RESEARCH ARTICLE

Urinary *N*-methylpyridinium and trigonelline as candidate dietary biomarkers of coffee consumption

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Scope: In order to validate the in vivo function of putatively healthy molecules in foods, human intervention studies are required. As the subject's compliance concerning intake or abstinence of a given food is considered mandatory to be monitored by biomarkers, the objective was to identify analytical markers for coffee consumption.

Methods and results: Urine samples collected from coffee drinkers were compared with those of non-coffee drinkers using hydrophilic liquid interaction chromatography (HILIC)/time-of-flight mass spectrometry-based metabolite profiling. Two urinary molecules, found to be contributing most to the dissimilarities between both groups, were identified as *N*-methylpyridinium (NMP) and trigonelline and their suitability as coffee-specific biomarkers was validated by means of a coffee intervention study. After the volunteers (five females and four males) consumed a single dose of coffee, morning urine was collected for 10 days while staying abstinent from any coffee. HILIC-MS/MS-stable isotope dilution analysis (SIDA) revealed elevated urinary concentrations of trigonelline and NMP for up to 48 ($p = 0.001$) and 72 h ($p = 0.002$), respectively, after coffee consumption when compared with non-coffee drinkers.

Conclusion: Analysis of urinary NMP allows to check for coffee consumption within a period of 3 days and is proposed as a dietary biomarker which might be used as an analytical probe to control compliance in human intervention studies on coffee.

Keywords:
Coffee / Compliance / Dietary biomarker / *N*-Methylpyridinium / Trigonelline

1 Introduction

Roasted coffee is a life style good of broad acceptance and appreciated by millions of people around the world for its alluring aroma, typical taste profile, as well as its stimulatory effect on the central nervous system. With increasing intensity over the last years, coffee moved into the focus of nutritional research since numerous epidemiological studies suggest that coffee consumption may help prevent several chronic diseases, including Alzheimer's disease [1], Parkinson's disease [2], type 2 diabetes mellitus [3, 4], and liver cirrhosis [5], respectively.

Although the responsible key coffee compounds are still unclear, various bioavailable and biologically active molecules have been identified in coffee beverages, such as caffeine, a competitive antagonist of adenosine able to excite the adenosine-sensitive sympathetic nervous system resulting in enhanced physical and cognitive performance when ingested (reviewed recently, [6]), chlorogenic acids showing antioxidative properties in vivo [7, 8], and the semi-vitamin nicotinic acid [9, 10]. Moreover, some evidence have been found for *N*-methylnicotinic acid, known as the second most abundant alkaloid trigonelline in coffee, to delay the development of diabetes type II [11, 12] in both animal and human studies, and for *N*-methylpyridinium (NMP), generated from trigonelline upon coffee roasting [13], to induce phase I/II detoxifying enzymes and exhibit chemopreventive properties [14]. Besides such health-beneficial

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Abbreviations: HILIC-MS/MS, hydrophilic liquid interaction chromatography/triple quadrupole mass spectrometry; **HILIC-UPLC/ToF-MS,** hydrophilic liquid interaction chromatography/time-of-flight mass spectrometry; **NMP,** *N*-methylpyridinium; **SIDA,** stable isotope dilution analysis

properties, roasted coffee contains molecules with putative detrimental effects such as the carboxylic acid 5-hydroxytryptamides, which are discussed as stomach irritants [15], and the furan diterpenes cafestol and kahweol, which are considered the most potent cholesterol raising agents in human daily diet, although the physiological mechanisms involved in vivo are still unclear [16].

Due to the presence of such putatively health beneficial and detrimental molecules, respectively, an increasing number of controlled human intervention studies are currently conducted in order to confirm one or the other physiological activity in vivo. In 2006, EU decision makers adopted a regulation laying down harmonized EU-wide rules for the use of health or nutritional claims on foodstuffs based on nutrient profiles. One of the key objectives of this regulation is to ensure that any claim made on a food label in the EU is clearly substantiated by solid scientific evidence which is to be verified by the European Food Safety Authority (EFSA). Recently, criteria for the scientific substantiation of such claims have been published, including detailed information on requirements of the design of clinical and intervention studies [17]. In particular, the subject's compliance concerning intake or abstinence of the food or food compound under investigation was considered necessary to be monitored by valid biomarkers [17]. In contrast to animal studies, where the diet is well defined, human studies are mainly dependent on dietary questionnaires asked of the subjects. Although improved education of the study subjects and physician involvement are known to improve compliance to some extend, this process can be fraught with error as it requires recall in great detail of the types and amounts of specific foods eaten. Although some biomarkers for the validation of dietary assessment methods have been developed, the monitoring of subject's compliance is still limited by the lack of suitable analytical biomarkers to reflect wider aspects of diet. For example, a group of alkylresorcinols, which are phenolic lipids almost exclusively present in the outer parts of wheat and rye grains in commonly consumed foods, have been recently proposed as specific dietary biomarkers of whole-grain wheat and rye intakes [18].

It is well agreed that there is the need to identify dietary biomarkers also for other foods as a prerequisite to assess metabolic profiles as measures of dietary exposure and indicators of dietary patterns, dietary changes, or effectiveness of dietary interventions. In particular, in nutritional human studies on the physiological effects of coffee ingredients the relative low accuracy of compliance is a major obstacle as it is usually rather challenging for coffee drinkers to stay abstinent in the wash-out phase of an intervention study.

Recently, urinary concentrations of *N*-feruloylglycine and dihydrocaffeic acid 3-*O*-sulfate have been proposed as possible biomarkers for coffee consumption [7]. However, the suitability of these molecules seems limited since they are metabolites of phenylpropenoic acid derivatives such as chlorogenic acids, which are not at all exclusively present in coffee but occur in a wide range of plant-derived food products [19]. Due to its abundance in soft drinks, tea, and cocoa, also the alkaloid caffeine seems not suitable as a coffee specific marker. The objective of the present study was, therefore, to locate and identify more specific urinary biomarkers for coffee consumption aimed at controlling compliance in human intervention studies.

