Using the Agilent 7000B Triple Quadrupole GC/MS for Parts per Trillion Detection of PAH Metabolites in Human Urine

Application Note

Environmental

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Abstract

A method was developed for sensitive and robust monitoring of very low levels of polycyclic aromatic hydrocarbons (PAH) metabolites in urine using the Agilent 7000B Triple Quadrupole GC/MS System. The resulting limits of detection (LODs) were as low as 0.7 parts per trillion (ppt), and limits of quantitation (LOQs) were as low as 2.5 ppt, with nearly 100% recovery for all 19 PAH metabolites. This method was developed for current use by the Canadian Health Measures Survey (CHMS).

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Introduction

PAHs make up a class of more than 100 chemicals composed of up to six benzene rings fused together such that any two adjacent benzene rings share two carbon bonds. Burning carbon-containing compounds is the primary source of PAHs. PAHs are in gasoline and diesel exhaust, soot, coke, cigar, and cigarette smoke. Foods that contain small amounts of PAHs include smoked, barbecued or charcoal-broiled foods, roasted coffees, and sausages.

PAHs are a human health concern because a number of studies have shown increased incidence of cancer (lung, skin, and urinary cancers) in humans exposed to PAH mixtures [1]. Many individual PAH compounds have been classified as probable or possible carcinogens by entities such as the U.S. DHHS National Toxicology Program, the U.S. Environmental Protection Agency (EPA) and Health Canada.

The effects on human health depend mainly on the extent of exposure (length of time, and so forth), the amount one is exposed to (or concentration), the innate toxicity of the PAHs, and whether exposure occurs through inhalation, ingestion, or skin contact. A variety of other factors can also affect health impacts from such exposure, including pre-existing health status and age.

Biological monitoring of exposure through the analysis of urinary levels of PAH metabolites has become an increasingly popular means of estimating the absorbed dose [2]. PAHs are rapidly hydroxylated in the liver (phase 1 metabolism) and then undergo conjugation, typically to the glucuronide, which makes them more water soluble for excretion in urine [3]. Several industrial hygiene studies have shown that 1-hydroxypyrene in urine is a valid biomarker for occupational exposure to PAHs. Furthermore, the determination of the benzo[a]pyrene metabolite concentration in human urine can be used as an indicator for PAH contamination [4, 5]. Since these compounds are considered priority contaminants in Canada, methods have been developed to assess environmental exposure to PAHs by monitoring the metabolized products in urine.

This application note describes development of a method for measurement of hydroxylated PAH metabolites in human urine using an Agilent 7890 GC coupled to an Agilent 7000B Series Triple Quadrupole GC/MS System, using gas chromatography and mass spectrometry with multiple reaction monitoring (GC/MS/MS). LOQs are as low as 0.0025 µg/L (2.5 parts per trillion; ppt), and do not exceed 12 ppt, with the exception of 2-napthol. Most recoveries are in the 94 to 104% range, and intra-day and inter-day precision (relative standard deviation) does not exceed 10% for any of the 19 metabolites analyzed.

Experimental

Standards and reagents

Hydroxybenz[a]anthracene, hydroxybenzo[a]pyrene, hydroxychrysene, hydroxyfluoranthene, and their respective isomers were obtained from Midwest Research Institute (Kansas City, MO). The hydroxyfluorene and naphthol isomers were obtained from Sigma-Aldrich (St. Louis, MO). Hydroxyphenanthrene, hydroxypyrene, and their respective isomers were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). All ^{13}C labeled standards were obtained from Cambridge Isotope Laboratories (Andover, MA) except for 3-hydroxyfluoranthene-$^{13}C_6$, which was obtained from Midwest Research Institute (Kansas City, MO). All deuterium labeled standards were obtained from Toronto Research Chemicals (Toronto, Canada). The β-glucuronidase enzyme (in water) was obtained from Sigma-Aldrich, and the urine samples were obtained from non-exposed volunteers. The derivatization agent N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Thermo Fisher Scientific. The internal standards used, and their respective analytes, are shown in Table 1.

