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RESEARCH PAPER

Deciphering the human urine matrix: a new approach to simultaneously quantify the main ions and organic compounds by ion chromatography/mass spectrometry (IC-MS)

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Abstract

Analyzing the composition of (human) urine plays a major role in the fields of biology and medicine. Organic molecules (such as urea, creatine) and ions (such as chloride, sulfate) are the major compounds present in urine, the quantification of which allows for the diagnosis of a subject's health condition. Various analytical methods have been reported for studying urine components and validated on the basis of known and referenced compounds. The present work introduces a new method able to simultaneously determine both major organic molecules and ions contained in urine, by combining ion chromatography using a conductimetric detector with mass spectroscopy. The analysis of organic and ionized compounds (anionic and cationic) was achieved in double injections. For quantification, the standard addition method was used. Human urine samples were pre-treated (diluted and filtered) for IC-CD/MS analysis. The analytes were separated in 35 min. Calibration ranges (0–20 $mg.L^{-1}$) and correlation coefficients (> 99.3%) as well as detection (LODs < 0.75 $mg.L^{-1}$) and quantification (LOQs < 2.59 $mg.L^{-1}$) limits were obtained for the main organic molecules (lactic, hippuric, citric, uric, oxalic acids, urea, creatine, and creatinine) and ions (chloride, sulfate, phosphate, sodium, ammonium, potassium, calcium, and magnesium) contained in urine. The intra- and inter-day accuracies of the analytes consistently ranged from 0.1 to 5.0%, and the precision was within 4.0%. For all analytes, no significant matrix effects were observed, and recoveries ranged from 94.9 to 102.6%. Finally, quantitative results of analytes were obtained from 10 different human urine samples.

Keywords Human urine analysis · Ion chromatography · Ion exchange · Mass spectrometry · Matrix effect · One-pot quantification

Abbreviations

DP	Dual pump
ESI	Electrospray ionization
HPLC	High-performance liquid chromatography
IC-CD/MS	Ion chromatography–conductivity detector/mass spectroscopy
ICH	International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
ME	Matrix effect
RE	Relative error
RF	Radio frequency
RSD	Relative standard deviation
SD	Standard deviation
UHPLC	Ultra-high-performance liquid chromatography

Symbols

a.u.	Arbitrary unit
B	Magnetic field (T)
K_1 and K_2	Calculated ratios to validate the human urine presence in a sample (dimensionless)

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$^{m}/_{z}$	Mass-to-charge ratio
n	Number of repetitions (dimensionless)
N	Size of the population
T_r	Retention time (min)
R^2	Correlation coefficient (dimensionless)
x_i	Each value from the population
μ	Population mean

Introduction

Using the human urine composition as a health indicator is an old method; first known as uroscopy and then as urinalysis, this practice has been in existence for about 6000 years ago. Early physicians considered urine as a revealing diagnostic tool, allowing them to understand various diseases like infections of the urinary tract, problems with the kidneys and bladder, and liver failure [1–4].

Produced by the kidneys and stored in the bladder between urinations, urine's composition depends on various factors, such as diet, lifestyle, and individual characteristics (i.e., gender, age, weight, eating habits). When freshly excreted from the bladder, urine contains more than 95*wt*% of water. Substances present in urine are split into organic compounds (~ 60*wt*%, composed of urea representing more than 60%, followed by ammonium salts ~ 20%, the rest including creatinine and other compounds) and inorganic compounds (~ 40*wt*%, salts such as sodium chlorides, potassium chlorides, sulfates, carbonates, and phosphates).

The work of Prof. D.F. Putnam in 1971 pioneered the determination of the human urine composition [5]. They provided, for the first time, a deep analysis demonstrating that human urine is an aqueous solution of urea, creatinine, uric acid, and various other species, such as chloride, sodium, potassium, sulfate, ammonium, and phosphate in smaller quantities. Proteins were also found to be present in urine but in trace amounts compared to their concentrations in blood plasma. Consequently and after a multitude of research works, Bouatra et al. [6] identified in 2013 more than 3000 components in human urine. Since then, over 90 compounds were found to be consistently present in urine samples, irrespective of gender or collection time. Later, various analytical methods have emerged to inspect the urine matrix [7, 8], and currently, multiple specific techniques are available to quantify traces of many "minor" molecules in urine, such as HPLC (hormones [9], antidepressants [10], ranitidine [11]), UHPLC/MS/MS (pyrithione metabolites [12], DL-cysteine [13], steroid hormones [14]) or LC-MS/MS (gluten-derived metabolites [15], THC metabolites [16], phytocannabinoids [17]).

However, any study has yet been developed allowing efficient quantification of the most concentrated compounds without sample preparation and in a short time. Among the different analytical techniques used to measure separately the majority molecules contained in urine, one can typically cite:

(i) For urea, electrochemical sensor [18], chemiluminometric [19], and spectrophotometric [20] methods;
(ii) For creatinine, electrochemical detection [21], HPLC-MS/MS [22], and colorimetric method [23];
(iii) For chlorides, spectrophotometric method [24].

Besides, the simultaneous determination of both uric acid and creatinine has already been reported using HPLC [25, 26] or LC/MS [27]. Takao et al. quantified urea, uric acid, and creatinine by LC/MS [28] in real human urine matrices, while Wang et al. focused on the simultaneous potentiometric detection of sodium and potassium [29] in artificial urine solutions. Using paper-based sensors in synthetic urine, Tasoglu et al. successfully detected, in physiological concentrations, the major ions contained in urine, such as sodium, potassium, calcium, chloride, and nitrite [30]. All these methods are undeniably accurate but do not allow for one-pot and short-time analysis of the majority of compounds in the urine matrix. To the best of our knowledge, none of them is able to simultaneously determine the concentrations of both ions and organic compounds.

For the first time, the present work proposes a simple, rapid, and sample preparation-free method for the simultaneous analysis of the main organic molecules (lactic acid, hippuric acid, citric acid, uric acid, oxalic acid, urea, creatine, and creatinine as biomarkers [31–36]) and the most concentrated ions (chloride, sulfate, phosphate, sodium, ammonium, potassium, magnesium, and calcium) contained in human urine. This method consists of coupling ion chromatography to mass spectroscopy, which relevancy, accuracy, and robustness will be demonstrated through a study involving samples obtained from 10 healthy human volunteers.

