:	30 pC

Carbohydrate Waveform

Time (s)	Potential (V)	Integration
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	–2.0	
0.42	–2.0	
0.43	+0.6	
0.44	–0.1	
0.50	–0.1	

Reference electrode in Ag/AgCl mode

Preparation of Solutions and Reagents

Eluent Solutions

Potassium Hydroxide (50 mM)

Generate the potassium hydroxide (KOH) eluent online by pumping high-quality degassed, deionized (DI) water through the Dionex EGC II KOH cartridge. Chromeleon software tracks the amount of KOH used and calculates the remaining lifetime. Although electrolytic eluent

generation delivers the best performance, manually prepared eluents can be used, if needed. For manually prepared eluent, use NaOH rather than KOH and prepare according to the general instructions for hydroxide eluents in Dionex (now part of Thermo Scientific) Technical Note 71.¹¹ This method requires the installation of the Dionex ICS-3000 EG Vacuum Degas Conversion Kit (P/N 063353) to allow sufficient removal of the hydrogen gas formed with the potassium hydroxide eluent.¹²

Stock Standard Solution

Prepare a stock solution of 2 mg/mL HMF by dissolving 10 mg in 5 mL DI water in a plastic volumetric flask. Store aliquots of stock solutions in plastic containers at 4 °C. Stock standards are stable for at least one month.

Working Standard Solutions

Prepare working standards at lower HMF concentrations by diluting appropriate volumes of the 2 mg/mL stock with deionized water. Prepare working standards daily. Store the standard solutions at <6 °C when not in use.

Sample Preparation

Honey and Syrup

Prepare honey and syrup samples by dissolving 1 g in 100 mL of DI water and sonicating for 10 min. Store solutions in plastic containers at <6 °C. Further dilute syrup samples twofold with DI water before injection.

Fructose

Prepare a stock solution by dissolving 100 mg in 100 μ L DI water. Dilute 500-fold with DI water before injection.

Corn Stover and Wood Hydrolysate

Centrifuge corn stover and wood hydrolysate samples at 14,000 rpm for 10 min and inject with DI water at a dilution of 1/1000 for analysis.

Precautions

Carryover can occur because the honey, syrup, and treated biomass samples have high concentrations of sugars such as glucose, xylose, and sucrose. A syringe flush of 1000 μ L is recommended between samples. Column washes at 100 mM KOH are recommended if gradual retention time loss is observed. The application of 100 mM KOH changes the system equilibrium; re-equilibration at 50 mM for ~2 h is recommended to achieve high precision.¹³

Results and Discussion

Figure 2 shows HMF in a thermally stressed honey sample. HMF elutes at 4.8 min and can be detected without interference from the other sugars. The HMF

Column:	Dionex CarboPac PA1, Analytical, 4 × 250 mm Dionex CarboPac PA1, Guard, 4 × 50 mm	Peaks: 1. Glycerol – μ g/mL 2. HMF 3.4 3. Glucose – 4. Xylose – 5. Fructose – 6. Sucrose –
Eluent:	50 mM KOH	
Eluent Source:	Dionex EGC II KOH Cartridge	
Flow Rate:	1.0 mL/min	
Temperature:	30 °C	
Inj. Volume:	10 μ L (full loop)	
Detection:	PAD (Au)	

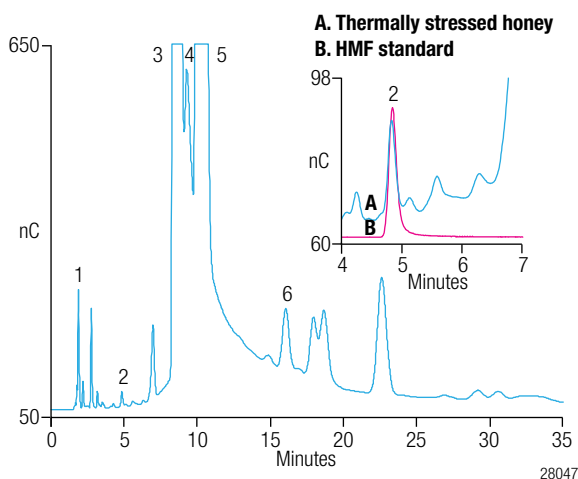


Figure 2. HMF in thermally stressed honey.

content in this sample was determined to be 330 mg/kg of honey. Typically, fresh honey has a low amount of HMF (<15 mg/kg). The HMF concentration increases as honey undergoes heat treatment to reduce viscosity and prevent crystallization to facilitate filling.¹⁴ The EU Directive (110/2001) and the Codex Alimentarius (ALINORM 01/2000) standards limit HMF to 40 mg/kg for honey produced under European conditions and 80 mg/kg for honey coming from tropical countries.⁴ In the fresh honey sample, HMF was determined to be 0.17 μ g/mL (which amounts to 17 mg HMF/kg of honey, Table 1).