Table 1. Labeled Internal Standards Associated with their Analytes for Quantitation

Internal standard	Analyte
1-napthol-$^{13}C_6$	1-napthol
2-napthol-$^{13}C_6$	2-napthol
2-fluorene-d_9	2,3, and 9-OH-fluorene
3-OH-phenanthrene-$^{13}C_6$	1,2,3, and 4-OH-phenanthrene
9-OH-phenanthrene-$^{13}C_6$	9-OH-phenanthrene
3-OH-fluoranthene-$^{13}C_6$	3-OH-fluoranthene
1-OH-pyrene-$^{13}C_6$	1-OH-pyrene
6-OH-chrysene-$^{13}C_6$	2,3,4, and 6-OH-chrysene 1 and 3-OH-benz[a]anthracene
3-OH-benzo[a[pyrene-$^{13}C_6$	3-OH-benzo[a]pyrene (3-OH-BaP)

Sample Preparation

Samples of 5 mL of urine were collected and urinary metabolites were digested with β-glucuronidase. All samples were then extracted with hexane at neutral pH and derivatized with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA).

Instruments

The study was performed on a 7890A gas chromatograph equipped with a Multi-Mode Inlet (MMI) and coupled to an Agilent 7000B Series Triple Quadrupole mass spectrometer, using Multiple Reaction Monitoring (MRM) and Electron Ionization (EI) acquisition modes. Table 2 shows the instrument conditions.

Results

Separation

The hydroxy-PAHs are positional isomers (for example, 4-OH-chrysene and 6-OH-chrysene and so forth) and the isomers, while differing in retention times, all have the same MS/MS transitions within each isomer group (Figures 1 and 2). Coeluting interferences would affect quantitation and detection limits, and thus good chromatographic separation is critical. The gas chromatographic method was optimized to ensure sharp, Gaussian peak shapes, and good separation of analyte peaks from interference peaks (Figure 3).

Table 2. Agilent 7890/ 7000 Gas Chromatograph and Mass Spectrometer Conditions

GC run conditions	
Analytical column	Agilent DB-XLB 30 m × 0.25 mm, 0.10 µm (p/n 122-1231E)
Injection	1 µL; pulsed splitless
Injector temperature gradient	130 °C for 0.1 minute 720 °C/min to 270 °C
Carrier gas	Helium, constant flow, 1 mL/min
Oven temperature gradient	65 °C for 1 minute
	15 °C/min to 160 °C
	5.3 °C/min to 270 °C
	40 °C/min to 320 °C
	2-minute hold
Transfer line temperature	250 °C
Total run time	31.67 minutes
MS conditions	
Acquisition mode	Electron Ionization (EI), Multiple Reaction Monitoring
Tune	atunes.eiex.tune.xml
EMV gain	15 for naphthols; 50 for 3-OH-B[a]P; 40 for remaining time segments
Collision gas flow	Nitrogen, 1.5 mL/min
Quenching gas flow	Helium, 2.5 mL/min
Source temperature	230 °C