Material and methods

Chemicals

Lactic acid, hippuric acid, citric acid, uric acid, oxalic acid, urea, creatine, and creatinine were purchased from Sigma-Aldrich (> 99.5%, St-Louis, USA) with a Normapur® grade. Anionic standards were prepared from a mixed solution from CPAChem (*Combined seven anions standard II–7 components*, Bogomilovo, Bulgaria), while cationic

standards were prepared from a mixed solution from SCP Science (*Standard for IC*, Baie-D'Urfé, Canada). Water was purified by a Milli-Q water purification system from Elga LabWater (*Medica*, Lane End, UK).

Glassware should be avoided for the preparation and storage of solutions and replaced by synthetic material in order to avoid any ionic salting out.

Preparation of standards and urine samples

Preparation of standards

Standard stock solutions of the analytes containing organic molecules at 0.5 $g.L^{-1}$ were prepared in Milli-Q water and stored in plastic bottles at 4 °C for no more than 1 week. Then, stock solutions were diluted to standard solutions at different concentrations.

Preparation of urine samples

Anonymous human urine samples were obtained from 10 healthy volunteers internally (7 male samples #1→#7 and 3 female samples #8→#10 in their 20s to 60s). The samples were stored at 4 °C until analysis. Urine samples were (i) filtered through 0.45-µm syringe filters, (ii) diluted 250-fold, and then (iii) injected to the analytical system.

Instrumentation and conditions

A scheme of the system configuration for IC-CD/MS urine analysis is provided in Online Resource SI 1.

Chromatography

Analyses were performed using an IC-CD system (Thermo Scientific Dionex™ ICS-5000$^+$) equipped with a DP analytical pump, an AS-AP auto-sampler, and a DC-5 module with double oven containing (i) Dionex™ CDRS 600 and Dionex™ ADRS 600 suppressors for cation and anion detections respectively as well as (ii) two conductivity detectors (CD). Ion suppression was ensured by applying a constant current of 31 and 24 mA to the anionic and cationic suppressors, respectively. The injection loop volumes were 25 µL and 10 µL, respectively, for cation and anion pathways (full loop). Thermo Scientific™ Chromeleon™ Chromatography Data System software 7.2.10 was used for IC control and data processing.

An IonPac CS16-4µm analytical column (2 mm × 250 mm) from Thermo Scientific™ was used for cation separation. The cationic eluent (methanesulfonic acid (MSA)) was generated at a flow rate of 0.16 $mL.min^{-1}$ by using a Thermo Scientific™ Dionex™ EGC 500 MSA Eluent Generator Cartridge. The column temperature was 40 °C.

Table 1 Concentration range (ordered by decreasing concentrations) used for each analyte to obtain the IC-CD/MS calibration curves and recoveries

Analyte	Concentration range for calibration curves ($mg.L^{-1}$)	Detection mode	Concentration range for determining recoveries ($mg.L^{-1}$)
Organic molecules		*MS*	
Urea	$0 \le [CH_4N_2O] \le 25$	+p	$5 \le [CH_4N_2O] \le 25$
Uric acid	$0 \le [C_5H_4N_4O_3] \le 20$	−p	$2.5 \le [C_5H_4N_4O_3] \le 20.0$
Creatine	$0 \le [C_4H_9N_3O_2] \le 10$	+p	$2.5 \le [C_4H_9N_3O_2] \le 10.0$
Creatinine	$0 \le [C_4H_7N_3O] \le 10$	+p	$2.5 \le [C_4H_7N_3O] \le 10.0$
Citric acid	$0 \le [C_6H_8O_7] \le 7.5$	−p	$2.5 \le [C_6H_8O_7] \le 7.5$
Lactic acid	$0 \le [C_3H_6O_3] \le 5$	−p	$1 \le [C_3H_6O_3] \le 5$
Hippuric acid	$0 \le [C_9H_9NO_3] \le 5$	−p	$1 \le [C_9H_9NO_3] \le 5$
Oxalic acid	$0 \le [C_2H_2O_4] \le 5$	−p	$1 \le [C_2H_2O_4] \le 5$
Ionic compounds		*CD*	
Chloride	$0 \le [Cl^-] \le 20$	⊖	$1 \le [Cl^-] \le 20$
Ammonium	$0 \le \left[NH_4^+\right] \le 15$	⊕	$2.5 \le \left[NH_4^+\right] \le 15.0$
Potassium	$0 \le [K^+] \le 10$	⊕	$1.25 \le [K^+] \le 10.00$
Sodium	$0 \le [Na^+] \le 7.5$	⊕	$1.25 \le [Na^+] \le 7.50$
Sulfate	$0 \le \left[SO_4^{2-}\right] \le 5$	⊖	$0.25 \le \left[SO_4^{2-}\right] \le 5.00$
Phosphate	$0 \le \left[PO_4^{3-}\right] \le 5$	⊖	$0.25 \le \left[PO_4^{3-}\right] \le 5.00$
Magnesium	$0 \le [Mg^{2+}] \le 2$	⊕	$0.25 \le [Mg^{2+}] \le 2.00$
Calcium	$0 \le [Ca^{2+}] \le 2$	⊕	$0.25 \le [Ca^{2+}] \le 2.00$