2 Materials and methods

2.1 Chemicals and reagents

Trigonelline (**1**, as hydrochloride), acetonitrile (LC-MS grade), formic acid, ammonium acetate solution (5 M), *N*-methylnicotinamide iodide (**3**), and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). d_3-Creatinine was obtained from CDN (Dr. Ehrenstorfer, Augsburg, Germany). The iodides of d_3-trigonelline (d_3-**1**), *N*-methylpyridinium (**2**, NMP), and d_3-*N*-methylpyridinium (d_3-**2**) were synthesized as reported recently [20]. Water for HPLC separation was purified by means of a Milli-Q water advantage A 10 water system (Millipore, Molsheim, France). Urine samples were collected in the morning prior to breakfast and aliquots (20 mL) were stored at −20°C until analysis. Pooled blank urine (PU) was prepared by pooling aliquots of urine samples (100 mL each) collected from non-coffee drinkers ($n = 9$). Artificial urine was prepared according to the literature [21].

Stock solutions of trigonelline hydrochloride (**1**), d_3-trigonelline hydroiodide (d_3-**1**), *N*-methylpyridinium iodide (**2**), d_3-*N*-methylpyridinium iodide (d_3-**2**), creatinine, and d_3-creatinine, respectively, were prepared in acetonitrile/water (1:1, v/v) at a concentration of 200 μmol/mL and kept at −20°C until use. To obtain a suitable internal standard working solution, appropriate aliquots of the internal standard stocks were combined and diluted with acetonitrile to give concentrations of 10 nmol/mL for d_3-creatinine and 1 nmol/mL for d_3-**1** and d_3-**2**, respectively.

2.2 Preparation of the Coffee Brew

Raw coffee beans (Arabica Brazil, harvested in 2008) were roasted for 165 s in a laboratory scale fluidized bed roaster (Novopack, Germany) operated with a gas temperature of 257°C and were then cooled with air (150 $m^3 \times h^{-1}$ for 120 s) to afford a medium dark coffee (67 SKT). Roast coffee samples obtained from five individual roast batches (300 g each) were blended, ground, packed in polyethylene bags, and stored at −20°C until use. An aliquot of coffee powder (48 g) was placed in a coffee filter (size 4, Melitta, Germany) and percolated with distilled water (900 mL) by means of a conventional filter drip machine (TCM, Germany). The

freshly prepared coffee beverage was used for the experiments.

2.3 Human urine samples

The group of coffee drinkers consisted of nine healthy volunteers (four males and five females; age 24–32 years) who were used to consume at least four cups of coffee on a daily basis (''coffee drinkers''). The control group consisted of nine healthy non-coffee drinking volunteers (four males and five females; age 23–34 years; ''controls''). Subjects were recruited from the Technische Universität München, Germany, without any exclusion parameters besides being in good health, non-smoking, and not under medication. All subjects gave informed consent to the work. With the exception of coffee and coffee-containing products, no further restrictions concerning intake of foods or drinks were made during the study. The study was approved by the Ethical Commission of the Technical University of Munich (project number 2496/09).

2.3.1 Collection and processing of urine samples for comparison of coffee group and control group

Aliquots (100 μL) of morning urine samples, collected prior to breakfast, were mixed with acetonitrile (900 μL), centrifuged (12 500 rpm) for 5 min at 4°C, and an aliquot (5 μL) of the clear supernatant was analyzed by means of hydrophilic liquid interaction chromatography/time-of-flight mass spectrometry (HILIC-UPLC/ToF-MS) without further sample pretreatment. Besides the individual samples, a pooled urine sample was prepared for the coffee group and the control group, respectively, by mixing aliquots (200 μL) of the individuals.

2.3.2 Collection and processing of urine samples for time–concentration profiles of excretion of trigonelline and NMP

After having collected the morning urine (time point 0), the coffee drinkers consumed a bolus dose of 350 mL of the freshly prepared coffee beverage containing 2.31 (±7.2%) μmol/mL trigonelline (**1**) and 0.49 (±6.5%) μmol/mL NMP (**2**) as quantitatively determined by means of a stable isotope dilution analysis (SIDA) using hydrophilic liquid interaction chromatography/triple quadrupole mass spectrometry (HILIC-MS/MS) [20]. After this bolus dose, the participants had to avoid coffee until the study was finished. Urine samples were collected from each volunteer 8, 24, 48, 72, and 240 h after coffee ingestion and aliquots (10 μL) were mixed with the internal standard working solution (1000 μL), vortexed, and equilibrated for 5 min. After centrifugation (12 500 rpm) for 5 min at 4°C, the clear supernatant was transferred to an autosampler vial for HILIC-MS/MS analysis by means of a AB Sciex 3200 triple quadrupole mass spectrometer.

2.4 Quantitative analysis

2.4.1 Calibration curves

Standard solutions were prepared by mixing analytes and internal standards in concentration ratios (nmol/nmol) of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 in artificial urine and subsequent dilution with acetonitrile (1/10, v/v). Calibration plots were constructed by plotting peak area ratios of analyte versus internal standard against concentration ratios followed by linear regression. The equations obtained from replicate analysis ($n = 4$) were $A(A/IS) = 0.977 \times c(A/IS)$ (**1**/d_3-**1**), $A(A/IS) = 0.781 \times c(A/IS)$ (**2**/d_3-**2**), and $A(A/IS) = 0.670 \times c(A/IS)$ (creatinine/d_3-creatinine).