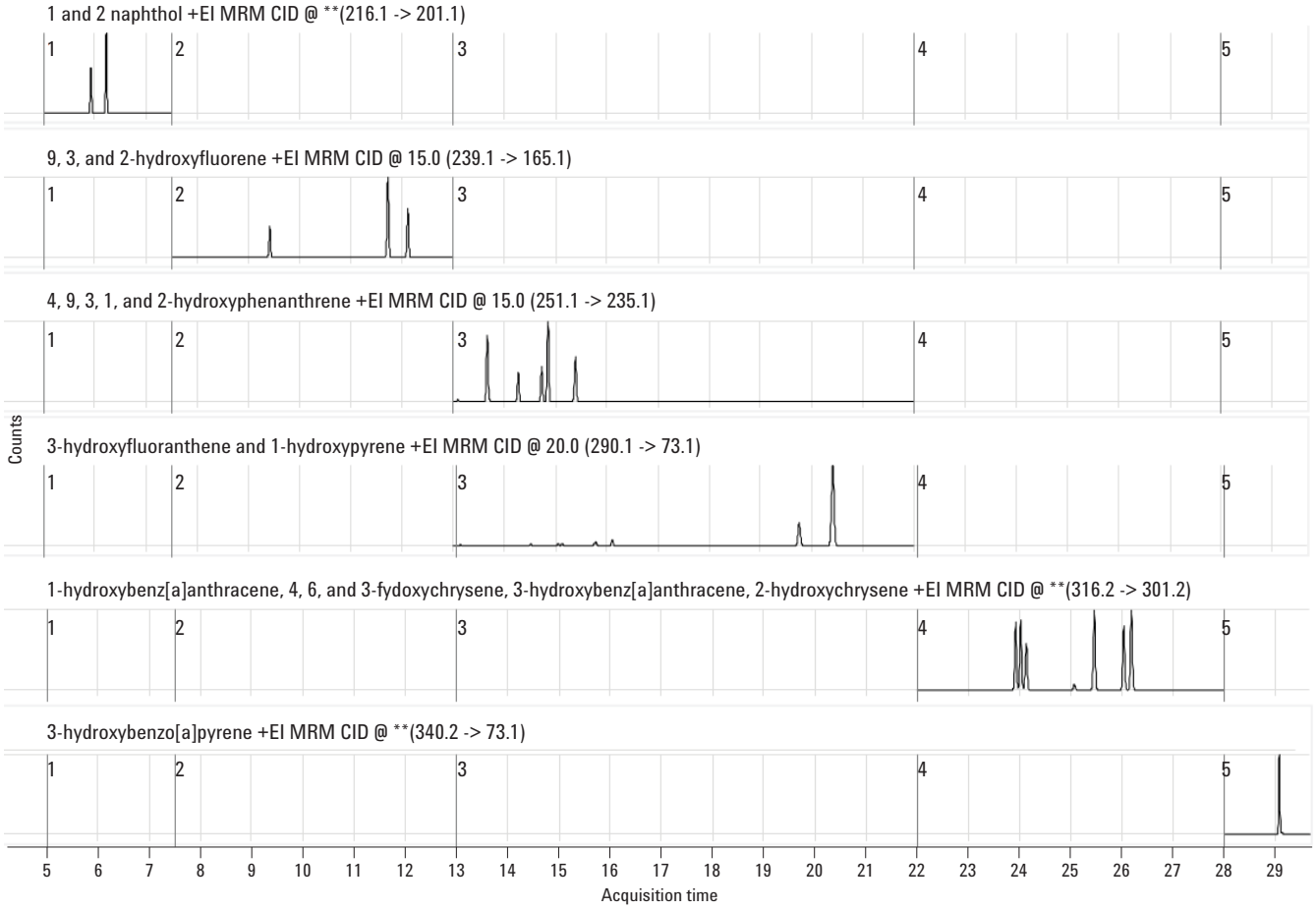

Figure 1. Chromatograms for all hydroxy PAHs in their respective time segments.

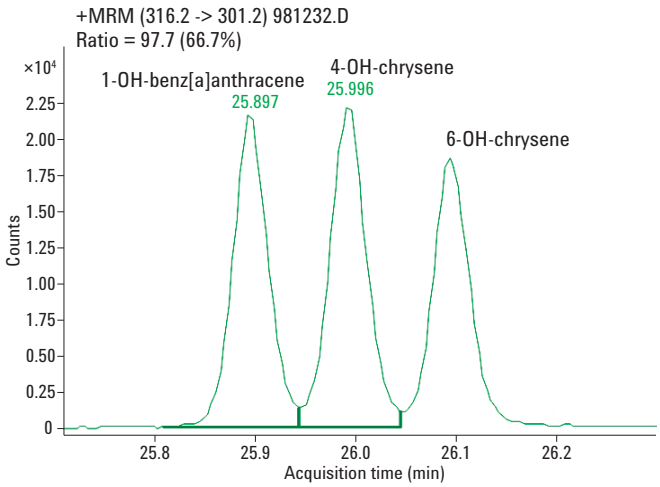

Figure 2. Representative chromatogram for the separation of three of the hydroxy PAHs.

