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Electrospray ionization mass spectrometric characterization and quantitation of xanthine derivatives using isotopically labelled analogues: an application for equine doping control analysis

Mario Thevis*, Georg Opfermann, Oliver Krug and Wilhelm Schänzer
Institute of Biochemistry, German Sport University Cologne, Carl-Diem Weg 6, Cologne, Germany

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Isotope-dilution mass spectrometry has been employed successfully in numerous fields of analytical chemistry enabling the establishment of fast and reliable procedures. In equine sports, xanthine derivatives such as caffeine and theobromine are prohibited, and doping control laboratories analyze horse urine specimens regarding these illicit performance-enhancing drugs. Theobromine has to exceed a threshold level of 2 μg/mL, hence a robust and reliable quantitation is required. Stably deuterated theobromine and caffeine were synthesized by the reaction of xanthine or theobromine with iodomethane-d_3 in the presence of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide or potassium carbonate in acetonitrile, respectively. Both compounds were characterized by nuclear magnetic resonance spectroscopy and electrospray ionization tandem mass spectrometry, and a robust and fast assay for the qualitative and quantitative analysis of theobromine in equine urine samples was validated. Urine specimens were extracted by means of solid-phase extraction cartridges, and concentrated extracts were analyzed by liquid chromatography interfaced to a triple-quadrupole mass spectrometer. In addition, the dissociation behavior of deuterated analogues to caffeine and theobromine allowed proposals for fragmentation routes of xanthine derivatives after atmospheric pressure ionization and collisionally activated dissociation. Copyright © 2004 John Wiley & Sons, Ltd.

Since the advent of atmospheric pressure ionization techniques such as electrospray ionization (ESI)[1] or atmospheric pressure chemical ionization (APCI) for mass spectrometric purposes, numerous analytical challenges have been solved or are still under investigation, ranging from applications including small as well as very large molecules. Also the interest in very common compounds such as methylated xanthines is still present for various reasons, for instance the determination of cacao usage by the earliest Maya civilization[2] or definition of specific enzyme activity.[3] Xanthine derivatives (Scheme 1) such as caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) have been investigated regarding their physiological properties for several decades, in particular for human medication purposes. By means of high-performance liquid chromatography (HPLC) as well as mass spectrometry (MS),[4] theobromine and theophylline were identified as metabolites of caffeine in 1977,[5] and both were found to be physiologically active. While theobromine is mildly diuretic, it also affects humans as a soft stimulant as well as a bronchodilator, and theophylline has been one of the most commonly used medications for the treatment of symptoms arising from chronic or exercise-induced asthma.

In addition, the trimethylated xanthine caffeine is considered the most widely administered psychoactive drug of abuse in the world[6] as it fulfils all characteristic criteria such as development of tolerance and dependence, combined with withdrawal symptoms upon abrupt cessation. Caffeine is a central nervous system stimulant that was banned by various international sports federations and the International Olympic Committee (IOC), owing to studies demonstrating performance-enhancing effects,[6–9] until it was removed from the list of prohibited substances and methods of doping established by the World Anti-Doping Agency (WADA) valid from 2004. But for equine doping controls, caffeine and its metabolites theophylline and theobromine remain prohibited according to appropriate guidelines established by the Federation Equestre Internationale (FEI)[10] and other responsible federations. While caffeine and theophylline are principally prohibited, theobromine has to exceed a threshold level of 2 μg per mL of urine in order to cause a positive test result. This requires reliable identification of these compounds and quantitation of theobromine in urine.

Several procedures based on gas chromatography/mass spectrometry (GC/MS),[11–13] high-performance liquid chromatography interfaced to UV detectors (HPLC-UV),[14–17]

**Correspondence to*: M. Thevis, Institute of Biochemistry, German Sport University Cologne, Carl-Diem Weg 6, Cologne, Germany.
E-mail: m.thevis@biochem.dshs-koeln.de
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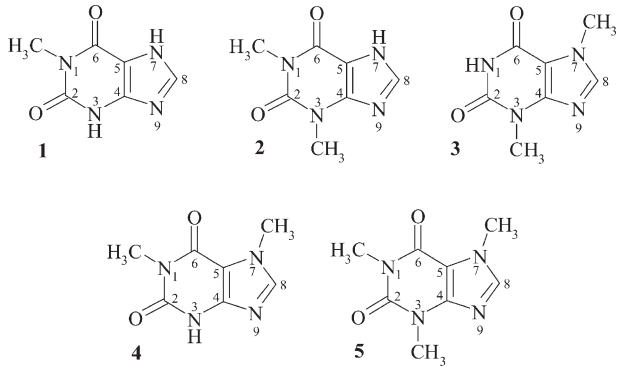

Scheme 1. Chemical structures of 1-methylxanthine (**1**); theophylline (1,3-dimethylxanthine **2**); theobromine (3,7-dimethylxanthine, **3**); paraxanthine (1,7-dimethylxanthine, **4**); and caffeine (1,3,7-trimethylxanthine, **5**).