2.4.2 Evaluation of recovery and precision

For precision and recovery evaluation, aliquots of pooled urine (PU, 900 μL) collected from non-coffee drinkers were spiked with a solution (100 μL) of the analytes to obtain urine samples spiked with a low and a high dose of creatinine (5000 and 20 000 nmol/mL), trigonelline (50 and 1000 nmol/mL), and NMP (10 and 100 nmol/mL), respectively. Sample aliquots (10 μL) were mixed with the internal standard working solution (1000 μL), vortexed, equilibrated for 5 min, centrifuged (12 500 rpm) for 5 min at 4°C, and the clear supernatant was transferred to an autosampler vial for HILIC-MS/MS analysis by means of a AB Sciex 3200 triple quadrupole mass spectrometer.

2.5 LC/MS

2.5.1 Hydrophilic liquid interaction chromatography/time-of-flight mass spectrometry (HILIC-UPLC/ToF-MS)

Urine samples collected from the coffee drinking group and the control group, respectively, were chromatographically separated on a BEH HILIC column (150 mm × 2 mm, 1.7 μm, Waters, Manchester, UK) using an Acquity UPLC core system (Waters, Bedford, MA, USA) hyphenated to a Synapt G2 HDMS Time of Flight mass spectrometer (Waters, Manchester). The ToF system was calibrated in the range of 50–600 Da using sodium formate (5 mM). Leucine enkephaline (Tyr-Gly-Gly-Phe-Leu) in acetonitrile/water (2 μg/mL, 1:1, v/v) infused at 20 μL/min served as lock mass (m/z 556.2771). Scan time for the lock mass was set to 0.3 s, an interval of 15 s, and three scans to average with a mass window of ±0.3 Da. The system operated in positive elec-

trospray with a resolution of 20 000. Voltages applied were 3 kV (capillary), 30 V (sampling cone), and 4 V (extraction cone), respectively. The source temperature was 150°C and desolvation temperature was 450°C. Cone gas and desolvation gas (both nitrogen) were set at flows of 30 and 850 L/h, respectively. Data were acquired in the range of 50–600 Da at a scan frequency of 20 Hz.

For chromatography, water/acetonitrile (10:90, v/v) and water/acetonitrile (1:99, v/v), each containing 1.5% formic acid and 5 mM ammonium acetate, were used as solvents A and B, respectively. Solvent A was increased from 0 to 80% within 10 min (curve 8), followed by 1 min of isocratic elution and re-equilibration for 4 min prior to the next injection. The flow rate was 0.45 mL/min, samples were kept at 10°C, column temperature was 40°C. The UPLC and Synapt G2 systems were operated with Mass Lnyx^{TM} software (Waters, Manchester), data processing and analysis were performed using Marker Lynx^{TM} software (Waters, Manchester).

2.5.2 Hydrophilic liquid interaction chromatography/triple quadrupole mass spectrometry (HILIC-MS/MS)

The Agilent 1200 Series HPLC-system consisted of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) and was connected to a API 3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt, Germany). Separation was carried out on a Kinetex HILIC 2.6 μm column (50 mm × 2 mm, Phenomenex, Darmstadt, Germany) equipped with a Krud Catcher guard column (Phenomenex) using a mixture (87.6:10.8:1.5, v/v/v) of acetonitrile/water/formic acid containing 5 mmol/L ammonium acetate as the mobile phase at a flow rate of 500 μL/min. Injection volume was typically 1 μL and chromatographic run time was 3 min. The ion source of the mass spectrometer was set to positive electrospray mode, nitrogen was used as the nebulizer gas (40 psi) as well as curtain gas (40 psi), heater gas was set at 60 psi, and the temperature was 550°C. In the multiple reaction monitoring (MRM) mode, the transitions from the parent ion $[M]^+$ and $[M+H]^+$, respectively, to the fragment ions formed after collision-induced dissociation were recorded (Table 2). The CAD gas was set to ''8'', the dwell time for each mass transition was 50 ms and the pause between the mass ranges was 5 ms. The quadrupoles operated at high resolution and the ion spray voltage was +5500 V. Sciex Analyst software v1.5.1 (Applied Biosystems, Darmstadt, Germany) was used for instrumentation control and data acquisition. Compound specific fragmentation was optimized by constant infusion of the individual compounds by means of a syringe pump ($10\,\mu L \times min^{-1}$) and software-assisted ramping of ion path parameters with Q1 and Q3 set to high resolution.

2.6 Calculations

The raw data obtained from HILIC-UPLC/ToF-MS analysis were processed with Marker Lynx XS (V4.1, Waters, Manchester) using ApexTrack peak integration to detect chromatographic peaks. Marker intensity threshold was set to 2000 cps, mass window was 0.02 Da, retention time window was 0.1 s, data were de-isotoped.

LC-MS/MS data were processed with Analyst 1.5.1 and exported to Excel 2003, Graphpad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and Origin 6.1.

3 Results

Aimed at discovering urinary metabolites as candidate dietary biomarkers for coffee consumption, urine samples collected from habitual coffee drinkers ($n = 9$) and non-coffee drinkers ($n = 9$), respectively, were analyzed individually as well as after pooling aliquots of the coffee drinkers' urine or non-coffee drinkers' urine, respectively, by means of HILIC-UPLC/ToF-MS using positive electrospray ionization and recording masses in the range of 50–600 Da. To make the coffee drinking group and the group of non-coffee drinkers (control group) as representative as possible, both groups consisted of male and female volunteers and no additional restrictions to food intake were done.

Using Marker Lynx^{TM} software, the collected data were processed to build a matrix comprising information on the exact mass, retention time, and intensity of each metabolite, and, after normalization, to conduct a principle component

Table 1. Calculated and found masses and postulated sum formulae of the marker compounds identified with UPLC/ToF-MS analysis of urine samples

Found mass (Da)	Calculated mass (Da)	Error (mDa)	Error (ppm)	Formula	Compound (no.)[a)]
138.0550	138.0555	−0.5	−5.1	$C_7H_8NO_2$	Trigonelline (**1**)
94.0655	94.0657	−0.3	−3.2	C_6H_8N	*N*-methylpyridinium (**2**)
137.0712	137.0715	−0.3	−2.2	$C_7H_9N_2O$	*N*-methylnicotinamide (**3**)

Sum formulae are calculated on the basis of the exact masses and the isotope pattern.
a) The chemical structures of **1**, **2** and **3** are given in Fig. 2B.