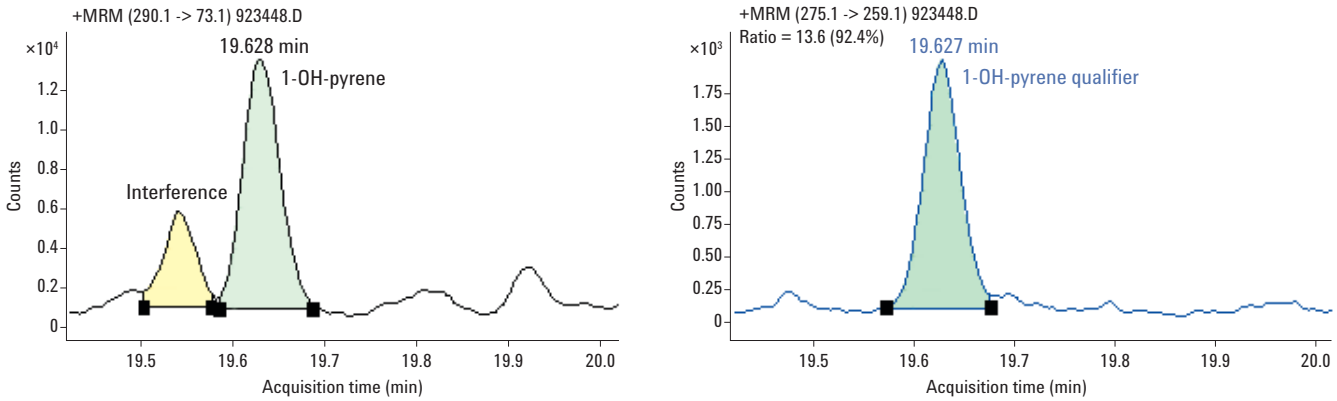

Figure 3. Good chromatographic separation of interference from 1-OH-pyrene quantifier and qualifier ions.

To meet the required sensitivity for the method, a minimum signal-to-noise ratio of 10 must be obtained for 3-OH-benzo[a]pyrene at a concentration of 0.01 µg/L (Figure 4). Hydroxylated benzo[a]pyrene was chosen because of its noted toxicity and because it is actively monitored in the environment. The extremely low detection limit in urine for 3-OH-benzo[a]pyrene is required because the major route of excretion is through feces (up to 90%) [6].

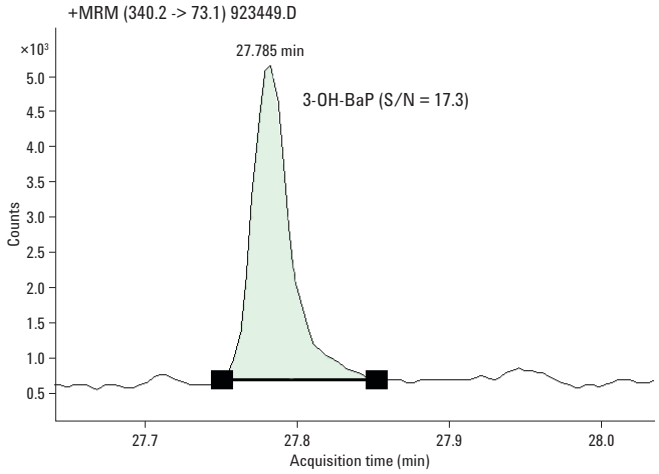

Figure 4. Sensitivity of 3-OH-BaP demonstrating 17.3 signal-to-noise (S/N) at 10 parts per trillion.

Quantitation

Calibration curves for hydroxy PAHs provided more accurate results when generated in urine rather than in a solvent such as benzene, presumably due to the possible adsorption of hydroxy-PAHs on the glass vial in benzene and possible interactions with active sites within the GC flow path. All samples, including calibration standards, were treated with the enzyme β-glucuronidase. It has been reported that the hydroxy-PAHs are more than 90% conjugated in urine [6]. However, curves determined with urine treated with or without β-glucuronidase were essentially identical (Figure 5). It is important to use the appropriate internal standard for each analyte to maximize accuracy of quantitation. For example, changing from the deuterated forms of internal standards (1 and 2-naphthol-d_7) to the ^{13}C forms (1 and 2-naphthol-$^{13}C_6$) helps to better monitor the native forms (Table 1).