or HPLC combined with fast atom bombardment (FAB),[18] have been developed for human and animal samples. The major shortcomings of these procedures are the necessity of a time-consuming derivatization for GC/MS analysis or marginal specificity of UV detectors used in HPLC-UV assays. With the improvements in coupling liquid chromatography to mass spectrometers by means of atmospheric pressure ionization techniques such ESI or APCI, very sophisticated tools are available enabling a fast, specific and sensitive determination of drugs in biological matrices, which have been applied to a range of doping control analysis issues in the past. Also for quantitation purposes, modern mass spectrometric instruments are very suitable, in particular if stable-isotope-labelled analogues[12,13] are available. In the present study, we describe a facile way to synthesize the six-fold deuterated analogue of theobromine and three-fold deuterated caffeine, and report their mass spectrometric behavior upon ESI and collisionally activated dissociation (CAD); fragmentation routes of methylated xanthines are proposed on the basis of labelled, unlabelled, and substituted analogues of caffeine and its metabolites. Also, a validated method for the determination and quantitation of theobromine in equine urine specimens is described.

EXPERIMENTAL

Chemicals

Xanthine (99–100%), ethanethiol (97%) and iodomethane-d_3 (99.5+ atom% D) were purchased from Sigma-Aldrich (Steinheim, Germany). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Chem. Fabrik Carl Bucher (Waldstetten, Germany) and distilled before use. 1-Methylxanthine (≥97%) and ammonium iodide were bought from Fluka (Buchs, Switzerland). *tert*-Butyl methyl ether was supplied by Kraemer & Martin (St. Augustin, Germany), and acetonitrile (HPLC grade) by J. T. Baker (Deventer, The Netherlands). Theobromine, theophylline, paraxanthine, and caffeine (all pure anhydrous powders), ammonium acetate (p.a.) and acetic acid (glacial) were purchased from Merck (Darmstadt, Germany), potassium carbonate (p.a.) from Riedel-de Haen (Seelze, Germany), and Oasis HLB solid-phase extraction (SPE) cartridges (60 mg, 3 mL) from Waters (Milford, MA, USA).

Synthesis of $^2H_6$-theobromine

In the past, several strategies for the synthesis of purines and substituted xanthines have been established.[19–23] In order to introduce two triply deuterated methyl residues into the xanthine nucleus at positions N-3 and N-7, a published procedure for the generation of 7-substituted 1-methylxanthines[24] was adapted and modified as follows: 156 mg of xanthine (1 mmol) were added to 40 mL of a mixture of MSTFA/ammonium iodide/ethanethiol (1000:2:6, v/w/v), which contains the *in situ* generated trimethyliodosilane (TMIS). The mixture was incubated at 70°C in a water bath under constant stirring for 1 h, yielding a clear solution. A volume of 130 µL (2 mmol) of iodomethane-d_3 was added, and the solution was kept at 60°C in a water bath. After 14 h, 100 mL of methanol were added, the reaction mixture was evaporated to dryness *in vacuo* at 50°C by means of a rotary evaporator, and the dry residue was dissolved in 40 mL of deionized water. The aqueous layer was extracted eight times with 200 mL of *tert*-butyl methyl ether, the combined extracts were evaporated to dryness, and the crude product was purified by HPLC fractionation. Therefore, the dry residue was dissolved in 5 mL of 0.03 N aqueous sodium hydroxide, and aliquots of 100 µL were injected into a Hewlett Packard 1090 HPLC instrument (Waldbronn, Germany) equipped with a Macherey-Nagel (Düren, Germany) VP 250/10 Nucleosil 100-7 C_{18} semipreparative column and a UV detector. The solvents used were A: 0.2% acetic acid, and B: acetonitrile, utilizing a gradient from 97% A to 50% A in 15 min followed by re-equilibration for 6 min. The flow was adjusted to 3.5 mL/min, and compounds were detected at a wavelength of 275 nm. The elution time of the target substance was determined by analyzing the unlabelled analogues of theobromine, theophylline and paraxanthine, and the appropriate fraction was collected into a 50 mL flask; 19.6 mg (10.5%) of the desired product were obtained by evaporation of the solvents at 60°C *in vacuo*.

Synthesis of $^2H_3$-caffeine

The deuteromethylation of the dimethylxanthine theophylline was accomplished by means of the traditional alkylation reaction described by Dünges[25] and Garst *et al*.,[26] employing an alkyl halide in the presence of a base. Briefly, 1 g of theophylline (5.6 mmol) was dissolved in 50 mL of acetonitrile, and 1 g of potassium carbonate (K_2CO_3) and 0.53 mL of iodomethane-d_3 (CD_3I, 8.33 mmol) were added. The mixture was refluxed for 14 h, filtered through a medium porosity frit, and the filtrate was evaporated to dryness. The residue was dissolved in 50 mL of deionized water, 1 g of sodium chloride and 100 mg of K_2CO_3 were added, and the aqueous layer was extracted four times with 220 mL of *tert*-butyl methyl ether. The combined extracts were evaporated to dryness and 628 mg (57.8%) of the desired product were crystallized from methanol.

