

Quantification of Caffeine Metabolites in Human Urine Via a Simple and Rapid Liquid Chromatography-Tandem Mass Spectrometry Method

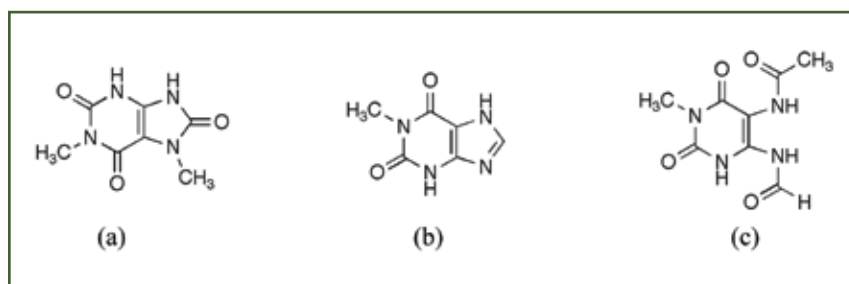
Authors

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Introduction

The ratios of caffeine metabolites from urinary collections can be used to assess the in vivo activities of caffeine metabolizing enzymes CYP1A2 and xanthine oxidase.¹ HPLC coupled with UV spectroscopy is commonly employed to quantify these metabolites with a lengthy chromatographic analysis (30-60 minutes) and a moderate quantification limit.² Here we report a simple sample preparation procedure combined with a rapid LC-MS/MS protocol to analyze three major caffeine metabolites: 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 1,7-dimethyluric acid (1,7-DMUA) and 1-Methylxanthine (1-Met). The method consists of a 2 minute LC separation followed by a 4 minute LC-column wash. The structures of these three metabolites are shown in Figure 1.

FIGURE 1. Chemical Structures of (a) 1,7-dimethyluric acid (1,7-DMUA); (b) 1-Methylxanthine (1-Met); (c) 5-acetylamino-6-formylamino-3-methyluracil (AFMU)



Method

SAMPLE PREPARATION:

1. Thaw samples in wet ice bath
2. Aliquot 50 μL of samples into a 96-well plate
3. Add 400 μL of ice-cold internal standard (500 ng/mL AFMU-D₃, 1,7-DMUA-¹³C₄, ¹⁵N₃ and 1Met-¹³C₄, ¹⁵N₃ in dilution buffer)
4. Vortex mix and centrifuge samples
5. Transfer 50 μL of supernatant into a clean 96-well plate
6. Add 400 μL dilution buffer
7. Transfer 50 μL of the diluted samples into a clean 96-well plate
8. Add 400 μL dilution buffer. Mix well.
9. Store the final sample plate at 1-8°C for analysis.

CHROMATOGRAPHIC CONDITIONS

Column:	Luna Silica, 5 μm
Mobile phase:	A: 10 mM Ammonium Acetate, pH unadjusted. B: Acetonitrile
C: Water:	Acetonitrile (10:90 v/v) (make up solvent for diversion)
Injection volume:	5-20 mL
Column temperature:	30°C
AS temperature:	1-8°C
Needle wash:	(10 mM Ammonium Acetate, pH unadjusted)/ MeCN (10:90 v/v)

MASS SPECTROMETER CONDITIONS

Instrument:	MDS API 4000
Ionization mode:	Turboionspray, Negative ion mode
Source Temperature:	500°C
SRM transitions:	AFMU 225.1 \rightarrow 127.1
	1,7-DMUA 195.1 \rightarrow 137.1
	1-Met 165.2 \rightarrow 108.1
	AFMU-D ₃ 228.1 \rightarrow 130.1
	1,7-DMUA- ¹³ C ₄ , ¹⁵ N ₃ 202.1 \rightarrow 142.1
	1Met- ¹³ C ₄ , ¹⁵ N ₃ 172.2 \rightarrow 113.1

