Quantification of xanthine- and uric acid-related compounds in urine using a "dilute-and-shoot" technique coupling ultra-high-performance liquid chromatography and high-resolution Orbitrap mass spectrometry

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ABSTRACT

Quantitative analysis of relevant metabolites in biofluids such as urine is often a tedious procedure, since it usually requires extraction, purification or preconcentration. For instance, in the analysis of methylxanthines in urine, a solid-phase extraction is often required. In the current work, a rapid and highly sensitive "dilute-andshoot" method combining ultra-high-performance liquid chromatography and high-resolution mass spectrometry (UHPLC/HRMS) was validated for urinary determination of twelve analytes: uric acid, hypoxanthine,
xanthine, 1-methyluric acid, 1,3-dimethyluric acid, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, theophylline, theobromine, paraxanthine and caffeine. theophylline, theobromine, paraxanthine and caffeine. These analytes are the major physiological metabolites of caffeine, theobromine or theophylline, or final products of purine catabolism. The separation was carried out on a core-shell Kinetek EVO C18 column coupled to a Q Exactive Orbitrap high-resolution mass spectrometer equipped with a heated electrospray ionization (HESI) probe, that operated both in positive and negative io-nization modes. The twelve analytes eluted from between 1.5 and 10.5 min. The lower limit of quantification (LLOQ) values ranged from 0.25 to 2.5 ng/mL, and the calibration curves were linear from the LLOQ to 100 ng/ mL. The only pretreatment needed was to dilute each urine sample (typically to 1/500) with 0.1% formic acid solution, and then filter the diluted sample before injecting it into the UHPLC system. With this high dilution, there were no significant matrix effects, and the intra- and inter-day precision and accuracy values were ac-ceptable (coefficients of variance and relative errors below 15%, except for the LLOQ, for which they were below 20%). Furthermore, the analysis of spiked urine samples with 25 ng/mL of the target analytes showed excellent recoveries and precision levels for the twelve analytes. To our knowledge, there is no other published method that allows for the simultaneous determination of the concentrations of these twelve compounds, nor has a previously reported method been indicated to show such low LLOQ values as we have for the majority of the analytes. We expect our protocol to be useful for nutritional assessments, interventional studies, kidney stone research, and purine metabolism studies.

1. Introduction

Caffeine, theophylline and theobromine are three well-known me-thylxanthines with health benefits. Caffeine enhances fundamental as-pects of cognitive performance, such as vigilance, attention and reac-tion time, and improves physical performance [1,2]. Theophylline is used for the treatment of asthma and apnea of prematurity [3], while

theobromine has been tested as a cough suppressant, and to protect the enamel surface [4,5]. Several methods have therefore been developed to determine these three methylxanthines and their metabolites in plasma, saliva and urine. The percentages of these metabolites excreted in 48-h urine after the intake of caffeine, theophylline and theobromine are shown in Table 1 [6]. Most of these methods are based on liquid chromatography, with the chromatography apparatus coupled to an ultraviolet detector, photodiode-array detector, or mass spectrometry detector, usually with prior solid-phase extraction or liquid–liquid ex-traction [7–14].

These methylxanthines and related compounds are also involved in the formation of kidney stones. For example, high concentrations of uric acid in urine, coupled to a low urinary pH, can lead to the precipitation of uric acid in the kidneys [15]. The determination of urinary xanthine and hypoxanthine levels is also important, since xanthinuria and hypoxanthinuria are serious rare alterations that can lead to de development of xanthine or hypoxanthine kidney stones [16]. Recently, we have shown theobromine to be an inhibitor of uric acid crystallization with potential application in the treatment and prevention of uric acid nephrolithiasis [17]. Caffeine intake also ap pears to be associated with a lower risk of kidney stone formation, al-though the effects are not clear [18].

These observations suggest that consuming methylxanthines may help prevent renal lithiasis. It may therefore be useful to have an analytical procedure to rapidly quantify the main natural methyl-xanthines and their major metabolites, as well as the different compo-nents of kidney stones.

In this paper, we describe and validate a simple and rapid metho-dology based on ultra-high-performance liquid chromatography cou-pled with high-resolution mass spectrometry (UHPLCeHRMS), to de-termine the urinary concentrations of 12 compounds related to xanthine and uric acid (Fig. 1): uric acid (UA), hypoxanthine (HX), xanthine (X), 1-methyluric acid (1-MU), 1,3-dimethyluric acid (1,3-DMU), 1-methylxanthine (1-MX), 3-methylxanthine (3-MX), 7-methyl-xanthine (7-MX), theophylline (TF), theobromine (TB), paraxanthine (PX) and caffeine (CF). Our proposed approach is to use a “dilute-and-shoot” technique, which simply consists in diluting the urine samples, and directly inject them into the UHPLC system.