The chromatogram of thermally stressed pancake syrup (Figure 3) shows the separation of HMF from other thermal degradation products. HMF is a product of thermal degradation of fructose, the main constituent of pancake syrup. HMF in high-fructose corn syrup (HFCS) is also a problem for beekeepers because they use HFCS as a source of sugar to feed bees when natural nectar sources are limited. Note that complex matrices like pancake syrup may have later-eluting peaks (e.g., pancake syrup has a peak at ~55 min, not shown), and the long retention time could interfere with subsequent injections if a shorter run time is used.

Table 1. Intraday and between-day precisions for honey and corn stover samples.

Sample	Amount (μ g/mL)	RT Precision (RSD)		Peak Area Precision (RSD)	
		Intraday	Between-Day	Intraday	Between-Day
Fresh honey	0.17	NC			
Thermally stressed honey	3.4	0.09	0.06	1.19	1.17
Corn stover	4.0	0.11	0.67	0.33	3.13

NC: Not Collected

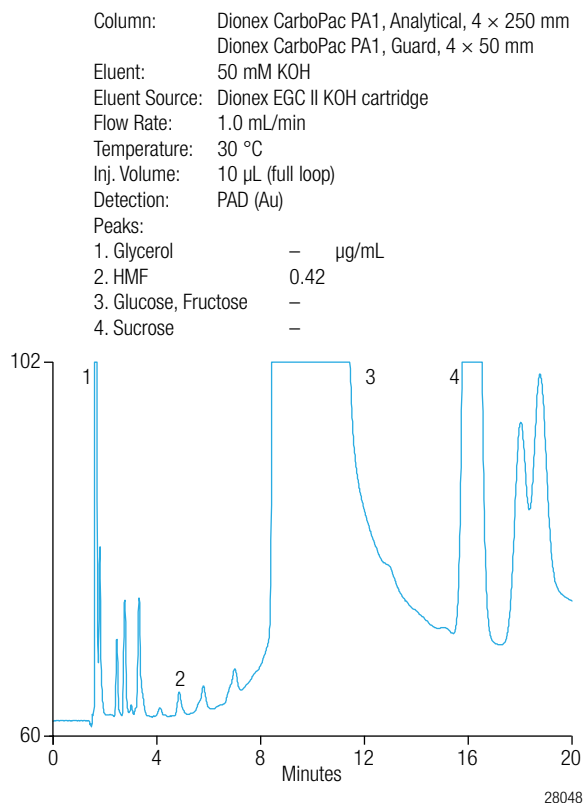


Figure 3. HMF in pancake syrup (high-fructose corn syrup).

The described HPAE-PAD method was applied for HMF detection in fructose. The United States Pharmacopeia (USP)¹⁵ and Food Chemicals Codex (FCC) have monographs¹⁶ for the analysis of HMF in fructose and fructose injections. The USP monograph is based on the Seliwanoff test which depends on the reaction of HMF with resorcinol to form a red-colored compound, and the FCC monograph is a spectrophotometric method based on UV absorbance of HMF at 283 nm. HMF is present as an organic impurity in fructose and must be quantified and meet USP requirements before use as a food substance or as infusion fluids. HMF is formed during sterilization and storage. The chromatogram of thermally stressed fructose solution (Figure 4) shows the separation of HMF from other thermal degradation products. HMF is also formed in aqueous dextrose solutions and a HPAE-PAD-based method has been reported for quantification of HMF in commercial dextrose solutions.¹⁷ Note that the thermally stressed honey, syrup, and fructose solutions were prepared by heating samples at 100 ± 10 °C for 4 h and cooling to room temperature before dilution and injection.

Table 2. Linear range and precisions for HMF standards.