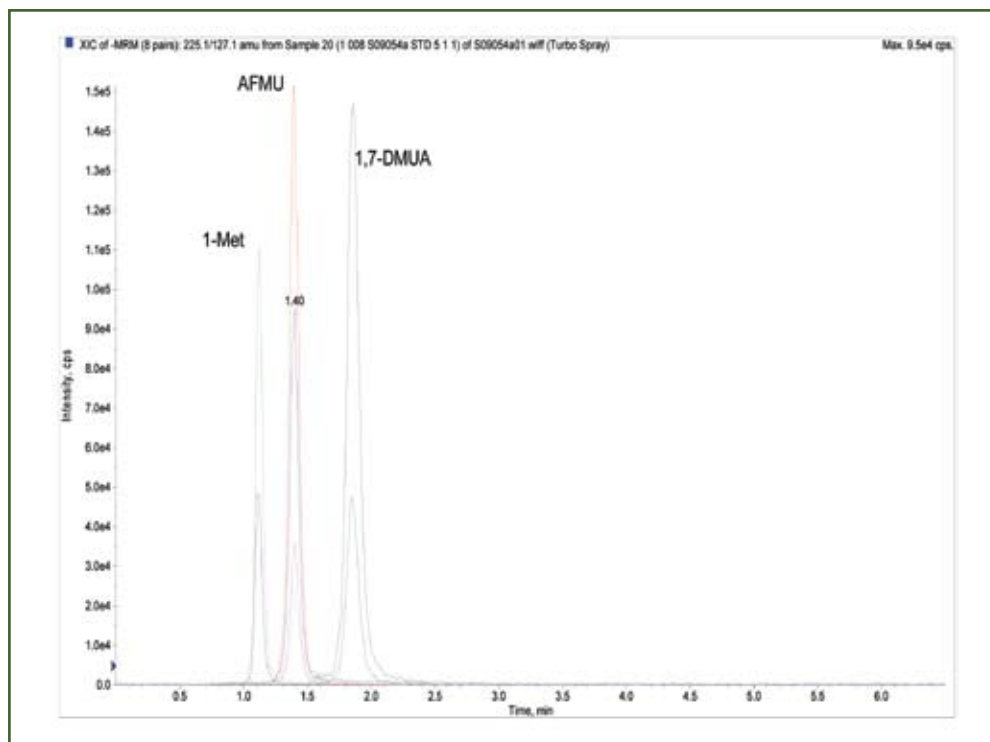
Results and Discussion

METHOD DEVELOPMENT & VALIDATION

1) Liquid Chromatography Development

The stock solutions for this combination assay were prepared separately. 1,7-DMUA and 1-Met are prepared in strong basic solvent while AFMU is prepared in slightly acidic solvent. Baseline separation was obtained for the three analytes within 2 minutes using a silica column at experimental conditions (Figure 2).

FIGURE 2. Chromatographic Separation of 1,7-DMUA, 1-Met and AFMU



Results and Discussion continued

2) Method selectivity and specificity

Six lots of pre-screened human urine were tested at blank and LLOQ level. The contribution of endogenous matrix elements to the analytes meets the acceptance criteria (Table 1). The specificity of the method was also verified by the interference test with a panel of caffeine compounds and small-molecule drugs (Table 2-3).

TABLE 1. Selectivity at the Lower Limit of Quantitation (500 ng/mL) for 1,7-DMUA, 1-Met and AFMU

	1,7-DMUA	1-Met	AFMU
Mean	500	541	538
S.D.	48.1	47.4	36.3
%CV	9.6	8.8	6.7
%Bias	0.0	8.2	7.6
n	6	6	6

TABLE 2. Interference Compounds and Concentrations

Interference Compound	Concentration (ng/mL)
Caffeine	10,000
1-Methyluric Acid	100,000
R-Warfarin	1000
S-Warfarin	1000
Omeprazole	1000
5-Hydroxy Omeprazole	1000
Midazolam	10.0
a-Hydroxymidazolam	10.0
Dextrophan	500
Dextromethorphan	500
Digoxin	50.0