Table 1. Excretion of caffeine, theobromine, theophylline and their metabolites in human subjects after the intake of these compounds. Results are expressed as percent excreted in 48-h urine [6].

	Caffeine intake	Theophylline intake	Theobromine intake
% of excreted caffeine	1.2	–	–
% of excreted theobromine	2	–	20
% of excreted theophylline	1	16	–
% of excreted paraxanthine	6.5	–	–
% of excreted 1-methylxanthine	19	1	–
% of excreted 3- methylxanthine	3	14	21.5
% of excreted 7- methylxanthine	7.5	–	36
% of excreted 1-methyluric acid	26.5	20	–
% of excreted 1,3- dimethyluric acid	2.6	47	–
% of excreted of other compounds	30.7	2	22.2.5

2. Methods and materials

2.1. Reagents and solutions

Uric acid, hypoxanthine, xanthine, 1-methyluric acid, 1,3-di- methyluric acid,1-methylxanthine, 3-methylxanthine, 7-methyl- xanthine, theophylline, theobromine,

paraxanthine and caffeine were purchased from Sigma-Aldrich (St Louis, MO, USA). Optima LC/MS-grade acetonitrile was obtained from Fisher Scientific (Loughborough, UK). All solutions were prepared in ultra-pure H2O from a Milli-Q system and filtered through 0.45 μm filters.

2.2. Standards and quality controls

From each of the twelve analytes, a solution of 50 μg/mL was prepared by dissolving the appropriate amount of the powder product in water. A standard stock solution containing 1 μg/mL of all analytes was prepared in 0.1% formic acid and serially diluted in 0.1% formic acid solution to prepare the standard solutions used in this study. The working solution concentrations were 0.25, 0.5, 1, 2, 5, 10, 25, 50, 75 and 100 ng/mL. All solutions were filtered through 0.22 μm filters. As it was not possible to obtain fresh real urine without some of the analytes, such as uric acid, xanthine or hypoxanthine, quality control (QC) samples were prepared in synthetic urine, whose composition is shown in Table 2. This synthetic urine contained the most abundant substances found in urine, such as sodium, calcium, citrate and creatinine, in concentrations similar to those usually found in human urine, as well as a pH around 6, which is also a normal pH found in urine. The QC were prepared in synthetic urine diluted 1/500, at the lower limit of quantification (LLOQ), and at medium (50 ng/mL) and higher (100 ng/mL) concentrations of the calibration curve, for each analyte.

2.3. Sample preparation

All urine samples used in this study were obtained from healthy volunteers, who were free of dietary restrictions. Urine was collected into sterile 150 mL plastic flasks, and stored at 4 °C until analysis. Before being injected into the UHPLC system, each urine sample was diluted 500, 5000 and 10,000 folds with 0.1% formic acid solution, filtered through 0.22 μm filters, and transferred into the vial. A volume of 5 μL of the diluted urine was then injected into the UHPLC-HRMS system. We obtained prior approval for the analysis of the urine samples by the ethical committee of the Balearic Islands.

2.4. Ultra-high-performance liquid chromatography/high-resolution mass spectrometry

The LC was performed on a Dionex Ultimate 3000 UHPLC system (Thermo Fisher, Dreieich, Germany) and separation was carried out at 30 °C on a core-shell Kinetek EVO C18 column (100 × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA, USA), protected with a security C18 guard cartridge. The UHPLC system was coupled to a Q Exactive Orbitrap high-resolution mass spectrometer equipped with a heated electrospray ionization (HESI) probe (Thermo Fisher, Dreieich, Germany). Mobile phase A was 0.1% formic acid in water, and mobile phase B 0.1% formic acid in acetonitrile. A constant flow rate of 0.25 mL/min was set and the gradient elution was programmed as follows: 1–8% B from 0 to 5 min, 8–20% B from 5 to 9 min, and held at 20% B from 9 to 10.5 min. Finally, to equilibrate the column, the system was returned to initial conditions from 20 to 1% B from 10.5–10.7 min, and held at 1% until 15.5 min to recondition the column and eliminate any potential carryover prior the next injection. To speed up this last part, a flow rate of 0.40 mL/min was used. The HESI source operated both in positive and negative

mode, with a capillary voltage of 3.90 kV and a capillary temperature of 350 °C. The sweep gas and auxiliary pressure rates were set to 35 and 10, respectively. The sweep gas flow rate was set to 0. The S-Lens RF level was 55%, and the auxiliary gas heater temperature was 150 °C. Full-scan mass spectra were acquired, and MS/MS was performed to examine the confirmation peaks.