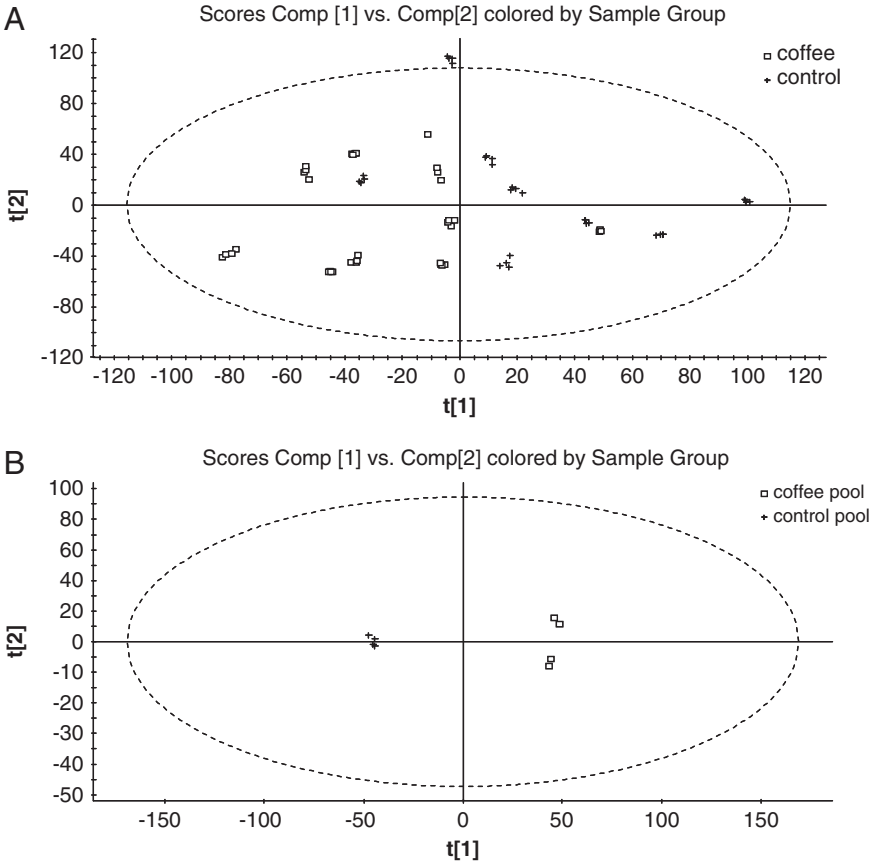

Figure 1. Scores plot obtained from Marker Lynx^{TM} analytical results for full scan analysis (50–600 Da, ESI+, resolution mode) of (A) urine collected from nine coffee drinkers (squares) and nine non-coffee drinkers (controls, black crosses), and (B) pooled coffee drinkers' urine (squares) and pooled non-coffee drinkers' urine (controls, black crosses), each injected in replicates ($n = 4$).

analysis. The scores plot, depicted in Fig. 1A, demonstrates that urine samples taken from individual coffee drinkers and those collected from individual non-coffee drinkers (controls) are largely scattered due to inter-individual variance in nutritional habits, age, and gender (panel A). In comparison, the scores plot of the data obtained for the pooled samples shows a clear differentiation between the coffee and the control group Fig. 1B). To visualize similarities and differences within the urine samples, S-plots, representing pairs of exact mass and retention time of each metabolite, were calculated from all 18 urine samples collected from coffee drinkers and non-coffee drinkers (controls) as well as from pooled coffee drinker and pooled non-coffee drinker urine (Fig. 2). As the *y*-axis of the S-plot denotes confidence of a biomarker's contribution to the group difference and the *x*-axis denotes the contribution of a particular biomarker to the group difference, both S-plots indicate the ions m/z 138.0550, m/z 137.0712, and m/z 94.0655 as masses of metabolites showing the highest difference in abundance in coffee drinkers and non-coffee drinkers.

On the basis of the exact mass data (Table 1), the sum formulae of the compounds showing m/z 138.0550, m/z 94.0655, and m/z 137.0712 were calculated to be $C_7H_8NO_2$, C_6H_8N, and $C_7H_9N_2O$, respectively, fitting well with the structures of trigonelline (**1**), NMP (**2**), and *N*-methylnicotinamide (**3**), depicted in Fig. 2B. Compounds **1** and **2** have recently been shown to be absorbed from coffee brew upon ingestion [20]. Comparison of the base peak ion chromatogram (Fig. 3A), the extracted ion chromatograms of m/z 138.0550 (Fig. 3B), m/z 94.0655 (Fig. 3C), and m/z 137.0712 (Fig. 3D) recorded for the pooled urine of coffee drinkers and the pooled control urine, respectively, demonstrated the absence of NMP (**2**) in the chromatogram recorded for the urine of the non-coffee drinkers and its presence in the urine of the coffee drinkers as confirmed by co-chromatography using the synthetic reference compound. Co-chromatography confirmed the compounds with m/z 138.0550 and m/z 137.0712 to be trigonelline (**1**) and *N*-methylnicotinamide (**3**). The compounds were detected in both pooled urine samples, but the peak area was about 6 and 2 times higher in coffee drinkers' urine compared with the control urine (Fig. 3B and D), respectively. As *N*-methylnicotinamide (**3**) is known as a primary metabolite of niacin, which is present in many foods, compound **3** was excluded from the group of putative coffee-specific biomarkers. Therefore, the following experiments were focused on compounds **1** and **2**.