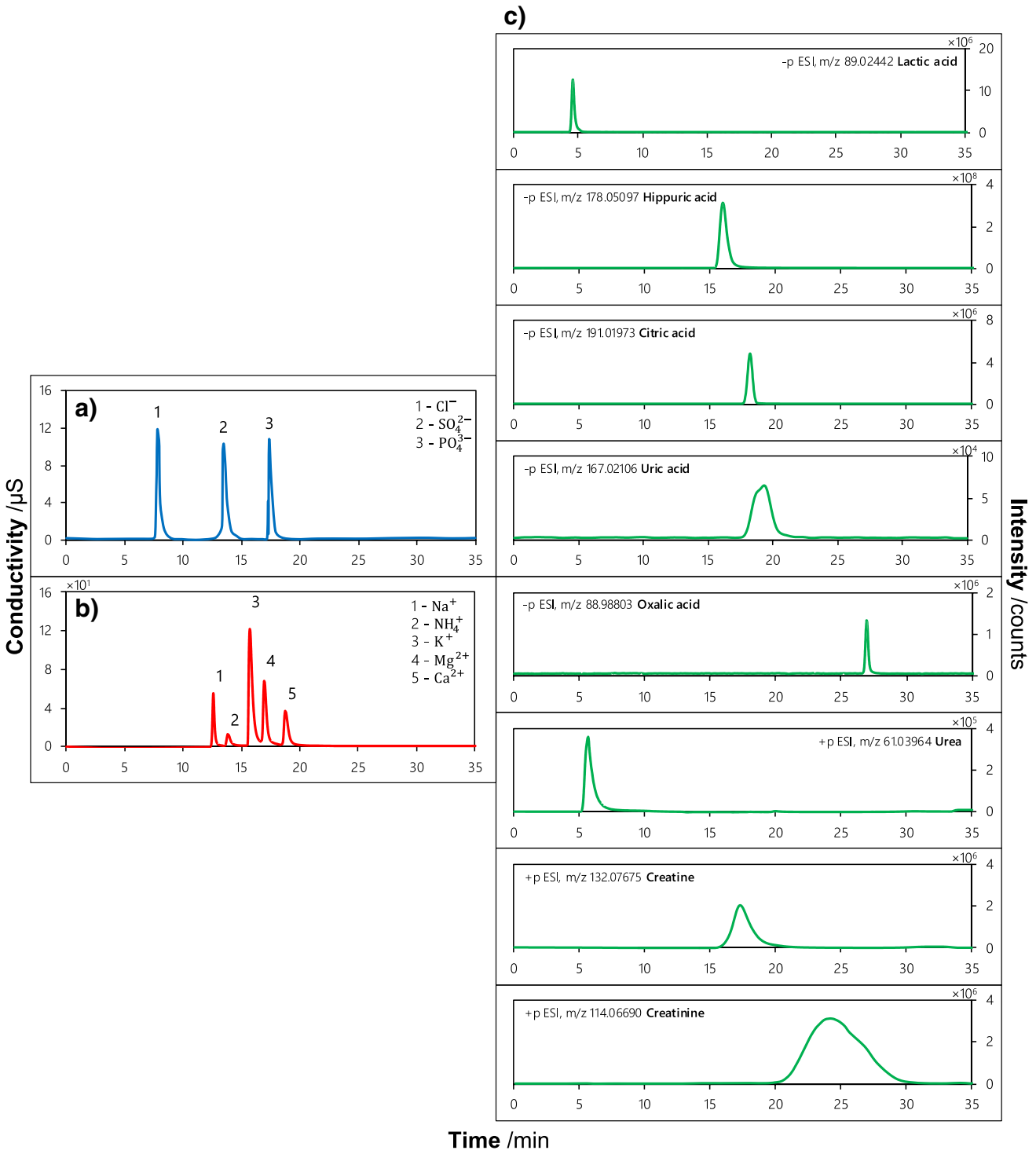

Fig. 1 **a** Anionic, **b** cationic, and **c** extracted ion chromatograms of a sample containing all the standards in water. Major compounds are identified and labeled

An IonPac AS11-HC-4µm analytical column (2 mm × 250 mm) from Thermo Scientific was used for anion separation. The anionic eluent (KOH) was generated at a flow rate of 0.25 mL.min^{-1} by using a Thermo Scientific™ Dionex™ EGC III 500 KOH Potassium Hydroxide Eluent Generator Cartridge. The column temperature was 25 °C. Both eluent concentration profiles are provided in Online Resource SI 2. The overall run time was 35 min.

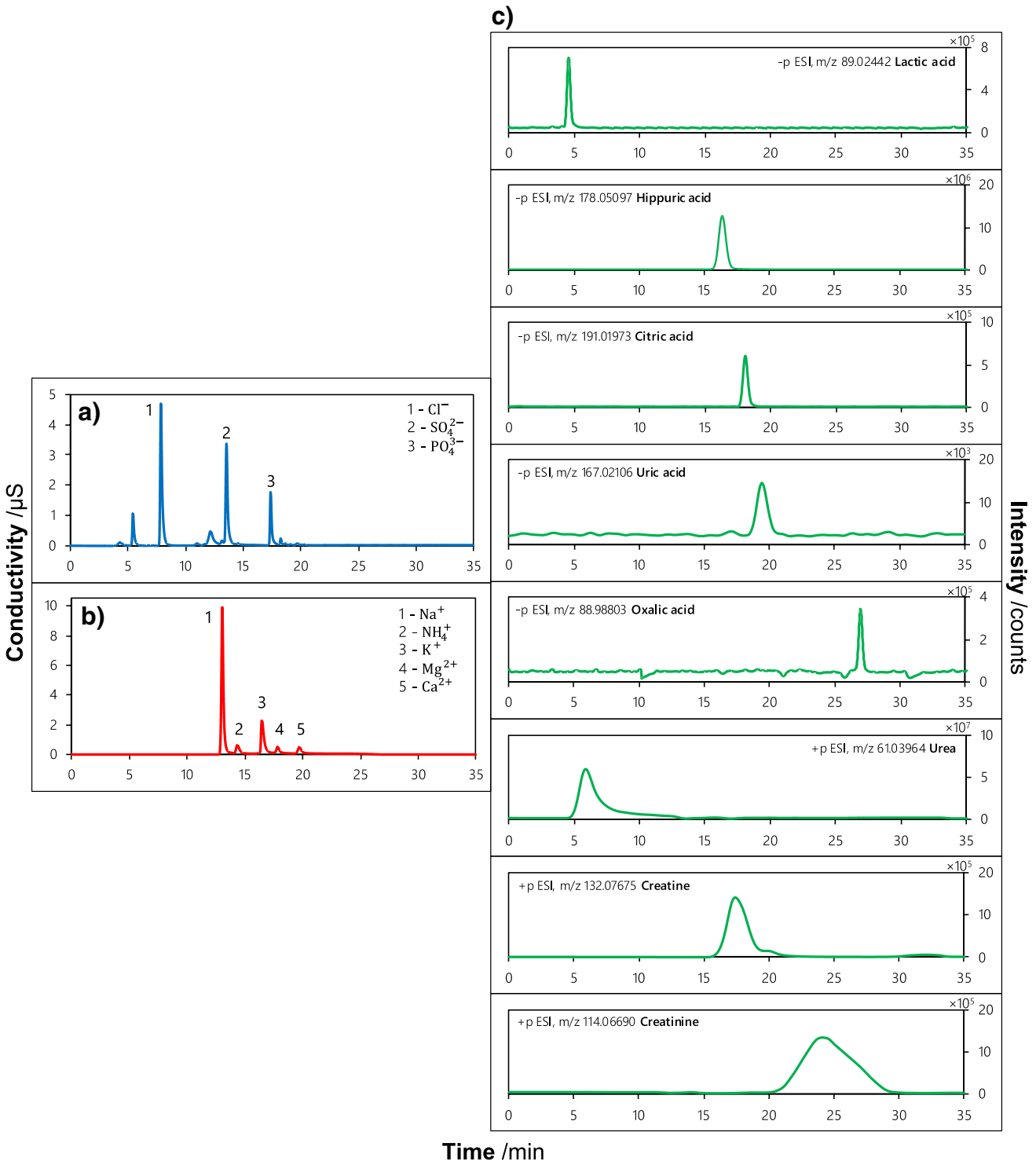

Fig. 2 **a** Anionic, **b** cationic, and **c** extracted ion chromatograms of a typical human urine sample. Major compounds are identified and labeled

Mass spectroscopy

The IC system was coupled to a mass spectroscopy analyzer Orbitrap (Thermo Scientific Q Exactive™ Focus). A Dionex™ AXP Auxiliary Pump (water, 0.25 mL.min^{-1}) was used to ensure a neutral pH at the mass spectrometry inlet by connecting it to the relevant suppressor. Thermo Scientific™ Q Exactive™ Plus software 2.11 was employed for MS control, and data processing was proceed by Thermo Scientific™ Chromeleon™ Chromatography

Data System software 7.2.10 and Thermo Scientific™ Xcalibur™ software.