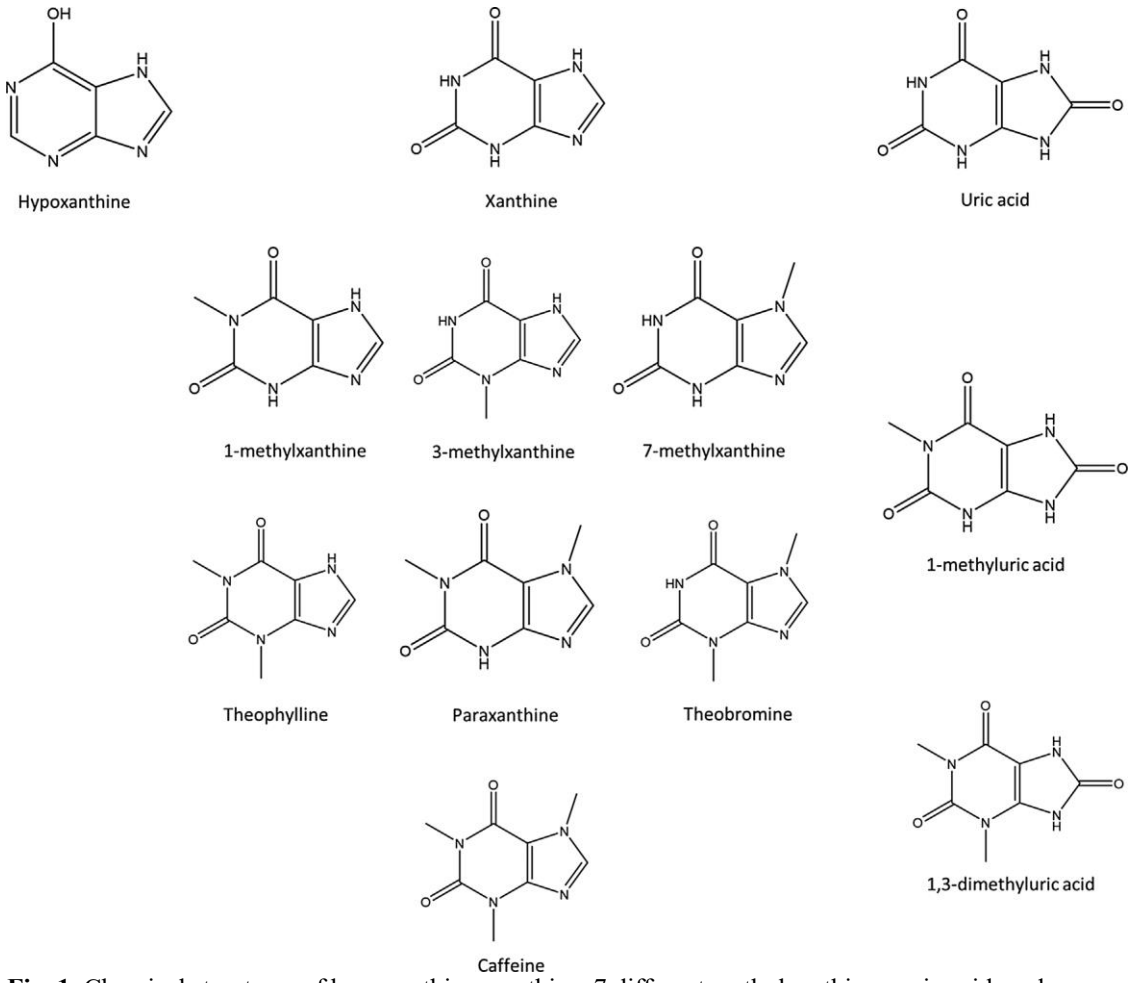

Fig. 1. Chemical structures of hypoxanthine, xanthine, 7 different methylxanthines, uric acid, and two different methyluric acid determined in this paper.

Table 2. Composition of the synthetic urine.

Solution A (g/L)		Solution B (g/L)	
Na2SO4 · 10H2O	6.23	NaH2PO4 · 2H2O	2.41
MgSO4· 7H2O	1.46	Na2HPO4 · 12H2O	5.60
NH4Cl	4.64	NaCl	13.05
KCl	12.13	Na2C2O4	0.092
CaCl2	0.83	Creatinine	0.2
Trisodium citrate dihydrate	2.5		

* Synthetic urine was obtained by mixing equal volumes of solutions A and B.

Table 3. Regression equations for the twelve analytes, for the selected ionization mode (positive or negative), retention times and LLOQ.