Characterization and purity of synthesized material

Structure confirmation and degree of purity of synthesized reference materials were obtained by nuclear magnetic resonance (NMR) spectroscopy (^1H, ^{13}C/DEPT), HPLC-UV and

LC/MS(/MS). NMR spectra of compounds dissolved in deuterated methanol (CD_3OD) or dimethyl sulfoxide (DMSO) were recorded using a Bruker DRX 500 spectrometer (Bremen, Germany). In order to determine purities of synthesized substances, HPLC-UV measurements were performed using a Hewlett Packard 1090 series II HPLC system equipped with a Nucleosil 120-5 C_{18} column (Macherey-Nagel) with a length of 120 mm and an i.d. of 4 mm. The solvents used were A: distilled water, and B: acetonitrile. A gradient was employed starting with 100% A for 3.5 min decreasing to 75% A in 13 min, at a flow rate of 1 mL/min. Wavelengths at 208 and 272 nm as well as full UV spectra were utilized for the detection of target compounds and contaminations. In addition, LC/APCI-MS analyses were performed by means of an Agilent 1100 liquid chromatograph coupled to an Applied Biosystems API 2000 triple-quadrupole mass spectrometer. Here, chromatography was accomplished by means of a Merck Purospher Star 18e column (55×4 mm, 3.5 μm particle size) utilizing the solvents A: 5 mM ammonium acetate/0.1% acetic acid (pH 3.5) and B: acetonitrile, at a flow rate of 800 μL/min (post-column split 1:10). A gradient was used from 95% A to 50% A in 6 min. The mass spectrometer was operated in the positive ion mode at an interface temperature of 400°C, and full scan mass spectra were recorded from *m/z* 50–500. Nitrogen was utilized as nebulizing and curtain gas delivered by a Whatman K75-72 nitrogen generator. LC/APCI-MS/MS was performed at a collision gas pressure of 2.0×10^{-5} Torr and a collision energy of 35 eV, and full scan product ion spectra of the six-fold deuterated precursor ion of theobromine at *m/z* 187 and of the triply deuterated precursor ion of caffeine at *m/z* 198 were recorded.

Studies of fragmentation pathways

In order to elucidate the fragmentation behavior of xanthine derivatives after ESI followed by CAD, solutions of $^2H_6$-theobromine, $^2H_3$-caffeine, their unlabelled analogues, theophylline, paraxanthine, ethyltheophylline or 1-methylxanthine, were prepared in 5 mM ammonium acetate/0.1% acetic acid and acetonitrile (1:1, v/v) containing 10 μg of analyte per mL, and introduced into an Applied Biosystems Qtrap mass spectrometer at 3 μL/min by means of a syringe pump. The ionization voltage was set to 5500 V, the declustering potential (DP) was 25 V, collision gas pressure was adjusted to 4×10^{-5} Torr, and a scan rate of 1000 Th/s of the linear ion trap was employed. Parameters such as collision energies in MS^2 or DPs in MS^3 experiments, respectively, varied in different measurements (*vide infra*). A solution of theobromine in 1 mM DCl (in D_2O) and acetonitrile (50:50, v/v) was utilized to probe for the elimination of the ionizing proton/deuteron during fragmentation processes.

Method validation for qualitative and quantitative analysis of theobromine in equine urine samples

Method validation for qualitative and quantitative determination of theobromine in urine specimens was performed according to EURACHEM ('The Fitness for Purpose of Analytical Methods')[27] or DIN guidelines.[28]

Sample preparation

Equine urine specimens were fortified with 2 μg/mL of the synthesized internal standard $^2H_6$-theobromine (20 μL of a 100 μg/mL solution) and extracted by means of Oasis HLB SPE cartridges (60 mg, 3 mL) that were pre-conditioned with 2 mL of methanol and 2 mL of distilled water. After loading 1 mL of urine, a washing step was performed with 2 mL of a mixture of water and methanol (95:5, v/v) according to the manufacturer's protocol,[29] and the cartridge was eluted with 2 mL of methanol. The eluted volume was evaporated to dryness by means of a rotary evaporator under vacuum, the dry residue was reconstituted in 100 μL of 5 mM ammonium acetate (containing 1% acetic acid), and 10 μL were injected into the LC/MS/MS system.

Analysis by LC/MS/MS

All urine samples were analyzed using an Agilent 1100 liquid chromatograph coupled to an Applied Biosystems API 2000 triple-quadrupole mass spectrometer. Chromatographic conditions were as described above (see 'Characterization and purity of synthesized material'). The mass spectrometer was operated in the positive ion mode at an interface temperature of 400°C employing multiple reaction monitoring (MRM) with dwell times of 50 ms. Nitrogen was utilized as nebulizing, curtain and collision gas delivered by a Whatman K75-72 nitrogen generator. MRM transitions for theobromine (*m/z* 181–138, 181–110, 181–69) and $^2H_6$-theobromine (*m/z* 187–144, 187–116, 187–72) were recorded enabling identification as well as quantitation of theobromine. For quantitation purposes, the ratios of peak areas for the therapeutic drug (transition 181–138) and the synthesized internal standard (transition 187–144) were calculated, and concentrations of theobromine were determined by means of a calibration curve.