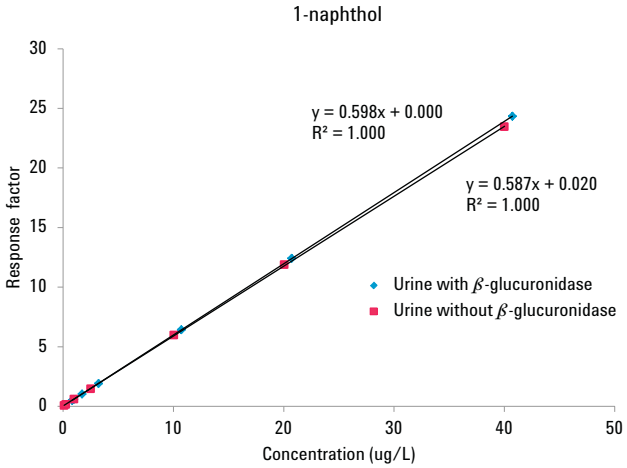

Figure 5. Representative calibration curves for 1-napthol, illustrating the nearly identical curves obtained in the presence or absence of β-glucuronidase.

Stability of Analytes in Urine Samples

Preliminary results suggest that all hydroxy-PAHs are stable for at least six weeks when stored below –20 °C. At room temperature, free hydroxy-PAHs appear to be less stable than the conjugated forms (Figure 6), and some of the hydroxy PAHs degrade over a three to six week period at room temperature, even in the conjugated form (Figure 7). Therefore, all urine samples were stored below –20 °C.

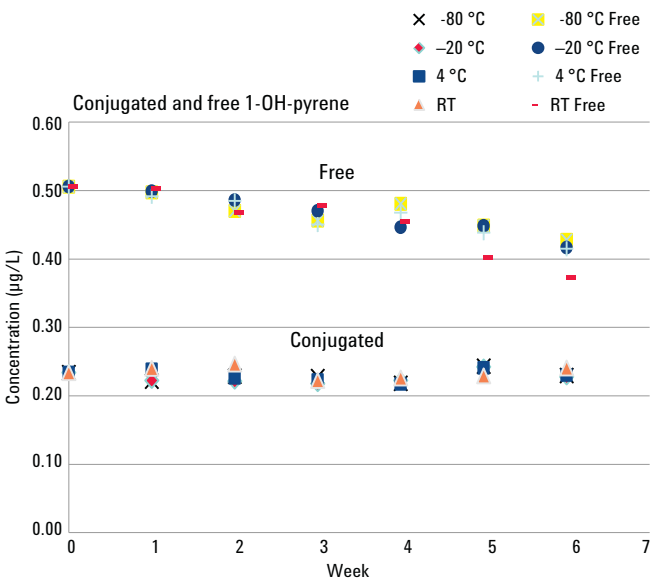

Figure 6. Reduced stability of the free form of a hydroxy-PAH versus the conjugated form.

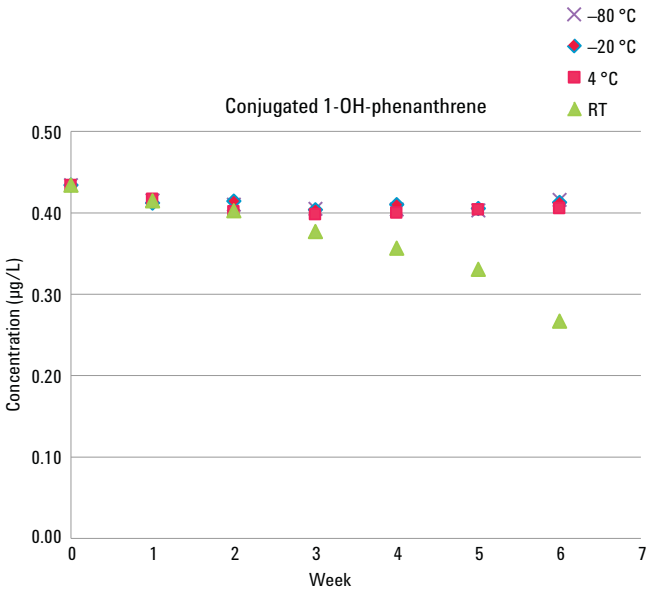

Figure 7. Stability as a function of time of the conjugated form of a hydroxy-PAH at various storage temperatures.