Analyte	Ret time (min)	Mode	Range (ng/ml)	Regression equation	R_2	LLOQ (ng/mL)
Uric acid	1.45	negative	2.5–100	y = 129900x + 108300	0.9997	2.5
Hypoxantine	1.48	positive	0.25–100	y = 554400x + 5943	0.9998	0.25
Xantine	1.74	positive	0.25–100	y = 286600x − 28830	0.9993	0.25
7-methylxanthine	3.26	positive	2.5–100	y = 382100x + 69840	0.9994	2.5
1-methyluric acid	3.47	negative	1–100	y = 168000x + 19950	0.9998	1
3-methylxanthine	3.78	negative	0.5–100	y = 192100x + 16930	0.9996	0.5
1-methylxanthine	4.35	negative	2.5–100	y = 185300x + 45240	0.9999	2.5
1,3-dimethyluric	6.15	negative	0.5–100	y = 197700x + 11520	0.9999	0.5
Theobromine	6.65	positive	2.5–100	y = 378200x − 113900	0.9996	2.5
Paraxanthine	8.20	positive	1–100	y = 604100x + 73520	0.9996	1
Theophylline	8.38	negative	1–100	y = 218000x + 25370	0.9997	1
Caffeine	10.43	positive	0.5–100	y = 870400x + 51550	0.9997	0.5

2.5. Method validation procedure

The method was validated with respect to selectivity, linearity, accuracy, precision, percent recovery and matrix effects, following the recommendations of the FDA text: Bioanalytical method validation [19]. Calibration curves were obtained by measuring peak areas, from the LLOQ of each analyte to 100 ng/mL. For each analyte, three QC samples were prepared, at the LLOQ, and at medium (50 ng/mL) and high (100 ng/mL) concentrations. The accuracy and precision of the measurements (intra and inter assay) were calculated by analyzing six replicates of each QC sample at the low, medium and high concentrations on three different days. Intra-day precision and accuracy were determined by analyzing six replicates of the QC three times in one day. Inter-assay precision and accuracy were calculated by analyzing the QC samples on three different days. Precision was expressed as the coefficient of variation (CV) and accuracy was calculated as the related error in the calculated mean concentration relative to the nominal concentration (RE). To be considered acceptable, the CV and RE at each QC level had to be less than 15%, except for the LLOQ, for which the CV and RE had to be less than 20%. Furthermore, to ensure precision and accuracy in the real urine samples, we analyzed four such samples, each diluted 500, 5000 and 10,000 fold and with each dilution spiked with 25 ng/mL of the analyte. Triplicates of each dilution were injected into the UHPLC-HRMS system, and CV and percent recovery were calculated. Finally, we carried out a stability sample study, in which we analyzed 4 urine samples recently collected, and after different storage condition: 36 h at room temperature; 10 days at 4 °C; 10 days at−20 °C and 10 days at −80 °C. Each urine sample was diluted with 0.1% formic acid solution prior storage, triplicates of these dilutions were analyzed, and the RE was calculated for each storage condition relative to fresh urine.