Calibration curve

A calibration curve was established from 0.4–4.0 μg/mL with ten calibration points at 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, and 4.0 μg of theobromine per mL of blank equine urine. Each specimen contained 2 μg of the internal standard $^2H_6$-theobromine, and samples were measured once by LC/MS/MS. Common phenomena affecting ESI-MS, such as ion suppression by co-eluting endogenous components, were taken into account as they would affect the analyte theobromine and the internal standard $^2H_6$-theobromine that co-elute with one another, and also matrix-matched standards were used.'

Selectivity

Ten blank urine specimens were prepared as described above in order to probe for interfering peaks in the selected reaction chromatograms at the expected retention time of theobromine. Also here, possible ion suppression effects are accounted for by the use of $^2H_6$-theobromine.

Limit of detection

The limit of detection (LOD) was defined as the 'lowest content that can be measured with reasonable statistical certainty'[28] at a signal-to-noise (S/N) ratio $\geq$3. Six blank urine samples spiked with internal standard (ISTD) only, and ten

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blank urine specimens fortified with 0.1 μg/mL of theobromine in addition to the ISTD, were prepared and analyzed according to the established protocol providing the data necessary to calculate the method LOD.

Lower limit of quantitation

According to DIN 38402 (Part 51, 1986), the limit of quantitation (LOQ) is defined as the analyte concentration generating a signal at the expected retention time and ion transition with a S/N ratio of at least 9. By extracting and analyzing ten urine samples containing 0.4 μg of theobromine per mL of equine urine as well as ten blank urine specimens, the lower limit of quantitation (LLOQ) was determined by the evaluation of S/N ratios.

Recovery

The recovery of theobromine from equine urine by SPE was determined at 2 μg/mL. Ten urine samples were fortified with theobromine before sample preparation, and another ten blank urine samples were extracted according to the described protocol and their extracts were then spiked with 2 μg of theobromine. To both sets of samples, 2 μg of $^{2}H_{6}$-theobromine were added to the volume eluted from the SPE cartridge before evaporation. Recovery was calculated by comparison of mean peak area ratios of analyte and ISTD of samples fortified prior to and after SPE.

Interday precision

On three consecutive days, ten urine samples of low (0.4 μg/mL), medium (2 μg/mL), and high (4 μg/mL) concentrations of theobromine were prepared, analyzed randomly, and the assay precision was calculated for each concentration level.

Table 1. ^{13}C chemical shifts of unlabelled and deuterated caffeine and theobromine

Carbon	Caffeine (**5**)	Theobromine (**3**)
2	155.7	154.9
4	148.8	149.8
5	107.7	107.1
6	152.2	151.0
8	143.0	142.8
N-1-CH_3	27.2[a]	—
N-3-CH_3	29.1	29.3[a]
N-7-CH_3	32.9	33.9[a]

[a] Deuterated position.

RESULTS AND DISCUSSION

Characterization and purity of synthesized material

Structure confirmation of synthesized compounds was obtained by ^{1}H and ^{13}C NMR spectroscopy in accordance with published data,[30] elemental analysis, as well as comparison of HPLC-UV and LC/APCI-MS(/MS) data of commercially available theobromine and caffeine to those obtained from chemical synthesis. ^{13}C chemical shifts of unlabelled and deuterated compounds are listed in Table 1. Retention times of synthesized materials on both liquid chromatographic systems (HPLC-UV and LC/APCI-MS) as well as UV spectra were identical with those obtained by analysis of commercially available caffeine and theobromine; by means of LC/APCI-MS the protonated molecules were determined enabling the confirmation of molecular weights of 197 u for $^{2}H_{3}$-caffeine and 186 u for $^{2}H_{6}$-theobromine.