TABLE 3. Interference Test for 1, 7-DMUA, 1-Met and AFMU

Analyte		Low QC 1500 ng/mL	High QC 40000 ng/mL
1,7-DMUA	Mean	1410	40900
	%CV	2.9	0.5
	%Bias	-6.0	2.3
1-Met	Mean	1450	41000
	%CV	2.9	1.8
	%Bias	-3.3	2.5
AFMU	Mean	1447	35900
	%CV	2.7	1.5
	%Bias	-3.6	-10.3

Results and Discussion continued

3) Linearity, precision and accuracy

- The method was validated for three analytes with a linear range of 500 to 50,000 ng/mL (Figure 2-6, Table 4).
- The precision and accuracy has been demonstrated at LLOQ, Low, Medium and High QC levels with n=6 for both 1,7-DMUA and 1-Met (Table 5-7).

FIGURE 2. Low Standard (500 ng/mL) for 1,7-DUMA

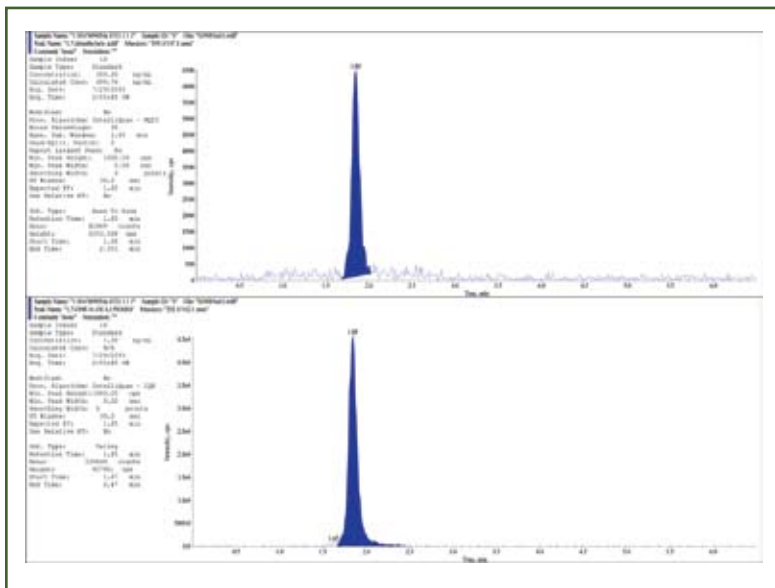
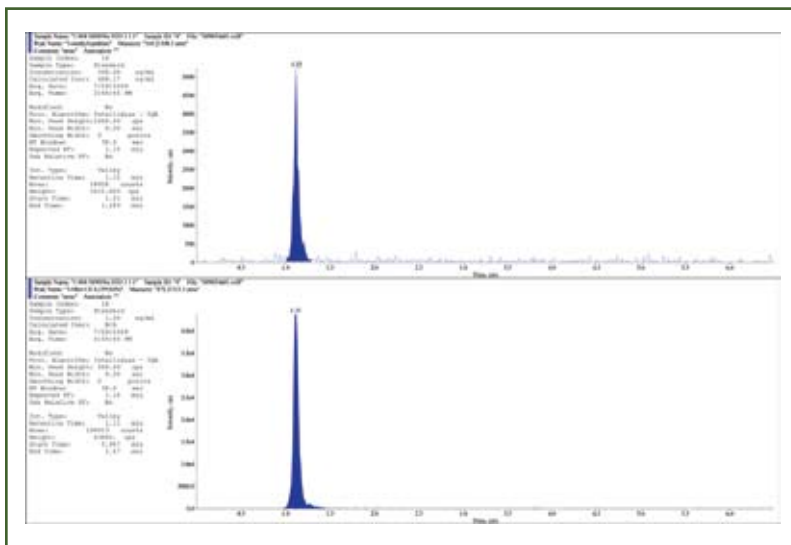
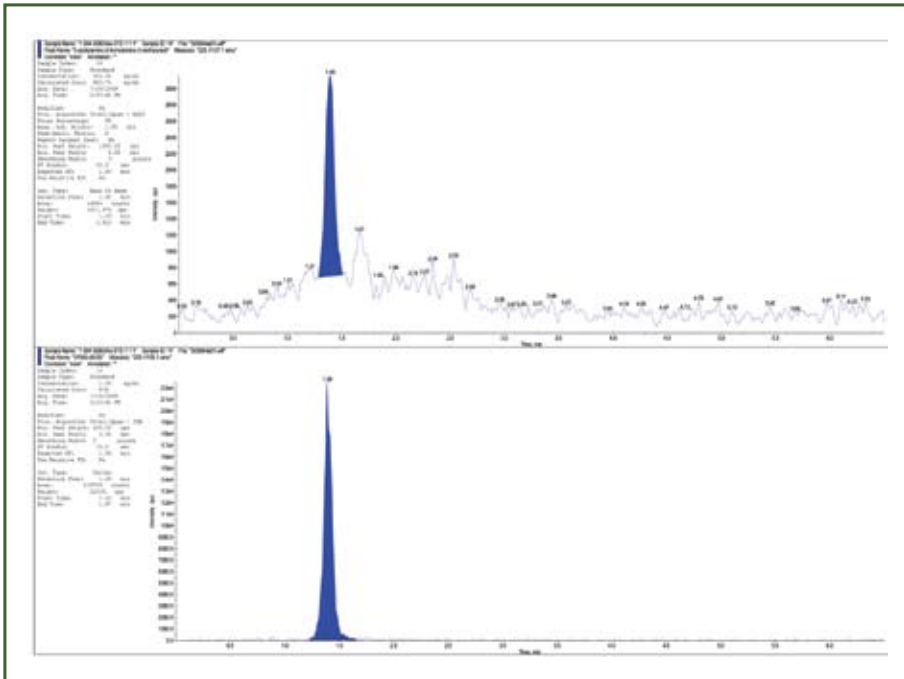