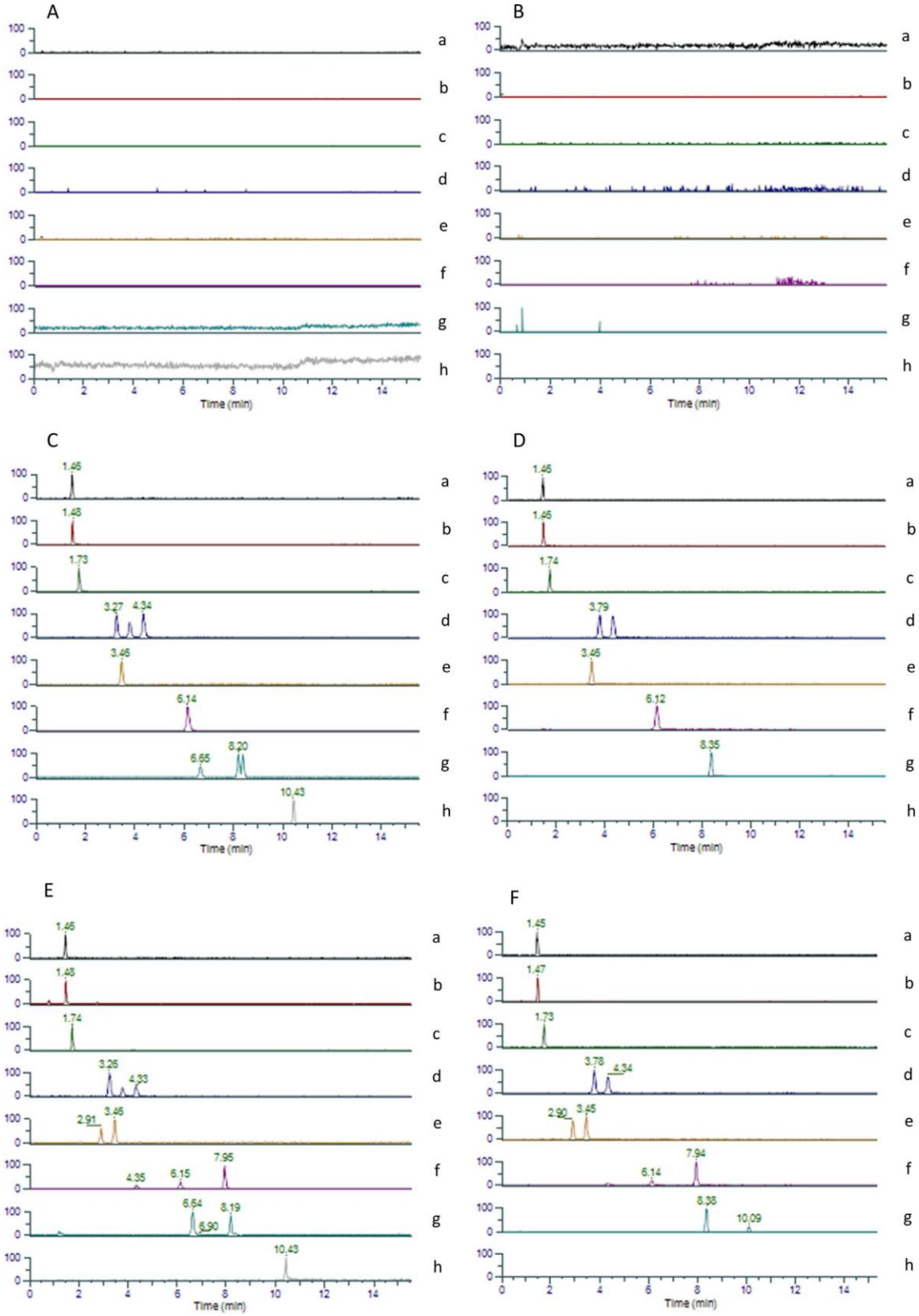

Fig. 2. Chromatograms of a blank in the (A) positive and (B) negative ionization mode; a quality control containing 50 ng/mL of all analytes in the (C) positive and (D) negative ionization mode; and a real urine sample diluted 1/500 in the (E) positive and (F) negative ionization mode. (a) Uric acid; (b) Hypoxanthine; (c) Xanthine; (d) 7-methylxanthine, 3-methylxanthine and 1-methylxanthine; (e) 1-methyluric acid; (f) 1,3-dimethyluric acid; (g) Theobromine, Paraxanthine and Theophylline; (h) Caffeine

Table 4. Accuracy (RE) and precision (CV) of intra- and inter-day assays (n = 6) for the QC samples at three different concentrations. Nominal concentrations are the concentrations in the quality control (synthetic urine dilute 1/500).

	Nominal concentration (ng/mL)	Intra-day assay			Inter-day assay		
		Mean calculated concentration (ng/mL)	Precision (CV; %)	Accuracy (RE; %)	Mean calculated concentration (ng/mL)	Precision (CV; %)	Accuracy (RE; %)
UA	2.5	1.99	7.3	20.3	2.00	0.5	20.0
	50	48.81	7.2	2.4	48.26	3.6	3.5
	100	98.78	6.6	1.2	96.85	5.3	3.1
HX	0.25	0.22	4.1	13.6	0.23	6.4	7.0
	50	53.53	1.0	−7.0	52.29	2.6	−4.6
	100	102.61	1.1	−2.6	101.90	1.1	−1.9
X	0.25	0.25	11.2	1.7	0.26	5.0	−2.3
	50	50.64	1.1	−1.3	49.73	2.3	0.5
	100	100.27	0.7	−0.3	100.23	0.3	−0.2
7-MX	2.5	2.88	5.6	−15.1	2.77	8.4	−11.0
	50	50.83	0.6	−1.7	49.97	2.0	0.1
	100	97.12	1.4	2.9	97.73	1.0	2.3
1-MU	1	0.87	9.8	12.6	0.85	6.5	15.2
	50	52.18	7.8	−4.3	51.00	4.4	−2.0
	100	104.65	6.3	−4.6	101.83	5.6	−1.8
3-MX	0.5	0.43	5.0	13.4	0.43	6.9	14.1
	50	51.68	5.8	−3.4	50.86	4.9	−1.7
	100	103.64	4.7	−3.6	101.54	4.8	−1.5
1-MX	2.5	2.38	7.6	4.6	2.33	7.5	6.8
	50	53.02	7.3	−6.0	52.02	5.9	−4.0
	100	105.96	6.1	−5.9	103.53	6.1	−3.5
1,3-DMU	0.5	0.44	7.6	12.9	0.43	6.1	14.5
	50	51.93	6.7	−3.8	50.77	3.4	−1.5
	100	103.74	5.3	−3.7	101.69	4.3	−1.7
TB	2.5	2.68	2.8	−7.1	2.80	6.1	−12.0
	50	49.67	1.2	0.7	49.11	1.0	1.8
	100	100.38	0.3	−0.4	99.66	0.9	0.3
PX	1	0.85	5.8	15.0	0.84	2.1	15.6
	50	48.32	1.8	3.3	48.21	1.9	3.6
	100	95.99	0.7	4.0	96.66	1.8	3.3
TF	1	1.18	2.7	−18.1	1.13	3.8	−13.4
	50	53.23	5.7	−6.4	52.07	4.0	−4.1
	100	104.47	4.6	−4.5	102.76	3.9	−2.8
CF	0.5	0.53	1.0	−5.3	0.51	11.1	−1.7
	50	48.32	3.6	3.3	47.80	2.2	4.4
	100	95.53	3.4	4.5	95.57	3.5	4.4