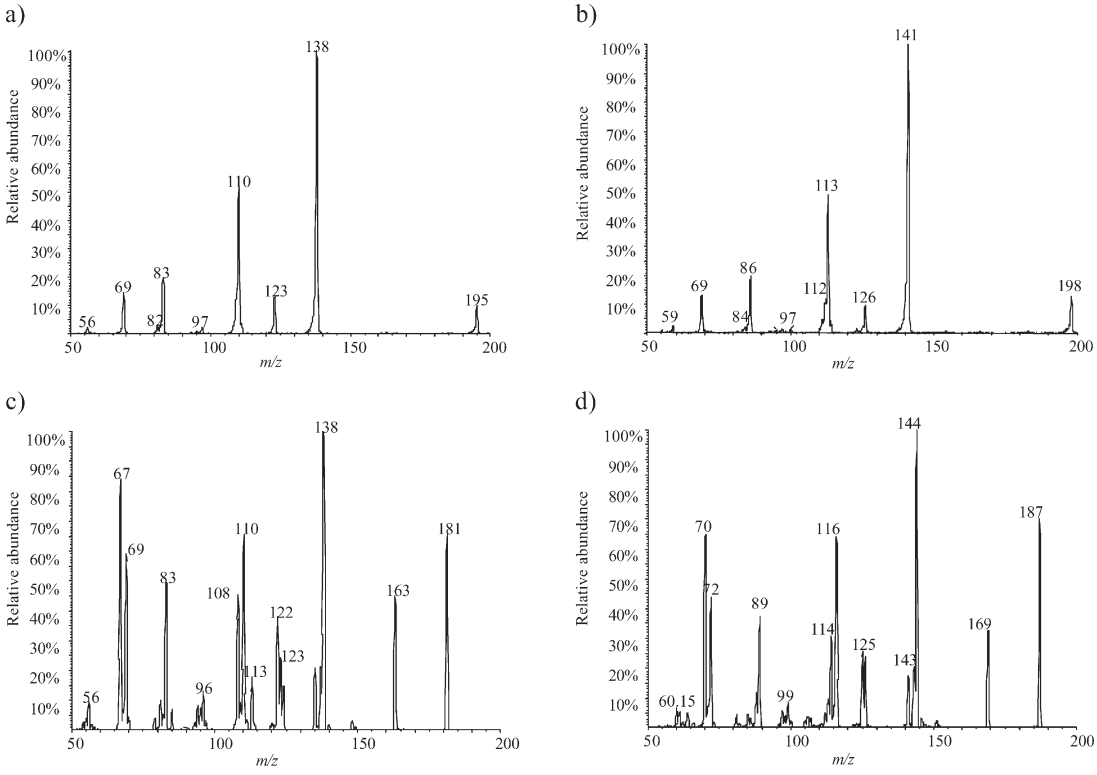

Figure 1. ESI product ion spectra of (a) *m/z* 195 of caffeine, (b) *m/z* 198 of $^{2}H_{3}$-caffeine, (c) *m/z* 181 of theobromine, and (d) *m/z* 187 of $^{2}H_{6}$-theobromine.

Table 2. Product ions of methylated xanthines after ESI and CAD

Compound	Precursor ion *m/z*	CE (eV)	DP (V)	Product ions: *m/z* (relative abundance %)											
				$[M+H]^+$ −18	$[M+H]^+$ −43	$[M+H]^+$ −18 −28	$[M+H]^+$ −43 −15	$[M+H]^+$ −43 −28	$[M+H]^+$ −43 −28 −27	$[M+H]^+$ −43 −28 −41	$[M+H]^+$ −57	$[M+H]^+$ −57 −15	$[M+H]^+$ −57 −28	$[M+H]^+$ −57 −28 −27	$[M+H]^+$ −57 −28 −41
1-Methylxanthine (**1**)	167 $[M+H]^+$	35	20	149 (2)	—	—	—	—	—	—	110 (100)	—	82 (24)	55 (27)	—
Theophylline (**2**)	181 $[M+H]^+$	30	20	—	—	—	—	—	—	—	124 (100)	109 (2)	96 (31)	69 (10)	—
Theobromine (**3**)	181 $[M+H]^+$	35	20	163 (44)	138 (100)	135 (21)	123 (25)	110 (66)	83 (50)	69 (58)	—	—	—	—	—
$^2H_6$-Theobromine	187 $[M+H]^+$	35	20	169 (34)	144 (100)	141 (18)	126 (23)[a]	116 (63)	89 (35)	72 (42)[b]	—	—	—	—	—
Paraxanthine (**4**)	181 $[M+H]^+$	30	20	163 (2)	—	—	—	—	—	—	124 (100)	—	96 (10)	69 (12)	55 (7)
Caffeine (**5**)	195 $[M+H]^+$	35	20	—	—	—	—	—	—	—	138 (100)	123 (14)	110 (52)	83 (20)	69 (15)
$^2H_3$-Caffeine	198 $[M+H]^+$	35	20	—	—	—	—	—	—	—	141 (100)	126 (14)	113 (51)	86 (20)	69 (13)[b]

CE = collision energy, DP = declustering potential.
[a] Loss of CD_3 (−18) instead of CH_3 (−15).
[b] Loss of CD_3NC (−44) instead of CH_3NC (−41).

Table 3. Product ions generated from theobromine by means of ESI in D_2O and CAD, and ions resulting from MS^3 experiments with labelled and unlabelled theobromine

	Precursor ion *m/z*	CE (eV)	DP (V)	Product ions: *m/z* (relative abundance %)						
Theobromine (**3**) in D_2O	183	35	25	163 (18)	139 (100)	135 (13)	124 (13)	111 (46)	84 (14)	70 (11)
Theobromine (**3**) MS^3	163	20	100	148 (3)	135 (14)	122 (3)	108 (70)	94 (31)	83 (5)	67 (51)
$^2H_6$-Theobromine (**3**) MS^3	169	20	100	151 (4)	141 (17)	128 (15)	114 (45)	97 (18)	89 (5)	70 (95)
Theobromine (**3**) MS^3	138	20	100	123 (66)	110 (67)	—	97 (15)	83 (100)	69 (61)	56 (16)
$^2H_6$-Theobromine (**3**) MS^3	144	20	100	126 (68)	116 (47)	113 (15)	100 (4)	89 (100)	72 (52)	61 (19)

CE = collision energy, DP = declustering potential.