Method Performance

This method provides excellent sensitivity and recoveries, as well as intra- and inter-day precision for 19 hydroxy-PAHs (Table 3). LODs are as low as 0.7 parts per trillion (ppt; 0.0007 µg/L), LOQs are as low as 2.5 ppt, and, with the exception of 2-napthol, only two exceed 11 ppt. The linearity of quantitation is nearly five orders of magnitude. All but one of the recoveries are in the 94 to 104% range. Intra-day precision relative standard deviation (RSD) does not exceed 6.5% and is as low as 0.8 to 0.9% for some hydroxyl-PAHs. Inter-day precision does not exceed 10% and is as low as 3–5% for several analytes.

Table 3. Method Performance

					Precision		
Analyte	**Limit of detection (µg/L)**	**Limit of quantitation (µg/L)**	**Linearity (µg/L)**	**Recovery (%)**	**Concentration (µg/L)**	**Intra-day (%RSD*)**	**Inter-day (%RSD*)**
1-OH-benz[a] anthracene	0.0023	0.0076	LOQ to 10	97	0.022	2.7	9.4
3-OH-benz[a] anthracene	0.0018	0.0061	LOQ to 10	98	0.020	3.2	7.2
3-OH-benzo[a]pyrene	0.0014	0.0048	LOQ to 10	102	0.020	4.1	8.8
2-OH-chrysene	0.0028	0.0095	LOQ to 10	103	0.030	4.1	8.8
3-OH-chrysene	0.0025	0.0082	LOQ to 10	102	0.022	4.3	7.0
4-OH-chrysene	0.0033	0.0110	LOQ to 10	98	0.020	5.3	9.1
6-OH-chrysene	0.0027	0.0091	LOQ to 10	94	0.024	5.3	6.6
3-OH-fluoranthene	0.0020	0.0067	LOQ to 10	104	0.039	6.3	9.9
2-OH-fluorene	0.0019	0.0062	LOQ to 10	97	0.120	0.8	4.0
3-OH-fluorene	0.0007	0.0024	LOQ to 10	96	0.044	0.9	5.2
9-OH-fluorene	0.0027	0.0089	LOQ to 10	98	0.120	0.8	9.7
1-OH-phenanthrene	0.0020	0.0680	LOQ to 10	101	0.100	1.7	2.7
2-OH-phenanthrene	0.0016	0.0052	LOQ to 10	99	0.062	2.1	5.3
3-OH-phenanthrene	0.0016	0.0054	LOQ to 10	98	0.057	1.5	3.8
4-OH-phenanthrene	0.0007	0.0025	LOQ to 10	98	0.015	2.7	5.2
9-OH-phenanthrene	0.0030	0.0100	LOQ to 10	114	0.030	5.3	9.0
1-OH-pyrene	0.0014	0.0048	LOQ to 10	98	0.044	1.8	6.5
1-naphthol	0.0260	0.0860	LOQ to 10	98	1.800	2.4	7.4
2-naphthol	0.0400	0.1300	LOQ to 10	100	1.900	0.9	6.5

*%RSD – percent relative standard deviation

Conclusion

A highly sensitive and reproducible GC/MS/MS method for reliable monitoring of PAH metabolite levels in human urine has been developed on the 7000 Series Triple Quadrupole GC/MS System. The method parameters have been optimized to provide maximum separation, freedom from matrix effect, five orders of magnitude dynamic range, high recoveries, and part per trillion sensitivity. This method is currently used for the CHMS.

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