3. Results and discussion

3.1. Liquid chromatography

The liquid chromatography conditions were established through a modification of a previous validated procedure to determine urinary concentration of theobromine in urine [8]. A previously unused UHPLC column was used throughout the validation procedure. We sought conditions that would separate the twelve compounds in a short period of time, and yield a good separation between the isomers TB, TF and PX, and also between 1-MX, 3-MX and 7-MX. The retention times for our analytes can be seen in Table 3 and Fig. 2. All of them eluted at some time period between 1.45 min (uric acid) and 10.43 min (caffeine). Uric acid and hypoxanthine had very similar retention times (1.45 and 1.48 min, respectively). However, due to their different chemical structures (Fig. 1), they could be quantified by using the mass spectrometer. The separation between the isomers paraxanthine and theophylline is usually a challenging part [11]. However, in our case, there was a good separation between them (8.20 and 8.38 min, respectively), and there were different confirmation peaks for both of them, which allowed us to unmistakably

identify and quantify the target analyte. To avoid retention time drift, three blanks were injected into the UHPLC system before each set of samples were injected. During all of the validation processes, the retention times were stable, except on one occasion, in which a change of the security guard cartridge was necessary.

3.2. Sample preparation

One of the benefits of the present method is that it does not require extraction, purification or preconcentration of the urine sample. The "dilute-and-shoot" method is an interesting approach and has been successfully used in doping control [20]. However, its use in quantitative analysis of compounds related to xanthines and uric acid is scarce. As the method is also extremely sensitive, we decided to assay three urine dilutions: 1/500, 1/5000 and 1/10,000. Our first approach was to dilute the urine samples with water or with ammonium formate buffer, but in both cases high matrix effects were observed for some of the analytes, mainly uric acid and methyluric acids. This matrix effect was overcome by diluting the urine samples in mobile phase A (0.1% formic acid). Thus, the sample preparation consisted of just urine dilution, followed by filtration through a 0.22 μm filter, and injection into the UHPLC system.

3.3. High-resolution mass spectrometry

Full-scan spectra were acquired both in positive and negative modes, with a resolution of 70,000. At the beginning of the validation process, we used the positive mode for all of the analytes, in order to adjust the chromatographic conditions and obtain well-defined peaks. Four of the twelve analytes (7-MX, TB, PX and CF) were not detectable in the negative ionization mode. The explanation may be that each of them have a methyl group instead of a hydrogen attached to the nitrogen in the seventh position of the xanthine, and therefore cannot lose a proton here to form a negative charge. For the rest of the analytes, we chose the positive or negative ion mode based on which mode gave the better sensitivity (Table 3). Chromatographic profiles of all the analytes both in the positive and negative modes (when available) are shown in Fig. 2. MS/MS was also performed, with a resolution of 17,500 in order to verify the confirmation peaks in case of doubt when retention times alone could not unambiguously identify the analyte. For example, this tool was very useful to distinguish between the isomers TF and PX, which had similar retention times. Their different confirmation peaks allowed us to identify each analyte.

Table 6. Stability of the analytes in urine samples at different storage conditions: 36 h room temperature and 10 days at 4 °C, −20 °C and −80 °C. Determination was performed in triplicate and accuracy was expressed as % of relative error (RE) related to the concentration of fresh urine.