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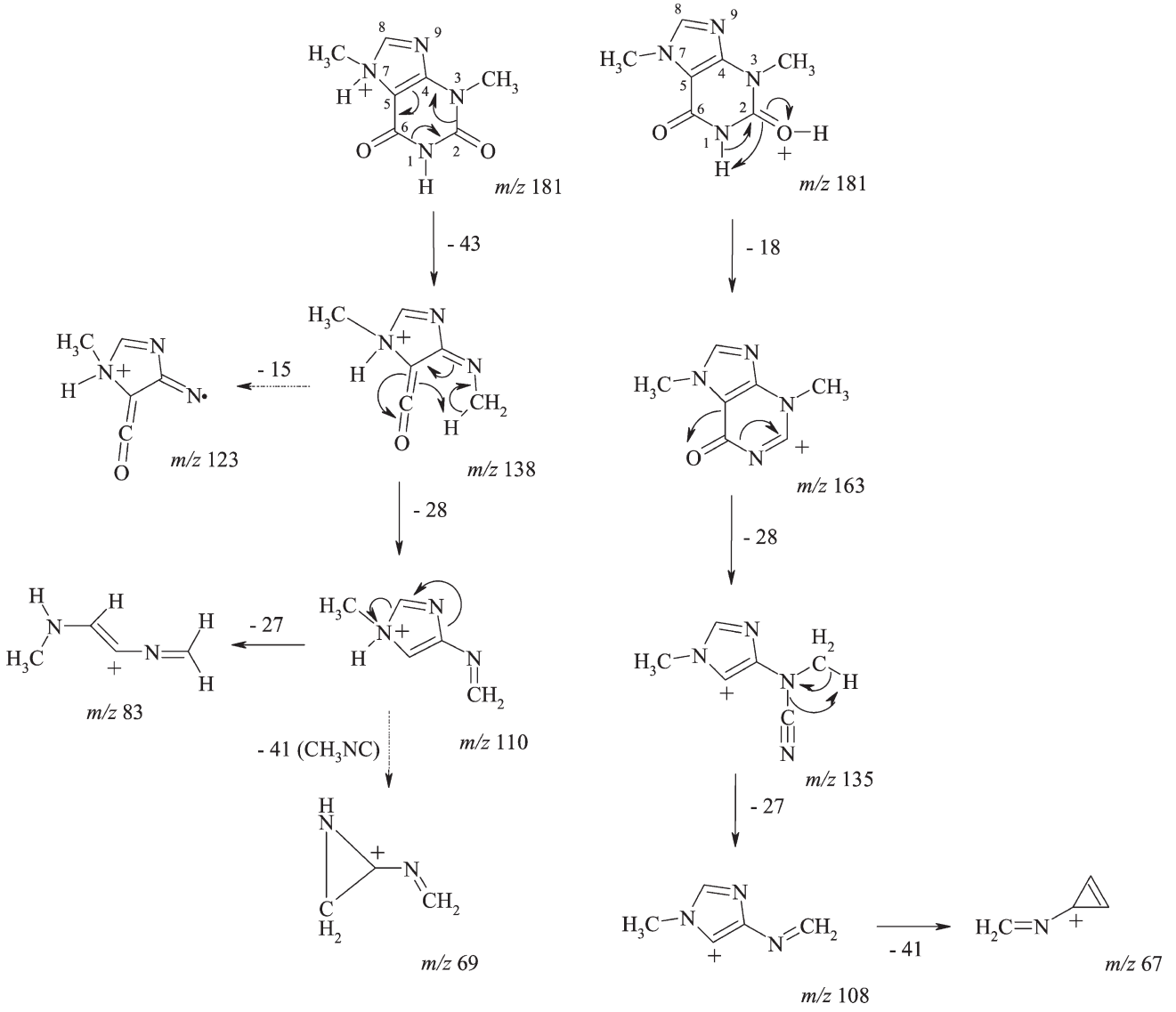
HPLC-UV analyses at 208 and 272 nm demonstrated purities of the synthesized compounds of >99%.