FIGURE 3. Low Standard (500 ng/mL) for 1-Met



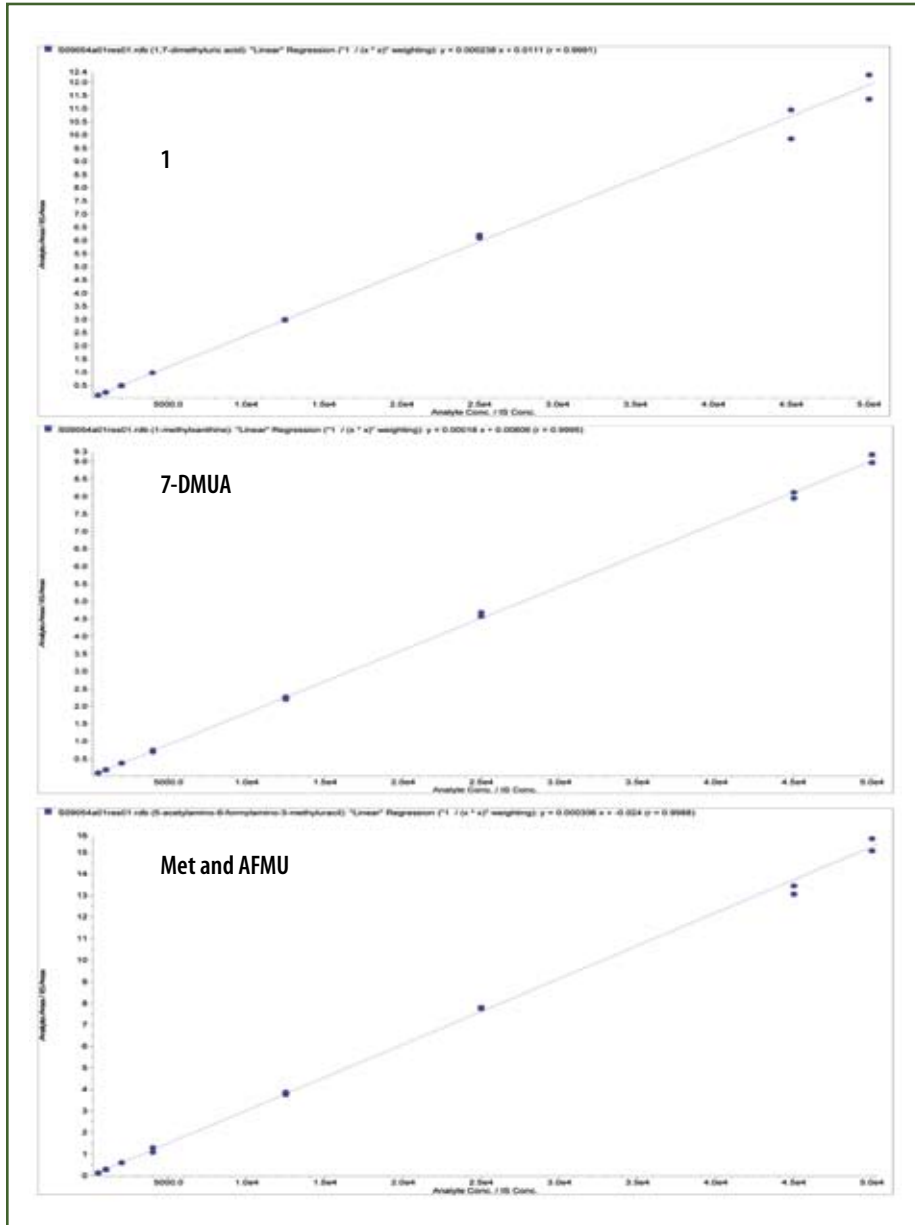
Results and Discussion continued

FIGURE 5. Low Standard (500 ng/mL) for AFMU



Results and Discussion continued

FIGURE 6. Representative Calibration Curve for 1,7-DMUA, 1-Met and AFMU



Results and Discussion continued

TABLE 4. *Back-Calculated Concentrations of Calibration Standards for 1,7-DMUA, 1-Met and AFMU (Linear weighted 1/x²)*

All concentrations are expressed as ng/mL.

Analyte		500	1000	2000	4000	12500	25000	45000	50000
1,7-DMUA	Mean	499	996	2020	4030	12800	25000	43100	50600
	%CV	4.4	3.6	2.9	4.9	2.5	3.9	5.7	3.8
	%Bias	-0.2	-0.4	1.0	0.8	2.4	0.0	-4.2	1.2
1-Met	Mean	505	982	1990	4010	12900	25000	44200	50100
	%CV	3.8	6.1	3.9	2.7	5.9	4.0	6.5	5.8
	%Bias	1.0	-1.8	-0.5	0.3	3.2	0.0	-1.8	0.2
AFMU	Mean	500	998	2005	3997	13000	24567	43900	49750
	%CV	2.4	5.1	2.0	7.8	2.8	3.6	2.5	2.6
	%Bias	0.0	-0.2	0.3	-0.1	4.0	-1.7	-2.4	-0.5

4) Stability

The stability data for 1,7-DMUA, 1-Met and AFMU are summarized in Table 8. AFMU only demonstrated 20 days of long-term matrix stability (LTMS) at -20 °C. Additional stability tests were performed at the High QC level which demonstrated that AFMU is not stable at -20 °C, but is stable at -70 °C (Figure 7).

Results and Discussion continued

TABLE 5. *Intra- and Inter-assay Accuracy and Precision for 1,7-DMUA*

Nominal Conc.	LLOQ QC 500 ng/mL	Low QC 1500 ng/mL	Medium QC 5000 ng/mL	High QC 40000 ng/mL
Mean Observed Conc.	458	1400	4800	39200
%Bias	-8.4	-6.7	-4.0	-2.0
Between Run Precision (%CV)	2.4	0.0	2.1	2.4
Within Run Precision (%CV)	6.1	4.5	5.1	2.1
Total Variation (%CV)	6.6	4.5	5.5	3.2
n	18	18	18	18
Number of Runs	3	3	3	3