MS was performed using the full-scan method in the negative mode (0–35 min, m/z 50–500) and positive mode (0–35 min, m/z 50–500) using a heated electrospray ionization (ESI) source. Concerning the negative mode, the optimal parameters were set as follows: sheath gas flow rate, nitrogen set to 40 *a.u.*; auxiliary gas flow rate, nitrogen set to 20 *a.u.*; spray voltage, 3.5 kV; capillary temperature, 300 °C; S-lens RF level, 60; and auxiliary gas heater temperature, 450 °C. Concerning the positive mode, the optimal parameters were set as follows: sheath gas flow rate, nitrogen set to 30 *a.u.*; auxiliary gas flow rate, nitrogen set to 10 *a.u.*; spray voltage, 4.5 kV; capillary temperature, 300 °C; S-lens RF level, 60; and auxiliary gas heater temperature, 320 °C.

Validation of the method

An example of the obtained chromatograms for various analyte standard concentrations is provided in Online Resource SI 3. The method was validated for selectivity, sensitivity, linearity, accuracy, and precision according to the ICH guidelines [37].

Selectivity

The method's selectivity to ensure a proper separation of the different ions was examined on standard solutions and blank solvent (Milli-Q water) via conductivity measurements.

For the organic molecules, the method's selectivity was examined by analyzing several standard solutions. The signal of the mass spectrum of each pure compound was then compared to the corresponding one obtained in the urine spectrum as illustrated in Online Resource SI 4.

Sensitivity

The method's sensitivity was determined under the operating conditions used through the LODs and LOQs determination at a signal-to-noise ratio of 3 and 10, respectively.

Table 2 Retention time, calibration curve ranges, regression equation, correlation coefficients, LODs, and LOQs obtained, for each analyte (ordered by increasing retention time)

Analyte	ESI polarity	T_r (min)	Analyte concentration range (ppm)	Regression equation ([*j*] in mg.L^{-1})	Correlation coefficient R^2	LOD (mg.L^{-1})	LOQ (mg.L^{-1})
Organic compounds (MS)				(*Peak area* in counts.min)			
Lactic acid	−p	4.6	0–5	Peak area = $3.84\times10^6\times[C_3H_6O_3]+9.40\times10^4$	0.9988	0.19	0.64
Hippuric acid	−p	16.0	0–5	Peak area = $5.08\times10^6\times[C_9H_9NO_3]+6.01\times10^5$	0.9958	0.37	1.22
Citric acid	−p	18.1	0–7.5	Peak area = $5.43\times10^4\times[C_6H_8O_7]+4.39\times10^3$	0.9990	0.30	1.01
Uric acid	−p	19.5	0–20	Peak area = $3.21\times10^3\times[C_5H_4N_4O_3]+8.41\times10^2$	0.9988	0.72	2.41
Oxalic acid	−p	27.0	0–5	Peak area = $3.98\times10^5\times[C_2H_2O_4]+3.37\times10^4$	0.9982	0.24	0.80
Urea	+p	5.9	0–25	Peak area = $1.22\times10^6\times[CH_4N_2O]+1.51\times10^5$	0.9999	0.32	1.07
Creatine	+p	17.3	0–10	Peak area = $3.86\times10^5\times[C_4H_9N_3O_2]+2.55\times10^4$	0.9979	0.59	1.96
Creatinine	+p	23.8	0–10	Peak area = $1.25\times10^6\times[C_4H_7N_3O]+2.59\times10^5$	0.9972	0.63	2.11
Ionic compounds (CD)				(*Peak area* in µS.min)			
Chloride	[-]	7.5	0–20	Peak area = $0.50\times[Cl^-]+0.06$	0.9999	0.12	0.41
Sulfate		13.1	0–5	Peak area = $0.40\times\left[SO_4^{2-}\right]+0.10$	0.9994	0.14	0.47
Phosphate		17.0	0–5	Peak area = $0.17\times\left[PO_3^{2-}\right]+0.01$	0.9982	0.25	0.84
Sodium		13.0	0–7.5	Peak area = $8.58\times[Na^+]+0.69$	0.9991	0.23	0.77
Ammonium		14.3	0–15	Peak area = $0.72\times\left[NH_4^+\right]+0.13$	0.9987	0.75	2.59
Potassium		16.5	0–10	Peak area = $0.64\times[K^+]+0.06$	0.9963	0.71	2.37
Magnesium		17.8	0–2	Peak area = $2.12\times[Mg^{2+}]+0.08$	0.9936	0.16	0.54
Calcium		19.7	0–2	Peak area = $1.09\times[Ca^{2+}]+1.05$	0.9950	0.14	0.48