Studies on fragmentation pathways

Mass spectrometry can provide detailed structural information about compounds in complex mixtures such as urine, plasma, etc. Hence, several investigations have been performed to gain data on fragmentation and dissociation of caffeine and its metabolites following ionization, in particular electron ionization (EI).[4] In order to unambiguously identify caffeine (**5**) and its metabolites such as theophylline (**2**), theobromine (**3**) or paraxanthine (**4**) in a biological matrix, knowledge of their behavior after ESI and CAD is of paramount importance. Figure 1 shows the product ion spectra of (a) *m/z* 195 of caffeine, (b) *m/z* 198 of $^2H_3$-caffeine, (c) *m/z* 181 of theobromine, and (d) *m/z* 187 of $^2H_6$-theobromine; Tables 2 and 3 list common fragment ions of all investigated methylated xanthines as obtained by ESI and various mass spectrometric experiments. Methylxanthines give rise to a series of fragment ions, for which the proposed mechanisms of formation are demonstrated for theobromine as an example.

CAD of theobromine

From the protonated theobromine molecule at *m/z* 181, an initial loss of water (−18 u) or isocyanic acid (−43 u) is observed, giving rise to the product ions at *m/z* 163 and 138, respectively (Fig. 1(c)). For the latter, a rearrangement like a retro-Diels-Alder reaction is postulated enabling the elimination of isocyanic acid as presented in Scheme 2. Evidence for the presence of nitrogen N-1 in the leaving group was obtained by the analysis of the compounds **1**, **2**, **4** and **5**, all of which eliminated methyl isocyanate (−57 u) instead of isocyanic acid, producing ions at *m/z* 110 (**1**), 124 (**2** and **4**) or 138 (**5**) (Table 2). MS^3 experiments demonstrated that the fragment of theobromine at *m/z* 138 subsequently expels a methyl radical from N-3 (−15 u) generating the ion at *m/z* 123 (Table 3). The fact that only the removal of the methyl residue at N-3 is involved is based on the analysis of the triply deuterated caffeine, for which the labelling is located at its methyl group at N-7. As shown in Table 2, the ion at *m/z* 123 of **5** is shifted to *m/z* 126 indicating the presence of three deuterium atoms in the remaining fragment ion and thus proving the loss of the unlabelled methyl unit at N-3.



Scheme 2. Proposed fragmentation pathways of the protonated molecule of theobromine after ESI and CAD.

Moreover, from the corresponding fragment of the six-fold deuterated theobromine at *m/z* 144, a loss of 18 u ($-CD_3$) is observed resulting also in a product ion at *m/z* 126 (Table 2, Fig. 1(d)). In a different fragmentation pathway, *m/z* 138 eliminates carbon monoxide (−28 u) generating *m/z* 110, which is followed either by the removal of HCN (−27 u) or methyl isocyanide (CH_3NC, −41 u) to *m/z* 83 or 69, respectively, as proposed in Scheme 2. Also here, the triply deuterated caffeine provides valuable information on the elimination processes (Fig. 1(b)). In the case of the liberation of HCN, all deuterium atoms remain in the charged fragment at *m/z* 86, while the removal of methyl isocyanide results in *m/z* 69, demonstrating the loss of the deuterated methyl residue (Table 2). A different series of fragment ions is generated from MS^3 experiments on **3** by CAD of the protonated molecule *m/z* 181 and subsequently of *m/z* 163 (formed by loss of water). Here, the elimination of carbon monoxide (−28 u) followed by removal of HCN (−27 u) are predominant giving rise to *m/z* 135 and 108, respectively. Moreover, the ion at *m/z* 108 can also expel methyl isocyanate (−41 u) producing the fragment at *m/z* 67 (Fig. 1(c)). A postulated mechanism is shown in Scheme 2, which is substantiated by the corresponding fragment ions generated from *m/z* 169 of $^2H_6$-theobromine (Table 3). While the product ions at *m/z* 141 (counterpart to *m/z* 135) and 114 (counterpart to *m/z* 108) still contain all six deuterium atoms, the ion at *m/z* 70 (counterpart to *m/z* 67) contains only three deuterium atoms as it eliminates one labelled methyl residue as methyl isocyanide (−44 u).

In order to elucidate the role of the ionizing proton during fragmentation processes, ESI-MS/MS experiments on **3** were conducted in 1 mM DCl in D_2O and acetonitrile (50:50, v/v). In addition to the exchange of a labile hydrogen at N-1 by a deuterium, charge was transferred to theobromine by adding a deuteron resulting in a positively charged molecule of *m/z* 183 (Table 3). In contrast to the CAD spectrum of **3** in Fig. 1(c), elimination of D_2O was observed upon CAD of *m/z* 183 demonstrating the immediate removal of the labile hydrogen/deuterium at N-1 together with the ionizing proton/deuteron in this elimination of water. Loss of water was not observed in product ion spectra of **2** and **5**, but compound **4** showed also a minor signal corresponding to $[(M+H)^+-18]$ (Table 2), supporting the assumption that protonation of oxygen next to a nitrogen bearing a labile proton is necessary for expulsion of water from methylated xanthines.

Assay validation for qualitative and quantitative analysis of theobromine in equine urine samples

Calibration curve

A linear calibration curve was obtained for theobromine from 0.4–4.0 μg/mL with $y = 0.47x + 0.023$, and $r^2 = 0.998$. Linearity was proven by ANOVA.