	Analyte	Urine dilution	Conc (ng/mL) Mean (SD) Fresh urine	Accuracy (% RE) 36 h room temp.	Accuracy (% RE) 10 days 4 °C	Accuracy (% RE) 10 days −20 °C	Accuracy (% RE) 10 days −80 °C
Urine 5	UA	1/10,000	71.57 (1.04)	−3.0	0.5	−1.2	−0.5
	HX	1/500	31.04 (1.67)	−4.8	4.8	4.2	1.3
	X	1/500	21.69 (1.24)	−3.6	5.1	2.7	2.0
	7-MX	1/500	49.34 (2.37)	−7.0	1.6	−9.5	0.5
	1-MU	1/10,000	5.97 (0.16)	−13.0	−4.3	5.3	−5.0
	3-MX	1/500	23.47 (0.23)	−0.07	1.4	0.9	0.0
	1-MX	1/500	66.92 (0.05)	−3.3	1.5	−8.3	0.8
	1,3-DMU	1/500	11.95 (0.54)	−2.8	3.4	1.4	1.8
	TB	1/500	33.20 (1.33)	−3.4	−1.1	−2.0	−2.9
	PX	1/500	24.03 (0.69)	−3.7	2.9	2.4	2.4
	TF	1/500	2.03 (0.14)	−3.9	−44.0	−42.3	−42.2

	CF	1/500	6.66 (0.01)	7.3	3.2	−1.3	3.8
	UA	1/10,000	31.10 (1.25)	−8.9	−5.0	7.2	−5.5
	HX	1/500	2.82 (0.12)	7.2	11.0	9.1	7.2
	X	1/500	4.42 (0.19)	−3.1	2.3	4.5	3.1
	7-MX	1/500	24.23 (0.42)	0.01	1.9	1.6	2.4
	1-MU	1/500	28.97 (0.15)	−2.8	0.0	−0.4	−0.7
	3-MX	1/500	12.77 (0.12)	1.9	1.2	−0.6	−0.6
	1-MX	1/500	24.40 (0.48)	−1.2	−0.7	0.1	−0.05
	1,3-DMU	1/500	3.61 (0.05)	−0.2	−1.3	−1.3	0.4
	TB	1/500	26.58 (0.96)	0.07	−1.2	−0.8	−4.2
	PX	1/500	21.74 (0.25)	0.6	0.4	−0.2	−0.2
	TF	1/500	1.82 (0.07)	−5.6	−11.4	−11.9	−8.9
	CF	1/500	9.09 (0.40)	−3.5	−8.7	−5.0	−6.8
Urine 7	UA	1/10,000	99.58 (1.91)	−5.8	1.1	1.0	0.7
	HX	1/500	61.56 (0.62)	3.2	11.5	4.2	7.7
	X	1/500	62.96 (1.73)	−3.2	2.3	1.1	4.8
	7-MX	1/10,000	14.11 (0.39)	1.1	1.4	2.1	4.1
	1-MU	1/10,000	6.67 (0.04)	−9.5	−0.1	−0.9	−0.8
	3-MX	1/10,000	10.56 (0.21)	0.9	−0.5	−0.4	1.6
	1-MX	1/500	57.95 (0.46)	−3.6	0.6	−4.6	0.5
	1,3-DMU	1/500	20.42 (0.15)	−1.8	3.7	−4.2	4.6
	TB	1/500	52.38 (1.45)	−0.4	1.4	−3.5	2.1
	PX	1/500	11.08 (0.21)	0.7	5.6	10.2	10.2
	TF	1/500	BELOW LLOQ				
	CF	1/500	4.21 (0.24)	4.8	0.9	−12.2	−6.1
Urine 8	UA	1/10,000	73.21 (1.21)	−9.0	1.7	−3.7	−4.9
	HX	1/500	26.77 (0.66)	2.0	15.3	10.3	−1.2
	X	1/500	23.46 (0.76)	−3.4	1.1	−2.3	−10.0
	7-MX	1/500	60.84 (0.80)	−5.4	4.4	2.9	−9.4
	1-MU	1/10,000	11.86 (0.12)	−18.5	−1.1	−5.1	−4.9
	3-MX	1/500	37.14 (0.60)	−1.2	0.9	−1.9	−12.9
	1-MX	1/10,000	6.29 (0.13)	3.0	7.4	1.0	1.1
	1,3-DMU	1/500	28.18 (0.07)	−2.3	3.7	1.3	−9.5
	TB	1/500	13.48 (0.11)	1.9	5.3	2.4	−7.9
	PX	1/500	28.37 (0.20)	−2.4	2.4	−10.8	−10.8
	TF	1/500	2.11 (0.08)	−1.8	−41.6	−39.9	−48.5
	CF	1/500	10.94 (0.22)	−1.2	2.6	0.5	−14.1