Table 3 Intra-day and inter-day precision and accuracy

Analyte	Concentration ($mg.L^{-1}$)	Intra-day ($n = 5$)		Inter-day ($n = 11$)	
		Accuracy RE (%)	Precision RSD (%)	Accuracy RE (%)	Precision RSD (%)
Organic molecules					
Lactic acid	1.00	1.2	1.5	4.3	0.6
	2.00	2.9	0.8	1.8	3.5
	3.00	3.5	3.1	0.2	2.2
	4.00	0.5	1.9	3.4	2.8
	5.00	3.3	2.6	2.5	3.4
Hippuric acid	1.00	3.6	3.2	0.9	1.8
	2.00	0.4	2.1	3.7	2.4
	3.00	1.1	1.4	4.2	0.3
	4.00	2.2	0.9	1.3	3.8
	5.00	1.7	1.2	2.0	2.0
Citric acid	2.50	1.1	1.4	2.6	1.5
	5.00	3.9	0.9	4.2	3.9
	7.50	0.9	2.2	1.1	0.2
Uric acid	2.50	2.5	0.5	3.7	2.8
	5.00	4.1	1.8	5.0	1.9
	7.50	3.9	1.2	2.4	3.1
	10.00	2.8	2.5	0.5	0.7
	15.00	0.6	0.3	3.8	3.7
	20.00	3.2	2.1	1.3	2.4
Oxalic acid	1.00	2.7	2.9	3.1	3.7
	2.00	4.1	1.7	2.6	0.4
	3.00	2.3	3.3	1.9	1.1
	4.00	1.5	3.6	4.4	1.6
	5.00	3.8	0.7	0.1	1.3
Urea	5.00	1.8	0.1	0.9	1.1
	10.00	4.5	1.7	4.1	3.4
	15.00	1.2	2.4	0.2	0.4
	20.00	3.6	1.0	3.3	3.0
	25.00	0.5	0.7	2.0	1.7
Creatine	2.50	1.7	1.6	4.6	2.5
	5.00	2.4	1.3	1.8	1.2
	7.50	3.8	0.4	3.9	4.0
	10.00	0.3	1.9	1.5	0.9
Creatinine	2.50	2.3	0.1	4.9	2.1
	5.00	3.7	2.3	0.0	3.6
	7.50	0.7	1.8	2.3	1.4
	10.00	1.5	2.0	3.1	2.9
Ionic compounds					
Chloride	1.00	1.1	0.6	1.1	0.8
	5.00	0.8	1.0	0.4	0.3
	10.00	0.3	0.4	0.9	1.0
	15.00	1.3	0.9	1.3	0.4
	20.00	0.1	0.2	0.5	0.7
Sulfate	0.25	0.6	0.8	1.0	0.2
	1.25	1.2	0.5	0.2	0.1
	2.50	0.9	0.3	1.2	0.9
	3.75	0.8	1.0	0.7	0.5
	5.00	0.8	0.0	0.1	0.7

Table 3 (continued)

Analyte	Concentration ($mg.L^{-1}$)	Intra-day ($n = 5$)		Inter-day ($n = 11$)	
		Accuracy RE (%)	Precision RSD (%)	Accuracy RE (%)	Precision RSD (%)
Phosphate	0.25	0.2	0.7	1.4	0.1
	1.25	1.7	0.3	1.0	0.9
	2.50	0.6	0.8	0.8	0.6
	3.75	0.3	0.5	0.0	0.2
	5.00	0.4	0.1	0.4	0.8
Sodium	1.25	1.9	1.0	1.1	0.4
	2.50	0.4	0.6	0.6	0.2
	5.00	0.1	0.9	1.3	1.0
	7.50	0.1	0.0	0.9	0.5
Ammonium	2.50	1.4	0.7	0.5	0.5
	5.00	0.8	0.2	1.1	0.6
	10.00	0.4	0.9	1.4	0.7
	15.00	0.6	0.4	0.2	1.0
Potassium	1.25	0.7	0.6	0.6	0.3
	2.50	1.9	0.3	1.2	0.3
	5.00	0.1	0.8	0.9	0.1
	7.50	0.3	0.5	0.1	0.9
	10.00	0.3	0.2	0.4	0.1
Magnesium	0.25	1.4	0.9	0.7	0.4
	0.50	0.7	0.2	1.0	0.7
	1.00	0.1	0.7	1.4	0.5
	1.50	0.7	0.5	0.2	0.1
	2.00	0.1	0.1	1.4	1.7
Calcium	0.25	0.2	1.0	0.6	0.9
	0.50	0.1	0.3	0.3	0.8
	1.00	1.6	0.6	1.2	0.5
	1.50	0.5	0.1	0.1	0.2
	2.00	0.8	0.8	1.3	0.1

Linearity

The linearity of the CD or MS signals of each compound against its concentration was examined by plotting the corresponding calibration curves. A set of solutions containing different concentrations of analyte was prepared, covering a wide range of values as reported in Table 1.

Accuracy

The method's accuracy was assessed by injecting standard solutions at different concentrations. By using calibration curves, the relative error (RE) between the theoretical and measured concentrations was then determined according to the Eq. (1).

$$\mathrm{RE} = \left| \frac{\text{Theoretical concentration value} - \text{Experimental concentration value}}{\text{Theoretical concentration value}} \right| \times 100 \quad (1)$$

The accuracy was also examined by means of the recovery of some known quantities of organic molecules and ionic compounds added to human urine samples. The latter parameter was already been studied by El Himri et al. [38] and was defined as Eq. (2):

$$\text{Recovery} = \frac{\text{Measured amount}}{\text{Spiked amount}} \times 100 \quad (2)$$

$$\text{Measured amount} = \text{Amount after spiking} - \text{Amount before spiking} \quad (3)$$

The dispersion of the measurements is assessed by evaluating the standard deviation (SD) as shown in Eq. (4):

$$\text{SD} = \sqrt{\frac{\sum \left(x_i - \mu\right)^2}{N}} \quad (4)$$

where x_i represents each value from the population, μ is the population mean, and N is the size of the population.

The recovery was determined by triplicate analysis of urine samples spiked with standards as shown in Table 1.

Precision

The intra-day precision was evaluated through the repeatability defined with relative standard deviation (RSD), as Eq. (5), and determined from 5 replicates at a minimum of 3 different concentrations on day 1. The inter-day precision was evaluated by injecting the same solution in triplicate on days 2 and 3:

$$\text{RSD} = \left|\frac{\text{SD}}{\mu}\right| \times 100 \quad (5)$$

Matrix effect

Calibration standards with equivalent concentration levels were prepared by introducing spiking into blank matrix extracts of the urine sample. Calibration curves were obtained by plotting the peak areas as a function of the concentrations of their respective calibration standards for each compound. The matrix effect (ME) was ascertained by comparing the slopes of the calibration curves formed from the matrix case and the ones formed from solvent-based calibration curves. The method used to quantify ME was derived from the equation proposed by Cho et al. [39] as described in Eq. (6). A value exceeding 100% (below 100%) means an increase (a decrease) in response signal. This analysis was performed on ten distinct samples:

$$\text{ME} = \frac{\text{slope of matrix calibration curve}}{\text{slope of solvent standard calibration curve}} \times 100 \quad (6)$$

Results

Implementation of the IC-CD/MS method

The operating parameters of the mass spectrometer were optimized (i.e., ESI voltage, gas temperature), corresponding to IC conditions (i.e., flow rate, eluent composition, and concentration) previously described. Under these conditions, all the analytes in this matrix could be efficiently separated and detected within an overall run duration of 35 min. The chromatograms of the mixed standards and urine samples obtained are shown in Figs. 1 and 2, respectively. The $[M-H]^-$ ions of lactic, hippuric, citric, uric, and oxalic acids were detected in the negative ion mode as lactate, hippurate, citrate, urate, and oxalate (their identification is also possible in CD but was not applied in this study). The $[M-H]^+$ ions of urea, creatine, and creatinine were detected in the positive ion mode.