In order to investigate the suitability of trigonelline (**1**) and NMP (**2**) as candidate urinary biomarkers for compliance monitoring of coffee ingestion in human trials, a robust high-throughput method needed to be developed and

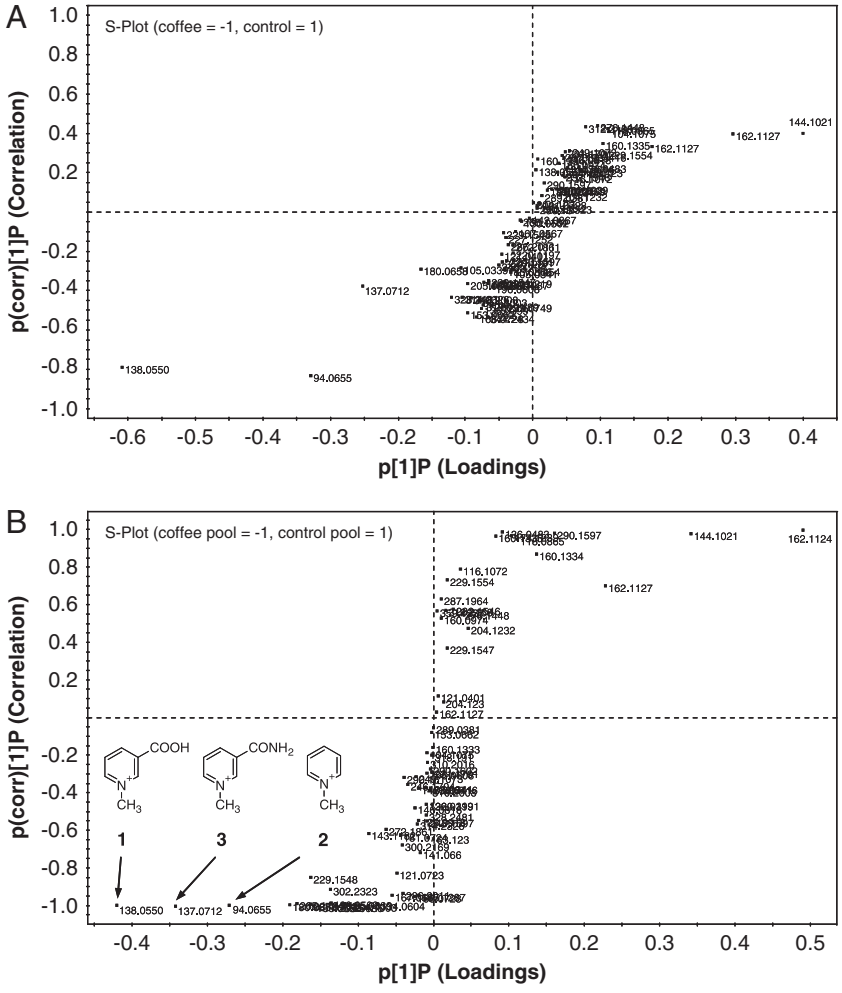

Figure 2. S-Plots of all 18 urine samples (A) collected from coffee drinkers and non-coffee drinkers (controls), and S-Plot of pooled coffee drinker urine versus pooled non-coffee drinker urine (B).

validated for the accurate quantitative analysis of the target metabolites **1** and **2** in urine samples. To achieve this, the recently developed HILIC-MS/MS method needed to be optimized and extended by the simultaneous analysis of creatinine for data normalization. Stepwise optimization of the chromatographic conditions enabled the base-line separation of the pyridines **1** and **2** as well as creatinine under isocratic elution conditions using 1.5% formic acid and 5 mM ammonium acetate in acetonitrile/water at high flow rates, thus making long-lasting equilibration time obsolete and decreasing inter-injection time from 18 min [20] to <3 min.

To analyze the target molecules with high selectivity by using tandem mass spectrometry operating in the MRM mode, solutions of the three analytes as well as their corresponding isotopologues were individually infused into the ESI source of the MS/MS system with a constant flow by means of a syringe pump to optimize ionization parameters and collision-induced fragmentation. At least two mass transitions of each compound were selected for the final acquisition method, typically the most intensive mass transition was used for quantitation, and a second transition was selected for unequivocal identification of the target analytes (Table 2). The chromatographic separation combined with the specific mass transitions of the analytes were sufficiently selective to allow their analysis of urine samples without the need of laborious and costly solid-phase cartridge clean-up procedures (Fig. 4).

For quantitative analysis by means of a SIDA, the urine samples were diluted with an internal standard working solution containing defined amounts of the internal standards d_3-trigonelline (d_3-**1**), d_3-*N*-methylpyridinium (d_3-**2**), and d_3-creatinine in acetonitrile/water. After equilibration, precipitated solids were removed by centrifugation and the clear supernatant was directly injected into the HILIC-MS/MS system without further sample clean-up. For the quantitative analysis of the target analytes, calibration curves were recorded for each analyte and peak area ratios of analyte and the corresponding internal standard were plotted against concentration ratios from 0.25 to 50 followed by