TABLE 6. *Intra- and Inter-assay Accuracy and Precision for 1-Met*

Nominal Conc.	LLOQ QC 500 ng/mL	Low QC 1500 ng/mL	Medium QC 5000 ng/mL	High QC 40000 ng/mL
Mean Observed Conc.	481	1440	4970	41100
%Bias	-3.8	-4.0	-0.6	2.8
Between Run Precision (%CV)	3.7	2.0	5.0	4.8
Within Run Precision (%CV)	7.1	5.4	4.8	3.5
Total Variation (%CV)	8.0	5.8	6.9	5.9
n	24	24	24	24
Number of Runs	4	4	4	4

TABLE 7. *Intra- and Inter-assay Accuracy and Precision for AFMU*

Nominal Conc.	LLOQ QC 500 ng/mL	Low QC 1500 ng/mL	Medium QC 5000 ng/mL	High QC 40000 ng/mL
Mean Observed Conc.	499	1470	4656	35294
%Bias	-0.2	-2.0	-6.9	-11.8
Between Run Precision (%CV)	8.4	1.1	2.5	9.0
Within Run Precision (%CV)	6.2	4.6	6.1	3.7
Total Variation (%CV)	9.7	4.9	6.6	8.7
n	18	18	18	18
Number of Runs	3	3	3	3

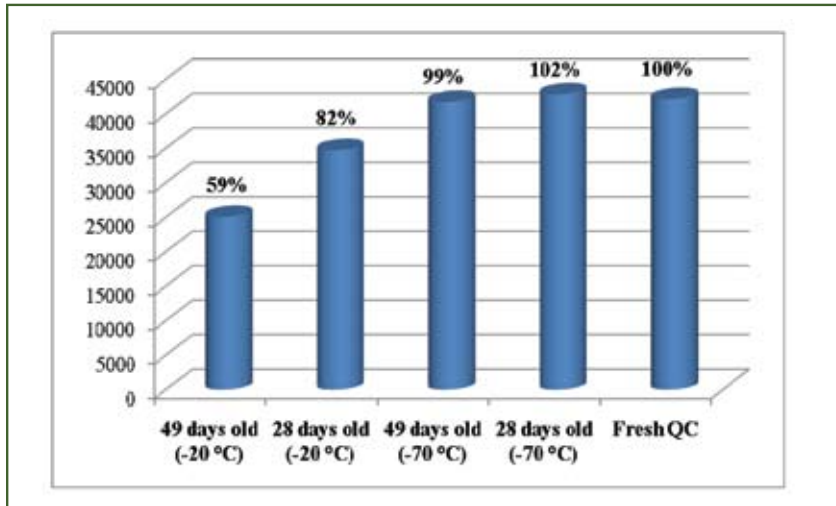


Results and Discussion continued

Table 8. Solution and Matrix Stability for 1,7-DMUA, 1-Met and AFMU

Stability Test	1,7-DMUA	1-Met	AFMU
Stock Solution	6 hours at RT 210 days at 1-8 °C	6 hours at RT 130 days at 1-8 °C	6 hours at RT 130 days at 1-8 °C
Stability in Human Urine	Freeze/Thaw, 4 cycles Wet ice bath, 6 hours LTMS at -20 °C /-70 °C, 213 days	Freeze/Thaw, 4 cycles Wet ice bath, 6 hours LTMS at -20 °C/-70 °C, 213 days	Freeze/Thaw, 4 cycles Wet ice bath, 6 hours LTMS at -20 °C/-70 °C, 20 days/ 49 days
Extract	222 hours at 1-8°C	222 hours at 1-8°C	222 hours at 1-8°C

FIGURE 7. Stability of AFMU at High QC Level vs. Storage Time and Temperature





Conclusions

- 1) A more rapid and robust LC/MS/MS method has been developed for analysis of three caffeine metabolites (1,7-DMUA, 1-Met and AFMU) compared to the existing method.
- 2) AFMU showed much shorter long-term matrix stability compared to 1,7-DMUA and 1-Met at -20 °C. However, additional long-term matrix stability for AFMU can be established at -70 °C.

References

1. Nyeki A. et. al. *Br. J. Clin. Pharmacol.* 2003, 55, 62-67.
2. Weimann A. et. al. *J. Mass Spectra.* 2005, 40, 306-317