Validation of the IC-CD/MS method

Linearity, LODs, and LOQs

Table 2 shows, for each analyte, the calibration curve ranges, regression equations, correlation coefficients, LODs, and LOQs. Whatever the analyte, a linear relationship is obtained between the peak area and the concentration of standards in water, with correlation coefficients above 0.993. In Online Resource SI 5, the calibration curves shows, for each standard, a good validation of the method by applying the Kohlrausch's law and the Kingdon's principle, respectively, for CD and MS detections [40, 41].

Precision

As shown in Table 3, the intra-day precisions for all concentrations of each standard were smaller than 2.5% and the RSDs for the inter-day analysis smaller than 4.0% for each analyte, thus demonstrating that the method can be used to obtain accurate results.

Accuracy

Table 4 shows the recovery of different analytes for various concentrations in spiked human urine samples, ranging from 94.9 to 102.6%. They indicate a good agreement between theoretical and experimental ones.

Matrix effect

As explained in the "Matrix effect" section, MEs were evaluated using ten human urine samples spiked with various analytes, with the objective of evidencing any

Table 4 Recovery of the analytes introduced into a human urine sample ($n = 3$)

Analyte	Spiked concentration ($mg.L^{-1}$)	Recovery rates	
		Mean value (%)	SD (%)
Organic molecules			
Lactic acid	1.00	95.8	3.2
	2.00	98.3	4.1
	3.00	100.5	5.0
	4.00	96.1	3.5
	5.00	101.4	4.8
Hippuric acid	1.00	101.7	5.1
	2.00	95.4	3.9
	3.00	99.9	2.9
	4.00	101.1	4.9
	5.00	97.3	3.7
Citric acid	2.50	95.3	3.4
	5.00	100.7	5.6
	7.50	101.5	4.8
Uric acid	2.50	99.2	4.0
	5.00	102.3	5.2
	7.50	97.8	3.6
	10.00	98.6	4.5
	15.00	100.1	5.7
	20.00	94.9	3.1
Oxalic acid	1.00	99.2	4.7
	2.00	102.1	5.6
	3.00	97.6	4.5
	4.00	100.8	5.3
	5.00	102.5	5.2
Urea	5.00	96.4	2.9
	10.00	102.6	5.1
	15.00	101.8	4.7
	20.00	97.1	3.8
	25.00	100.9	5.8
Creatine	2.50	95.6	3.3
	5.00	98.0	4.3
	7.50	99.7	5.0
	10.00	96.9	3.7
Creatinine	2.50	101.1	4.9
	5.00	95.9	3.0
	7.50	100.4	5.5
	10.00	97.5	3.2
Ionic compounds			
Chloride	1.00	99.3	0.4
	5.00	100.8	1.2
	10.00	101.5	1.4
	15.00	100.1	0.7
	20.00	99.7	0.9
Sulfate	0.25	101.2	1.1
	1.25	101.8	1.6
	2.50	99.9	0.3
	3.75	100.4	1.3
	5.00	100.6	0.6

Table 4 (continued)

Analyte	Spiked concentration ($mg.L^{-1}$)	Recovery rates	
		Mean value (%)	SD (%)
Phosphate	0.25	101.1	1.5
	1.25	99.6	0.2
	2.50	101.4	0.8
	3.75	100.0	1.1
	5.00	99.1	0.5
Sodium	1.25	101.7	1.0
	2.50	100.9	1.3
	5.00	100.2	0.1
	7.50	99.5	1.6
Ammonium	2.50	101.3	0.8
	5.00	99.8	1.2
	10.00	100.5	0.3
	15.00	99.4	1.5
Potassium	1.25	100.7	0.9
	2.50	101.0	1.4
	5.00	101.6	0.7
	7.50	99.2	1.0
	10.00	100.3	0.5
Magnesium	0.25	101.9	1.6
	0.50	99.0	0.4
	1.00	101.7	1.1
	1.50	100.4	0.2
	2.00	100.8	1.4
Calcium	0.25	100.1	0.7
	0.50	101.5	1.3
	1.00	99.3	0.6
	1.50	99.8	1.0
	2.00	100.9	0.2

suppression or enhancement of their signals. A typical example for a human urine sample spiked with commercial urea is presented in Fig. 3a. The y-shift shows the presence of urea initially contained in the human urine sample. As illustrated in Fig. 3b, the analyte concentration in the urine matrix can be obtained by applying the standard addition method. The same study was performed for all analytes, and the results are reported in Table 5. As described in Eq. (6), the comparison of the peak areas indicates any significant ME.

Analysis of human urine samples

Ten human urine samples obtained from healthy volunteers have been analyzed using the proposed method, and the quantitative results are reported in Table 6. The sixteen analytes, present in all samples, are quantified at a concentration within the linear ranges of their calibration curves.

Discussions

To the best of the authors' knowledge, the analytical technique described above (i.e., IC-CD/MS) is applied for the first time for simultaneously determination major compounds in urine. This allows a double analysis and direct dilute-and-shoot approach in 35 min without sample preparation.

The validation results demonstrate the reliability of this method for the simultaneous quantification of lactic acid, hippuric acid, citric acid, uric acid, oxalic acid, urea, creatine, creatinine, and ions in human urine samples (with a precision within 4.0%, accuracy ranging from 0.1 to 5%, and recovery rates varying from 94.9 to 102.6%).

Both external calibration and standard addition approaches enable the quantification of the analyte. Following this procedure, the proposed method provides satisfactory quantitative results (compared to the literature's values presented in Online Resource SI 6), without the need for expensive isotope-labeled compounds [42], thereby supporting its

applicability to daily testing of human urine samples. The difference in values can be attributed to different lifestyle practices (such as eating habits, sleep, sex, sporting activities).

According to various studies [28, 43, 44], the identification and characterization of a human urine solution can be performed by evaluating some concentration ratio according to the two following coefficients K_1 and K_2:

$$K_1 = 20 \times \left\{ [\text{Uric acid}] / \left[\text{Urea}_{\text{eq nitrogen}} \right] \right\} \quad (7)$$

$$K_2 = [\text{Uric acid}] / [\text{Creatinine}] \quad (8)$$

where concentrations are given in $g.L^{-1}$.