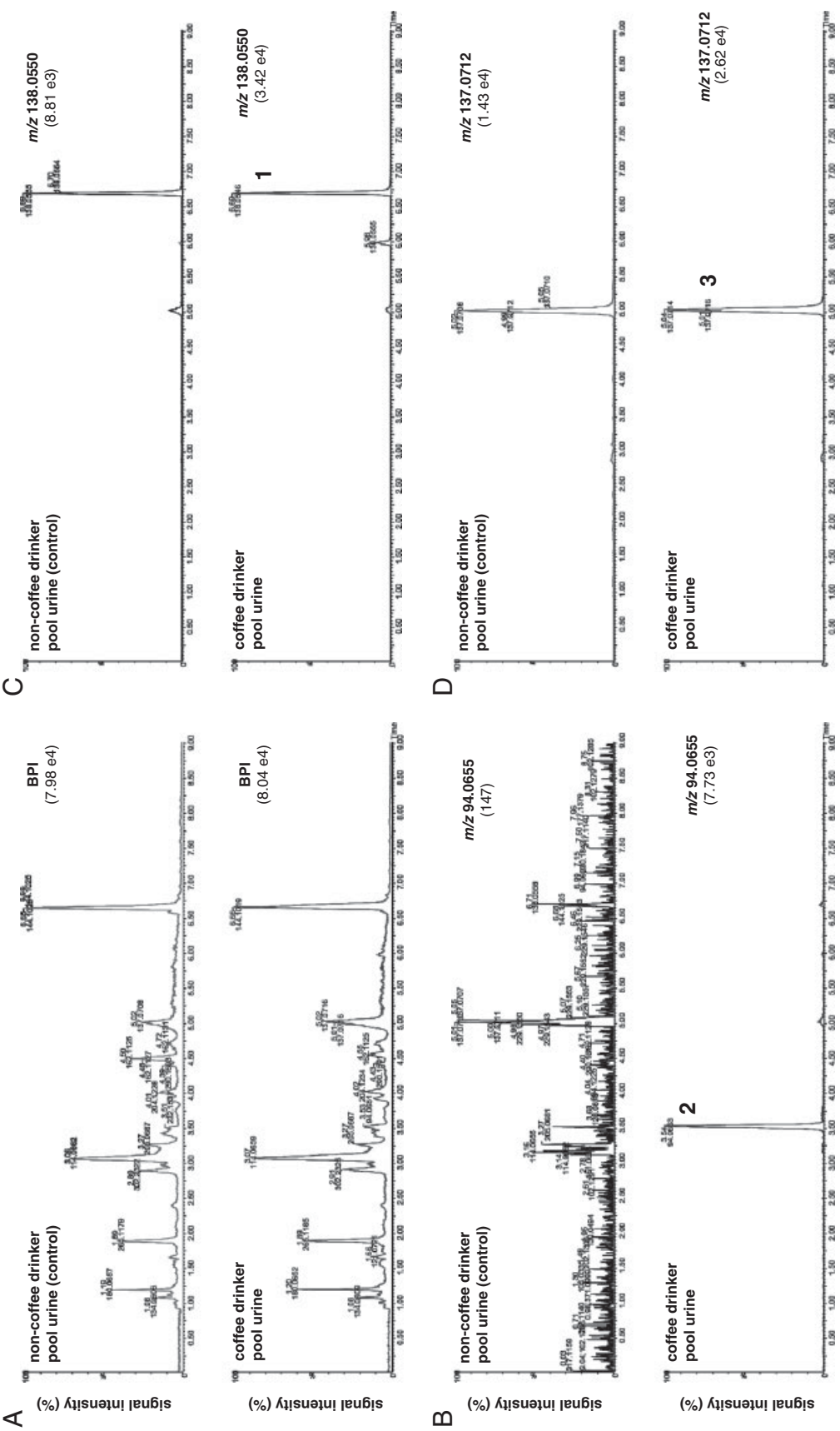

Figure 3. Base peak ion mass chromatogram of pooled non-coffee drinker urine (A, upper chromatogram) and pooled coffee drinker urine (A, lower chromatogram), extracted ion chromatograms of *m/z* 138.0550 in pooled non-coffee drinker urine (B, upper trace) and pooled coffee drinker urine (B, lower trace), extracted ion chromatograms of *m/z* 94.0655 in pooled non-coffee drinker urine (C, upper trace) and pooled coffee drinker urine (C, lower trace), and extracted ion chromatograms of *m/z* 137.0712 in pooled non-coffee drinker urine (D, upper trace) and pooled coffee drinker urine (D, lower trace).

Table 2. Retention time, precursor and fragment ions, and precision and accuracy of calibration curves prepared in artificial urine of creatinine, trigonelline (**1**) and *N*-methylpyridinium (**2**) in quantitative analysis using HILIC-SIDA-LC-MS/MS

Compound	Rt (min)[a)]	Q1 (*m/z*)	Q3 (*m/z*)	R^2	Weighing	RSD (%)	Accuracy (%)
Creatinine/d_3-creatinine	0.71/0.71	113.9/117.0	44.0,[b)] 86.0/47.0,[b)] 89.0	0.9998	–	1.00–10.3	94.0–109.3
1/d_3-**1**	2.40/2.38	138.0/141.0	94.0,[b)] 92.0/97.1,[b)] 95.0	0.9994	$1/x$	0.91–2.76	96.5–102.9
2/d_3-**2**	1.65/1.62	94.0/97.0	79.0,[b)] 78.0, 52.0/79.0,[b)] 78.0, 52.0	0.9994	$1/x$	0.57–2.12	90.9–105.0

RSD, relative standard deviation.
a) Retention time.
b) Quantifier transition is indicated.