Analyte	Sample (Range µg/mL)	Corr. Coeff. (r^2)	RT (min)	Concentration Used for Precision Injections (µg/mL)	RT Precision (RSD)	Peak Area (nC*min)	Peak Area Precision (RSD)
HMF	Honey (0.1–50)	0.9998	4.85	0.5	0.12	0.55	0.50
	Corn Stover (0.5–1000)	0.9965	4.85	5.0	0.10	4.91	0.14

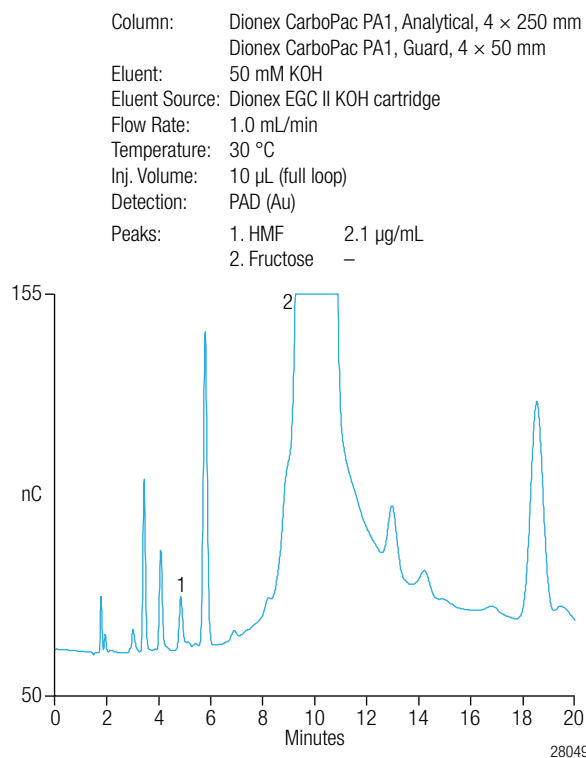


Figure 4. HMF in a thermally stressed fructose solution.

Figures 5 and 6 show the chromatograms of acid-hydrolyzed corn stover and a wood acid hydrolysate. HMF was detected without interference from the other sugars in both samples. The total run time for these samples was 15 min, providing high sample throughput, suggesting that this method can be used for online monitoring of HMF during biomass processing.

Linear Range, Limit of Quantitation, Limit of Detection

To determine the linearity of the method, calibration standards were injected in triplicate covering the expected concentration range of HMF in food and biomass samples. Calibration plots produced correlation coefficient (r^2) values of 0.9998 in the range 0.1 to 50 µg/mL for food applications and 0.9965 in the range 0.5 to 1000 µg/mL for biomass applications (Table 2). A least squares regression fit with weighting was used to accurately represent the lower values of the calibration curve.

The limit of detection (LOD) was determined by measuring the peak-to-peak noise in a representative one-minute segment of baseline where no peaks elute, followed by analyzing a standard at a concentration expected to provide a chromatogram with a signal-to-noise (S/N) ratio

Column: Dionex CarboPac PA1, Analytical, 4 × 250 mm
 Dionex CarboPac PA1, Guard, 4 × 50 mm
 Eluent: 50 mM KOH
 Eluent Source: Dionex DGC II KOH cartridge
 Flow Rate: 1.0 mL/min
 Temperature: 30 °C
 Inj. Volume: 10 µL (full loop)
 Detection: PAD (Au)
 Traces: A. Treated corn stover
 B. HMF standard

Peaks:
 1. Glycerol – µg/mL
 2. Fucose –
 3. HMF 4.5
 4. Arabinose –
 5. Glucose –
 6. Xylose –
 7. Fructose –

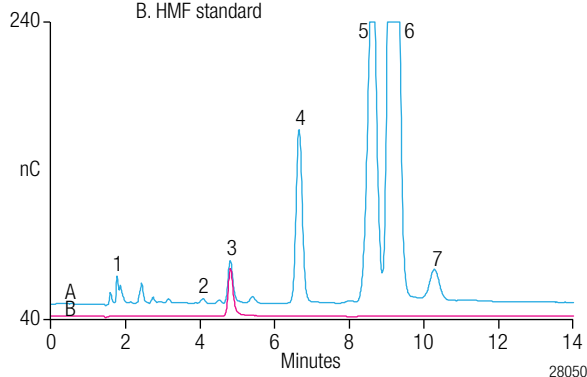


Figure 5. HMF in acid-hydrolyzed corn stover.

of 3. Similarly, the lower limit of quantitation (LOQ) was determined by injecting a standard at a concentration that resulted in a *S/N* ratio of 10. Typical baseline noise for this method was 20 to 40 pC. The LOD and LOQ for this method were 0.04 µg/mL and 0.10 µg/mL, respectively.