These two factors have been evaluated and reported in the Online Resource SI 7. Sakurai et al. [28] reported values of K_1 ranging from to 0.69 to 2.25, which are consistent with those obtained in this study, ranging from 1.63 to 3.45. As for K_2, Kwon et al. [27] and Sakurai et al. [28] reported values ranging from 0.28 to 0.66, similar to those obtained in this study (from 0.21 to 0.68). However, the pioneering technique of ion chromatography coupled with mass spectroscopy allows efficient characterization of the more significant analytes contained in human urine.

Conclusions

This work investigated a new IC-CD/MS method for the simultaneous quantification of the 8 main organic molecules (biomarkers) and 8 ions naturally present in human urine, in two runs and within 35 min. Chromatographic separation was performed using ion exchange columns coupled to a mass spectroscopy detector. Quantitative concentration values were obtained using the external standard method without requiring the use of expensive isotope-labeled compounds. The results were validated according to ICH guidelines (through selectivity, sensitivity, linearity, accuracy, and precision) and showed that the proposed method

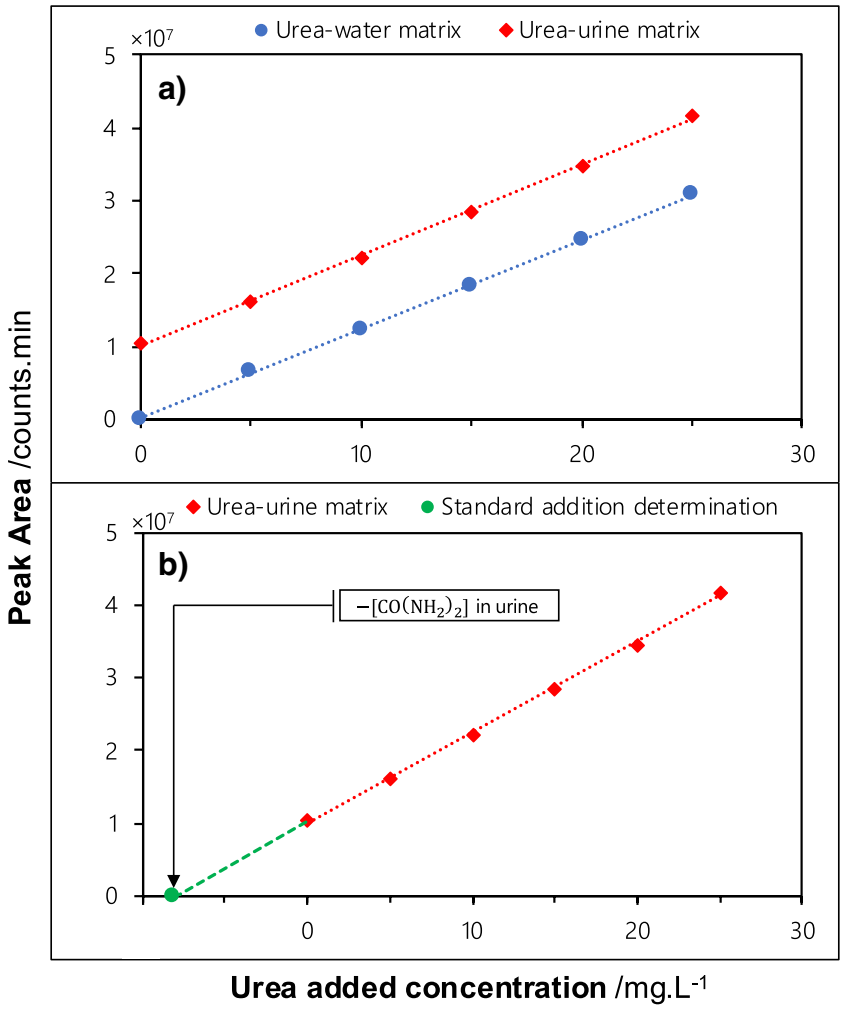

Fig. 3 **a** Variation of the urea MS signal magnitude with the urea added concentration, into water or into human urine and **b** illustrative example of the application of the standard addition method for the determination of the urea concentration in a human urine sample

Table 5 MEs of the analytes introduced into a human urine sample ($n = 3$)

Sample	*Organic molecules*															
	Lactic acid		Hippuric acid		Citric acid		Uric acid		Oxalic acid		Urea		Creatine		Creatinine	
	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)
#1	97.4	1.7	108.1	8.3	98.7	2.7	97.2	1.5	100.8	4.9	96.7	3.6	94.8	4.8	103.9	7.9
#2	102.6	9.6	104.7	3.1	101.5	7.6	101.8	6.2	101.9	7.5	108.2	10.1	106.3	9.3	99.5	6.5
#3	99.1	5.4	98.7	6.8	97.9	1.9	99.5	4.7	107.8	0.4	91.5	1.9	103.2	8.2	101.6	8.6
#4	104.2	4.1	103.5	2.9	99.6	5.3	96.7	0.6	97.6	8.7	104.3	7.3	97.7	2.7	104.8	9.8
#5	101.3	7.2	106.9	10.5	100.2	8.1	100.3	5.3	103.8	5.9	99.8	5.8	95.6	3.6	96.2	3.2
#6	105.7	3.5	99.5	0.8	102.1	4.2	102.1	2.4	98.3	9.2	100.1	8.4	110.9	7.9	98.3	4.3
#7	106.2	2.6	102.1	6.1	97.4	0.6	98.4	3.9	104.9	8.9	92.5	2.5	109.1	10.1	110.0	11.0
#8	100.1	9.9	107.3	4.5	100.9	6.9	101.0	6.7	102.4	1.3	109.3	9.3	99.2	5.2	92.7	2.7
#9	98.6	10.3	101.7	3.8	101.7	3.5	96.9	4.0	108.3	7.1	97.4	4.4	105.4	6.4	105.1	5.1
#10	103.1	0.6	100.3	5.2	100.4	7.3	100.7	6.5	97.9	2.1	102.6	6.2	90.8	0.8	110.4	6.4
Sample	*Ionic compounds*															
	Chloride		Sulfate		Phosphate		Sodium		Ammonium		Potassium		Magnesium		Calcium	
	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)	Mean value (%)	SD (%)
#1	95.1	3.1	98.8	6.8	93.4	2.4	98.1	6.1	97.3	4.3	98.6	5.6	97.8	8.3	101.3	3.5
#2	108.6	10.6	96.5	3.5	102.3	7.3	104.0	9.0	100.7	7.7	102.5	7.5	96.1	2.7	93.7	8.2
#3	94.7	4.7	104.1	9.1	100.8	6.8	111.5	8.5	102.8	9.8	100.2	6.2	105.3	5.0	99.0	6.9
#4	106.9	9.9	101.7	8.7	97.2	4.2	93.9	2.9	94.4	3.4	93.1	1.1	100.5	10.1	108.1	2.3
#5	99.3	6.3	95.9	2.9	110.0	11.0	107.6	10.6	103.1	8.1	106.1	9.1	109.2	9.2	104.2	7.1
#6	101.2	8.2	107.4	10.4	96.9	3.9	95.2	3.2	108.9	10.9	95.8	3.8	98.0	3.3	96.3	10.8
#7	109.8	10.8	93.6	1.6	105.7	9.7	109.7	11.7	95.5	2.5	108.4	10.4	94.9	4.0	95.0	4.6
#8	90.6	0.6	102.9	7.9	104.6	8.6	92.1	1.1	99.9	5.9	114.7	8.7	92.8	11.0	110.0	0.9
#9	97.9	2.9	109.5	11.5	99.1	5.1	106.8	9.8	91.1	0.1	99.6	5.6	101.9	1.6	92.6	9.4
#10	104.5	7.5	100.0	5.0	91.8	1.8	90.3	0.3	105.0	6.0	91.4	0.4	97.8	6.1	97.1	5.7