3.4. Method validation

The retention times, the ion mode (positive or negative), the LLOQ values, and the calibration curves resulting from triplicates of all the standards are shown in Table 3. The calibration curves were acquired from the LLOQ to 100 ng/mL. Since we tested urine dilutions of 1/500, 1/5000 and 1/10,000, the validation procedure was performed using as QC a synthetic urine diluted to 1/500. This dilution was found to have acceptable accuracy and precision, as well as lack of interference and matrix effects, which were also assumed for the more diluted urines, i.e., 1/5000 and 1/10,000. The LLOQ was set as the lowest concentration that allowed the concentration of the analyte to be determined with a precision (CV) and accuracy (RE) below 20%. The LLOQ values varied from 0.25 to 2.5 ng/ mL, depending on the analyte. Of all the analytes tested, HX and X showed the lowest LLOQ values, followed by 3-MX, 1,3-DMU and cf. To the best of our knowledge, these LLOQs are the lowest yet published for these analytes. This result is very useful, especially for HX and CF (Table 3): HX is further metabolized by xanthine oxidase to xanthine, and then to uric acid, and the concentration of HX in real urine is thus usually low; caffeine is also metabolized, and only around 1.2% of the caffeine intake is excreted in urine, and thus sensitive methods are necessary to quantify caffeine, especially if its intake is not very high. Four analytes, UA, 7-MX, 1-MX and TB, showed an LLOQ of 2.5 ng/mL. This high value does not pose such a problem since these compounds are usually excreted in the urine in relatively high amounts anyway. UA is always excreted in high amounts in urine (around 200–650 mg in 24-h urine) [21], and a dilution of 1/10,000 is always required. 7-MX and TB are excreted in considerable amounts if theobromine is consumed (Table 1), and 1-MX is a major metabolite of caffeine. The

intra-day and inter-day precision and accuracy values at three concentration levels are shown in Table 4. The intra-day precision values were determined from the means of three runs of six replicates analyzed in the same day, and inter-day precision values were calculated from the means of three runs of six replicates on three separate days. In all of the cases, the precision and accuracy values were calculated to be below 15%, except for some analytes at the LLOQ level, which were all below 20%. These results indicated an acceptable precision and accuracy for the present method. Since the method was validated in synthetic urine, four real urine samples, each diluted to 1/500, 1/5000 and 1/10,000, were analyzed. All of them were injected into the UHPLC system three times, with and without the addition of 25 ng/mL of the twelve analytes. For the twelve analytes, the dilution whose concentration was nearest to the middle of the calibration curve was chosen as the best dilution for that analyte in a particular urine sample. Results for four urine samples are shown in Table 5. In all of these four cases, for the twelve analytes, both the precision (CV) and accuracy (% recovery) were found to be acceptable. For eleven of the analytes, a 1/500 dilution was the best choice. However, for uric acid, a dilution of 1/10,000 was needed, due its concentration in the urine being much higher than the concentrations of the other analytes. Thus, to determine the concentrations of the twelve analytes in urine, we recommend to dilute each urine sample to 1/500 and 1/10,000. The concentration of theophylline was below the LLOQ in two of the samples. This result is probably due to the lack of theophylline intake, since it is only present in low amounts in cocoa and in tea. The main limitation of the present validated method is that it requires the use of an Orbitrap system, which is not available in most laboratories. This method yielded very low LLOQ values for the majority of the analytes, but as a urine dilution of at least 1/500 was used, the concentrations that could be determined in the urine were 500 times higher. However, due to the sensitivity of the method, and the concentrations of these analytes commonly found in urine, this issue seems not to be a drawback whatsoever. If needed, the method can be revalidated for lower dilutions of analytes in urine samples, in order to be able to determine lower concentrations. The results of the stability of urine samples under different storage conditions can be seen in Table 6. All the RE are below 15% in all analytes for the four urine samples, with the exception of theophylline. In the case of this analyte, when urine was stored for ten days, the RE was too high to be acceptable (around 40% in some cases). This is probably due to the low theophylline concentration (near the LLOQ), but also has to be taken into account that low amounts of theophylline may be lost by adsorption or degraded. So, for theophylline, we would not recommend to store urine samples more than two days.

4. Conclusion

In conclusion, we have validated a rapid, highly sensitive, specific and reliable dilute-and-shoot UHPLC/HRMS method for the determination of hypoxanthine, xanthine, uric acid, methyluric acids, and methylxanthines in urine. The concentration of the twelve analytes can be determined within a run time of 10.5 min, and with no sample pretreatment. This method allows the determination of the concentration of the main metabolites of caffeine, theobromine and theophylline, so it can be useful in interventional studies or nutritional assessments of these compounds. Furthermore, the analysis of xanthine, hypoxanthine and uric acid can also be useful in kidney stone research and purine metabolism studies. To the best of our knowledge, there is no other published method that allows for the simultaneous determination of the concentrations of these twelve compounds. Nor has a previously reported method been indicated to show

such low LLOQ values as we have for the majority of the analytes. If needed, the method can be easily revalidated for plasma samples or other biofluids such as saliva.

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Conflict of interest
All authors declare that they do not have any conflict of interest

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