Selectivity

None of the ten different blank urine specimens generated interfering peaks at the selected MS/MS transitions for theobromine (*m/z* 181–138, 181–110, 181–69) at the expected retention time. Figure 2 shows typical MRM chromatograms for theobromine in a blank (a) as well as (b) a fortified urine sample at 0.4 μg/mL, demonstrating the selectivity of the assay.

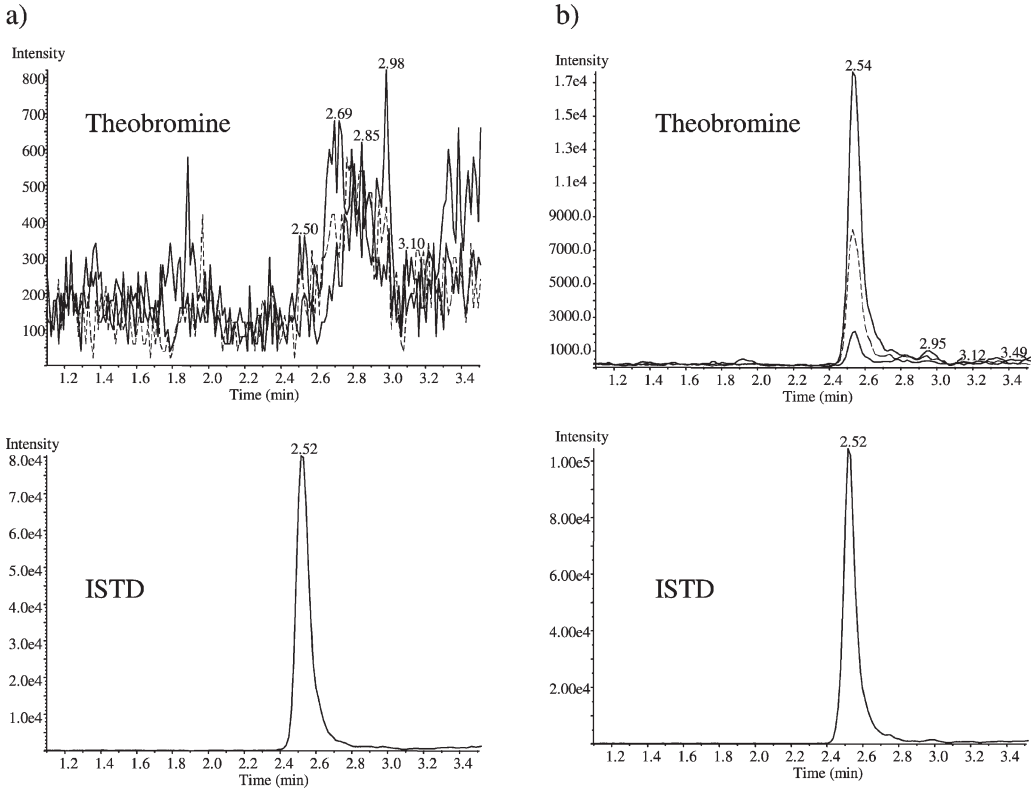


Figure 2. Extracted ion chromatograms of a blank urine specimen (a) and a urine sample fortified with 0.4 μg theobromine per mL (b), at the MS/MS transitions *m/z* 181–138, 181–110, 181–69. In all MRM chromatograms, distinct signals are absent for the blank (a) and present for the spiked (b) urine sample.

Limit of detection

Despite the fact that theobromine is prohibited in equine urine samples only when exceeding a threshold level of 2 μg/mL, the LOD of the assay presented here was determined. With urinary concentrations of theobromine of 0.1 μg/mL, S/N ratios ≥4 were obtained for all MS/MS transitions selected for qualitative evidence (*m/z* 181–138, 181–110, 181–69).

Lower limit of quantitation

Ten blank urine samples, as well as ten urine samples containing theobromine at 0.4 μg/mL, were analyzed. A S/N ratio >10 was obtained for the selected MS/MS transition at *m/z* 181–138, indicating a LLOQ of at most 0.4 μg/mL.

Recovery

The comparison of peak area ratios for urine samples fortified with theobromine prior to and after SPE demonstrated a recovery of 72% of the analyte from equine urine.

Interday precision

The assay interday precision was calculated for three concentrations at 0.4 (low), 2.0 (medium), and 4.0 μg/mL (high), with values of 9.8, 9.7, and 9.9%, respectively.

CONCLUSIONS

Isotope-dilution mass spectrometry has proven to be a reliable and powerful tool for quantitation purposes in various fields of analytical chemistry. In the present study we described a facile synthesis of triply deuterated caffeine and its six-fold deuterated metabolite theobromine, and the use of the latter for quantitative measurements of theobromine in equine urine. The established assay demonstrates robustness, and provides excellent qualitative information on the analytes of interest. Also, on the basis of elucidated dissociation behavior and fragmentation pathways of theobromine and analogues, structures of related alkylated xanthine derivatives can be investigated.

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