Table 6 Analytical results of organic compounds and ions contained in humane urine samples ($n = 3$)

Sample	*Organic molecules*															
	Lactic acid		Hippuric acid		Citric acid		Uric acid		Oxalic acid		Urea		Creatine		Creatinine	
	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)
#1	1.62×10^{-2}	0.17	0.78	0.18	0.92	0.60	0.92	0.60	3.16×10^{-2}	2.81	20.37	0.22	0.16	2.80	3.01	0.47
#2	7.17×10^{-3}	0.64	0.42	0.19	0.81	3.38	0.81	3.38	4.12×10^{-2}	0.75	21.34	0.62	1.59	0.35	3.85	0.77
#3	9.67×10^{-3}	2.38	0.09	0.26	0.52	0.21	0.52	0.21	<LOD	-	7.79	2.78	0.14	1.94	1.00	3.38
#4	4.25×10^{-3}	2.16	0.19	0.17	0.33	0.83	0.33	0.83	<LOD	-	6.20	2.33	<LOD	-	0.72	1.38
#5	1.50×10^{-2}	0.21	0.61	0.57	0.78	1.42	0.78	1.42	5.11×10^{-2}	1.74	9.65	1.50	0.05	3.63	2.24	1.07
#6	<LOD	-	0.16	2.24	0.59	0.93	0.59	0.93	3.73×10^{-3}	2.38	11.55	0.13	17.54	0.52	0.91	1.75
#7	3.46×10^{-3}	0.53	0.14	2.23	0.38	1.46	0.38	1.46	3.75×10^{-4}	1.18	5.84	0.99	<LOD	-	0.86	0.76
#8	5.53×10^{-2}	0.02	0.46	0.21	0.77	1.42	0.77	1.42	1.37×10^{-2}	0.06	15.56	0.09	0.65	0.71	1.58	1.81
#9	3.37×10^{-2}	0.38	0.61	0.46	0.72	0.38	0.72	0.38	2.17×10^{-2}	0.10	14.48	1.00	1.73	0.64	2.21	0.26
#10	1.72×10^{-2}	0.54	0.19	0.18	0.93	0.59	0.93	0.59	4.43×10^{-2}	0.20	23.14	1.93	6.88	0.67	1.37	0.11
Sample	*Ionic compounds*															
	Chloride		Sulfate		Phosphate		Sodium		Ammonium		Potassium		Magnesium		Calcium	
	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)	Mean value ($g.L^{-1}$)	RSD (%)
#1	3.37	0.42	1.31	1.01	2.70	0.76	0.32	0.51	0.73	3.86	4.13	1.07	0.08	0.10	0.19	0.61
#2	4.97	0.07	1.42	0.62	4.87	0.11	0.61	0.73	0.51	3.83	4.15	0.73	0.14	1.85	0.08	1.92
#3	3.16	0.11	0.38	2.31	0.44	2.35	0.24	0.14	0.10	2.34	2.98	0.65	0.03	0.25	0.05	1.76
#4	1.51	0.23	0.38	1.17	0.78	1.31	0.09	0.18	0.15	1.51	2.38	1.28	0.04	2.38	<LOD	-
#5	5.85	0.06	0.75	1.18	1.29	0.79	0.34	0.75	0.83	0.94	4.23	0.78	0.05	3.61	<LOD	-
#6	2.67	0.01	1.07	1.64	0.48	2.14	0.26	0.34	0.16	1.42	2.58	0.64	0.05	1.55	0.10	0.17
#7	2.60	0.14	0.25	1.74	0.25	1.22	0.16	0.42	0.20	0.00	2.80	0.49	0.03	2.86	0.05	1.60
#8	5.49	0.13	1.06	0.41	1.15	0.89	0.67	0.83	0.38	0.63	1.68	0.33	0.16	3.67	0.26	1.24
#9	2.53	0.42	1.16	0.38	2.23	0.46	0.28	0.32	0.64	0.39	2.60	1.07	0.10	1.72	0.09	3.42
#10	3.39	0.10	1.68	0.26	3.84	0.27	0.49	0.67	0.63	1.59	2.58	0.97	0.13	0.07	0.12	1.34

provided quantitative values with precision and accuracy higher than 95%. Furthermore, no matrix effects were observed. In the future, the proposed method could be thus used as a basic technique in any analytical field.

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Author contribution Guillaume Hopsort: conceptualization; methodology; validation; formal analysis; investigation; data curation; writing - original draft; and visualization. Laure Latapie: conceptualization; methodology; validation; formal analysis; investigation; resources; writing - review and editing; and funding acquisition. Karine Groenen Serrano: writing - review and editing, and supervision. Karine Loubière: writing - review and editing, and supervision. Théodore Tzedakis: writing - review and editing; supervision; project administration; and funding acquisition.

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Declarations

Ethical approval This is an observational study; no ethical approval was needed for the study. Before gathering any information, we ensured to obtain written approvals from each participant with notice of information and consent form. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments. This study was conducted with healthy individuals aged 18 and above, who had no previous record of kidney-related ailments. The study excluded those who were currently suffering from a urinary tract infection. Furthermore, female participants were instructed to undertake the test on non-menstrual days.

Conflict of interest The authors declare no competing interests.

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