Precision

The peak area and retention time (RT) precisions were determined for seven replicate injections of an HMF standard. The concentrations used for precision injections were 0.5 µg/mL for food applications and 5.0 µg/mL for biomass applications (Table 2). The retention time precisions (RSD) for the two concentrations were 0.12 and 0.10, respectively. The corresponding peak area precisions were 0.5 and 0.14. The high retention time precisions were attributed to consistent generation of high-purity KOH using the eluent generator.

Intraday and between-day precisions for HMF in honey and corn stover were evaluated over three consecutive days. The RT precisions were in the range of 0.06 to 0.67%, and peak area precisions were in the range of 0.33 to 3.13%. The high precisions suggest that this method can be used to measure HMF in complex matrices.

Accuracy

The accuracy of the method was verified by determining recoveries of HMF in spiked honey and acid-hydrolyzed corn stover samples over three consecutive days. The

Columns: Dionex CarboPac PA1, Analytical, 4 × 250 mm
 Dionex CarboPac PA1, Guard, 4 × 50 mm
 Eluent: 50 mM KOH
 Eluent Source: Dionex DGC II KOH cartridge
 Flow Rate: 1.0 mL/min
 Temperature: 30 °C
 Inj. Volume: 10 µL (full loop)
 Detection: PAD (Au)

Peaks:
 1. Glycerol
 2. Fucose
 3. HMF
 4. Arabinose
 5. Glucose
 6. Xylose
 7. Fructose

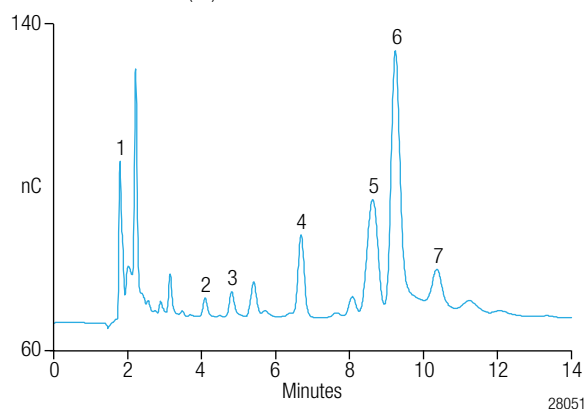


Figure 6. HMF in wood hydrolysate.

amount of HMF in a fresh honey sample was 0.17 µg/mL (Table 1: this equates to 17 mg of HMF per kg of honey). The thermally stressed honey had 3.4 µg/mL HMF (Figure 2), and was spiked with 2.9 µg/mL HMF. The treated corn stover sample (at 1000-fold dilution) had 4 µg/mL HMF and was spiked with 5.3 µg/mL HMF. Recoveries were calculated from the difference in response between the spiked and unspiked samples. Intraday concentration RSD was 1.7% for both honey and corn stover. The average recovery of HMF in honey was 103% and in corn stover was 112% (Table 3).

Conclusion

This study describes a HPAE-PAD method for the accurate determination of HMF in foods like honey and in biomass like acid-hydrolyzed corn stover. The method uses the Dionex CarboPac PA1 column with electrolytically generated hydroxide eluent. The method is shown to have a broad linear range, high precisions, and low detection limits. The disposable Au working electrode provides consistently high detector response, assuring greater instrument-to-instrument and lab-to-lab reproducibility. This configuration needs only addition of deionized water for continuous operation. In summary, the described HPAE-PAD-based HMF analysis method is accurate and reliable, and should be applicable to online monitoring of HMF levels in food and biomass applications.

Table 3. Recoveries of HMF in spiked honey and corn stover samples.

Analyte	Sample	Amount Found (µg/mL)	Amount Added (µg/mL)	Average Recovery ([%]n = 3 days)
HMF	Thermally stressed honey	6.4	2.9	103
	Corn stover	10.0	5.3	113

Suppliers

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A., Tel: 800-932-5000.

Sigma-Aldrich Chemical Co., P.O. Box 2060, Milwaukee, WI 53201, U.S.A., Tel: 800-558-9160.

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