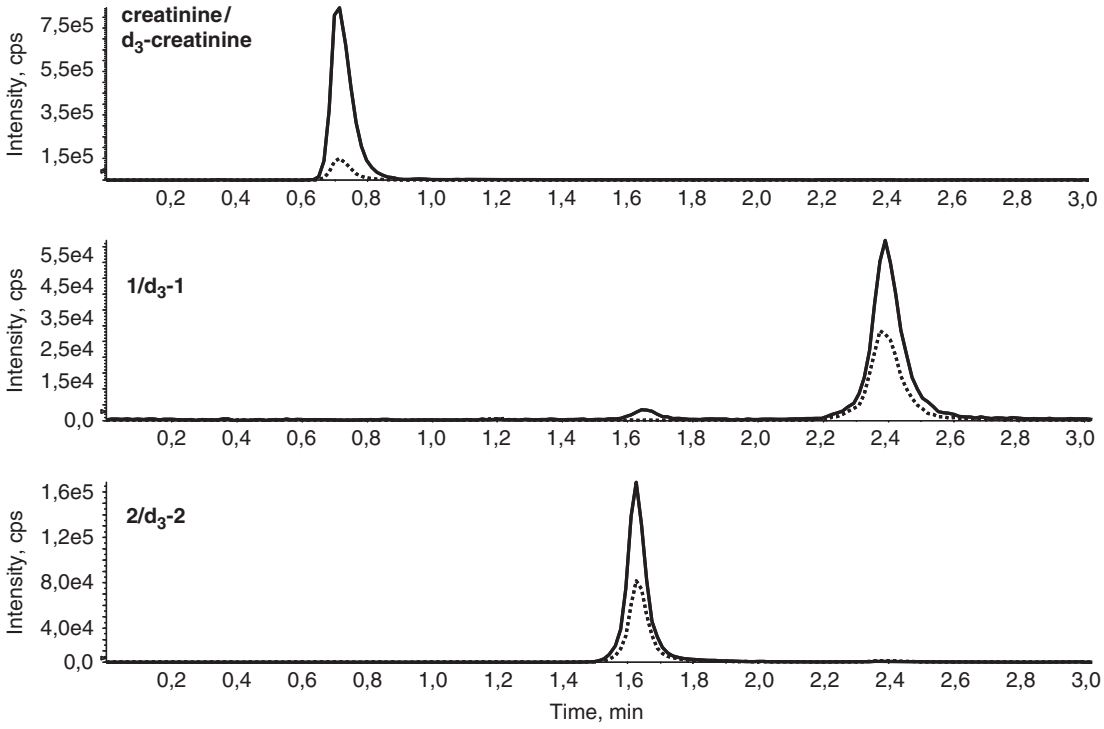

Figure 4. Optimized HILIC-MS/MS analysis of creatinine, trigonelline (**1**), and *N*-methylpyridinium (**2**) in a urine sample collected from a coffee drinker. Solid lines show analyte quantifier traces, whereas dotted lines show internal standard quantifier traces.

linear regression. For all compounds analyzed, excellent linearity was found with $R^2 \geq 0.999$, and RSD and accuracy of back calculated standards were appreciable (Table 2).

For evaluation of recovery and precision, pooled non-coffee drinker urine was spiked with the analytes creatinine, trigonelline, and NMP in a low and a high dose. The precision of the SIDA, expressed by the coefficient of variance (CV, %) obtained by replicate analysis ($n = 5$) of each addition level was $<5\%$ for each analyte. Recovery was between 90 and 103% (Table 3). The instrumental reproducibility, assessed by replicate ($n = 6$) injection of the same urine sample, was 3.71, 1.46 and 0.63% for creatinine, **1**, and **2**, respectively. These data demonstrate the developed SIDA as a reliable tool enabling an accurate simultaneous high-throughput quantitation of the target metabolites **1** and **2** as well as creatinine in human urine samples.

To evaluate the suitability of urinary concentrations of trigonelline and NMP as candidate markers for coffee consumption, a pilot human study was conducted with a group of four male and five female healthy volunteers (coffee drinkers) who were used to consume coffee on a daily base. After having collected the morning urine (time point 0), the coffee drinkers consumed a bolus dose of 350 mL of a coffee beverage containing 2.31 ($\pm$7.2%) μmol/mL trigonelline (**1**) and 0.49 ($\pm$6.5%) μmol/mL NMP (**2**). The urine samples collected from each coffee drinking volunteer before (0 h) and 8, 24, 48, 72, and 240 h after coffee ingestion were then analyzed for **1**, **2**, and creatinine by

Table 3. Recovery and precision of the HILIC-SIDA-LC-MS/MS quantitation of creatinine, trigonelline (**1**), and *N*-methylpyridinium (**2**) in urine matrix

	Low spiking level experiment[a)]			High spiking level experiment[a)]		
	Creatinine	**1**	**2**	Creatinine	**1**	**2**
Addition (nmol/mL)	5000	50.0	10.8	20 000	1000.0	108.0
Addition found (nmol/mL ± SD)	4890 (±44)	50.6 (±1.4)	10.9 (±0.3)	20 610 (±866)	907 (±21.9)	106.2 (±3.0)
CV (%)	0.9	2.8	2.9	4.2	2.4	2.9
Recovery (%)	97.8	101.2	101.8	103.0	90.7	98.3

a) Data are the means of replicate sample workup and analysis ($n = 5$).

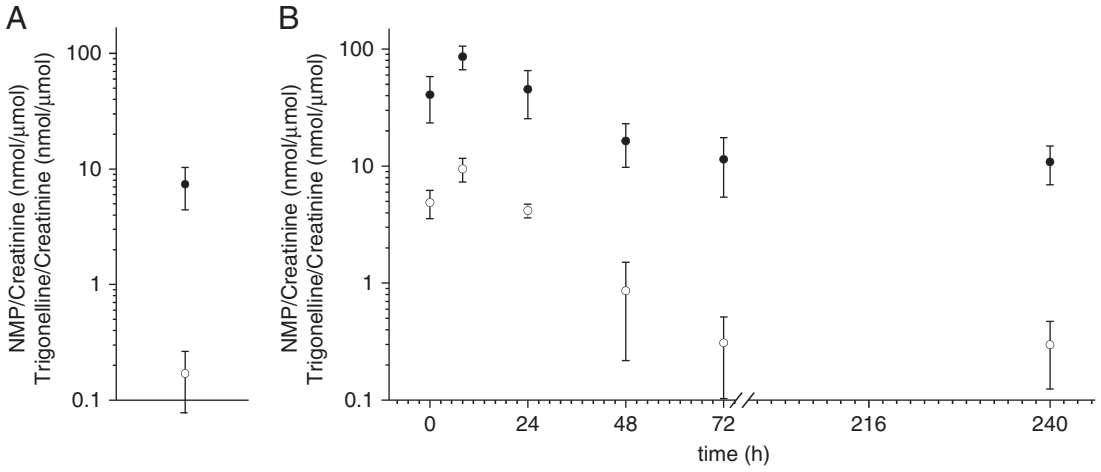

Figure 5. (A) Urinary concentrations of trigonelline (**1**, dots) and *N*-methylpyridinium (**2**, circles) in non-coffee drinkers (controls) and (B) time course of trigonelline (**1**, dots) and *N*-methylpyridinium (**2**, circles) levels in urine collected from coffee drinkers before (0 h) and 8, 24, 48, 72, and 240 h after ingestion of a single dose of 350 mL roast coffee brew. Concentrations (±SD) are given as the means of the individuals ($n = 9$ coffee drinkers, $n = 9$ non-coffee drinkers) and are normalized to individual urinary levels of creatinine (nmol/μmol).

means of the developed HILIC-MS/MS method. For comparison, the morning urine was collected from five female and four male healthy, non-smoking volunteers (non-coffee drinkers), who did not consume any coffee or coffee products at least over the last 5 years and was analyzed by means of HILIC-MS/MS. After normalization to creatinine (nmol/μmol), the concentrations of trigonelline (**1**) and NMP (**2**) were plotted against time, thus demonstrating a significant difference in the levels of both **1** and **2** in the morning urine collected from non-coffee drinkers and coffee drinkers (Fig. 5). The normalized levels of trigonelline were 6.88 (±3.17) and 37.37 (±10.54) nmol/μmol, and those of NMP were 0.18 (±0.10) and 4.70 (±1.06) nmol/μmol in the urine collected from non-coffee drinkers and coffee drinkers, respectively, thus demonstrating that the levels of **1** and **2** in the morning urine of coffee-drinkers were 5.4 and 26.1 times above those found for the non-coffee drinkers. After coffee ingestion, the levels of **1** and **2** in the coffee drinker's urine increased twofold after 8 h, followed by a decline within the following 3 days to finally reach levels of 10 and 0.3 nmol/μmol creatinine, respectively, measured 240 h after coffee consumption (Fig. 5B). At this time point, the concentrations of **1** and **2** resembled those found in urine samples collected from non-coffee drinkers (Fig. 5A).

Comparison of the creatinine-normalized data of trigonelline (**1**) and NMP (**2**) at each time point with the respective ratios determined in the urine of the non-coffee drinkers by means of an unpaired, two-tailed *t*-test showed that urinary NMP levels were significantly above those found for the non-coffee drinkers up to 72 h ($p = 0.0021$) after coffee consumption. In comparison, the last significant difference in urinary trigonelline levels between coffee and non-coffee drinker was detectable after 48 h ($p = 0.0011$).

4 Discussion

Comparative HILIC-UPLC-ToF-MS analysis of urine samples collected from coffee drinkers and non-coffee drinkers led to the discovery of two metabolites contributing most to the dissimilarities between both sample groups.

Based on the analysis of the exact mass, isotope pattern, retention time, and co-chromatography, these metabolites were unequivocally identified as trigonelline (**1**) and NMP (**2**). While trigonelline is a phytohormone present in many foods such as legumes (http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Trigonelline.pdf accessed via the Internet on 14/12/2010), the occurrence of NMP (**2**) in our daily diet seems to be restricted to roasted coffee [13, 14]. HPLC-MS/MS screening of a series of fresh and thermally treated foods did not show any significant amounts of NMP in any other food with the exception of coffee (data not shown). The trace amounts of NMP detected in the urine collected from non-coffee drinkers (Fig. 5A) might be explained by the fact that **2** is a natural metabolite of pyridine [22], which is present as a trace constituent in thermally processed foods and tobacco smoke [23]. Recent quantitative studies revealed that **1** and **2** are readily absorbed during ingestion of coffee brew with rather low inter-individual variance in maximal plasma concentration and peak time [20]. Among both compounds, NMP (**2**) seems to be excreted without further metabolism via the urine [[20], http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Trigonelline.pdf accessed via the internet on 14/12/2010, [22], http://monographs.iarc.fr/ENG/Monographs/vol77/mono77-21.pdf accessed via the internet on 8/2/2010]. Taking all this information into account, the elevated levels of NMP in urine collected from coffee drinkers seem to be due to the habitual consumption of coffee.

In order to investigate the persistence of elevated urinary levels of trigonelline and NMP, a pilot scale study was conducted with coffee drinkers, and data were compared with those found for non-coffee drinkers. Quantitative studies on **1** and **2** in morning urine samples collected from non-coffee drinkers and habitual coffee drinkers, respectively, by means of HILIC-MS/MS in combination with a SIDA revealed both pyridinium derivatives in significantly higher levels in the latter than in the former group of volunteers. However, after a 10-day period of coffee abstinence similar low concentrations of **1** and **2** were observed in the urine of coffee and non-coffee drinkers. Analysis of the samples of morning urine revealed that NMP was significantly higher compared with the concentration found in non-coffee drinker urine for up to 72 h. As elevated trigonelline levels came back to normal levels of non-coffee drinkers already after 48 h after coffee consumption, NMP was identified as a more suitable dietary biomarker for coffee consumption when compared with trigonelline. As the HILIC-MS/MS-SIDA method reported in this paper allows the precise quantitative analysis of NMP (**2**) besides trigonelline (**1**) and creatinine in 20 urine samples per hour, it can be considered a non-invasive, high-throughput technology enabling the rapid and accurate analysis of **2** as a coffee-specific candidate biomarker (Fig. 4).

In conclusion, the data reported here will help to monitor the subject's compliance in future nutritional human studies as it is usually rather challenging for coffee drinkers to stay abstinent in the wash-out phase of an intervention study. Moreover, NMP measurement can help to contribute to the establishment of a tool box of valid dietary biomarkers reflecting wider aspects of diet to assess metabolic profiles as measures of dietary exposure and indicators of dietary patterns, dietary changes, or effectiveness of dietary interventions.

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5 References

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