Research Article

Rapid Communications in Mass Spectrometry

Received: 22 October 2012 Revised: 19 January 2013 Accepted: 21 January 2013 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2013**, 27, 885–895
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6520

Collision-induced dissociation studies of caffeine in positive electrospray ionisation mass spectrometry using six deuterated isotopomers and one N1-ethylated homologue

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RATIONALE: In order to deepen the understanding of electrospray ionisation collision-induced dissociation (ESI-CID) fragmentation reactions of xanthine derivatives for the identification of metabolites using low-resolution liquid chromatography/mass spectrometry (LC/MS) analysis, basic experiments using caffeine (1,3,7-trimethylxanthine) as model compound have been performed.
METHODS: Six deuterium isotopomers and one N1-ethylated homologue of caffeine have been synthesized and their ESI fragmentation spectra have been obtained by using LC/MS in combination with either standard or perdeuterated eluent mixtures.
RESULTS: One result of these studies is the finding that the positive charges of the ESI-CID caffeine fragments are caused by the addition of protons. Furthermore, the performed experiments allow the determination of all molecular formulae of each ESI-CID caffeine fragment.
CONCLUSIONS: As basic CID reactions of caffeine have been elucidated in this work, the developed fragmentation scheme may serve as a valuable tool for the interpretation of ESI-CID fragmentation spectra of more complex xanthine derivatives and their respective metabolites.

The xanthine (3,7-dihydro-purine-2,6-dione) structure is an ubiquitous heterocyclic scaffold and has, *inter alia*, been used extensively as a lead template for the design and development of adenosine receptor ligands.[1,2] Our main research is focused on the development of new radiolabelled small molecules for the non-invasive *in vivo* imaging of adenosine receptors in the human brain using positron emission tomography (PET).[3] Some years ago we reported on the synthesis, radiosynthesis, and preclinical evaluation of the potent and selective A_1 adenosine receptor (A_1AR) antagonist 8-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine (CPFPX), and its fluorine-18 radiolabelled isotopomer [^{18}F]CPFPX.[4] CPFPX is a fluorinated bioisistere of the well-established A_1AR antagonist 1,3-dipropyl-8-cyclopentylxanthine DPCPX.[5]

The clinical routine use of this radioligand is limited by some unfavorable physiological properties, one being its liability to fast enzymatic degradation. Consequently, new projects aim at the development of 'second-generation' radioligands which should overcome the disadvantages of [^{18}F]CPFPX without concomitantly impairing the essential binding properties such as affinity and selectivity for the A_1AR subtype. Thus, the main improvement of follow-up radioligands will be a refinement of metabolic *in vivo* stability which should enhance radiotracer availability in plasma and hence brain uptake. In order to achieve this goal it is crucial to elucidate the metabolism of CPFPX and to unequivocally identify the main metabolite(s).

Because the amount of radiotracer administered to humans is so small, typically 0.5–5 nmol, or even less,[6] it is extremely difficult or even impossible to detect those compounds with the most advanced mass spectrometric (MS) methods. In order to identify metabolites of such compounds in human studies, additional steps are required.

Those radiolabelled – nearly massless – compounds are analytically accessible only by radio-chromatographic methods. Chromatographic comparison of radioactive metabolites from human blood with compounds generated by a suitable model system (e.g. *in vitro* metabolites by human liver microsomes (HLM)) give a first indication of their chemical identity. Compounds obtained from a suitable model system can be further analysed applying high-performance liquid chromatography (HPLC)/MS techniques.

Our previous work has demonstrated that spectra of simple collision-induced dissociation (CID) fragmentation reactions principally allow to obtain reliable information about the identity of metabolites *without* using a collision chamber.[7] The fragmentation scheme used to obtain structural information about the metabolites of CPFPX was derived from MS spectra of reference compounds CPFPX and DPCPX. An elaborated study could reveal that the chemical structure of the main metabolite of CPFPX in

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human blood, tentatively identified by HPLC/MS, is in full agreement with that of a reference compound obtained via a multistep organic synthesis.[8]

From a retrospective point of view the identification of metabolites originating from the synthetic xanthine CPFPX should theoretically have been possible with the knowledge extracted from existing publications which deal with various MS-fragmentation mechanisms of caffeine.[9–13] However, the published experiments were performed either by using advanced spectrometers in combination with additional ionisation modes like collision-activated dissociation (CAD) or by *a priori* using different ionisation methods like electron ionisation (EI). It seemed not reasonable to us to directly translate the published results into simple CID fragmentation.

It cannot be excluded that, depending on the particular fragmentation mode (CID or collision chamber), fragments having identical *m/z* values but belonging to different chemical species (radicals or ions), may be obtained. Thus, the aim of the present study was to investigate the ESI fragmentation of caffeine as a reference xanthine in deeper detail. From our point of view it was most likely that the results obtained with caffeine might help to elucidate and understand the fragmentation pathways of other synthetic xanthines.

Regardless of the successful results of the studies mentioned above a more general fragmentation scheme for chemically different xanthines would be desirable. Such a tool could be helpful to minimise time-consuming and cost-intensive preparative organic syntheses of putative metabolites.

In order to expand the scope in the present work six differently deuterated isotopomers (**2–7**) and one N-ethylated derivative (**8**) of caffeine (**1**) have been synthesized with two derivatives, namely **6** and **7**, being new compounds (Fig. 1). By performing HPLC/MS experiments using those derivatives **2–7** in combination with deuterated eluents and computer-based analyses it has been possible to clearly determine the molecular formulae of all caffeine fragments found by low-resolution CID. One recently published study used a collision fragmentation cell and a deuterated caffeine isotopomer for the clarification of possible fragmentation reactions and was the motivation for the present work.[12]

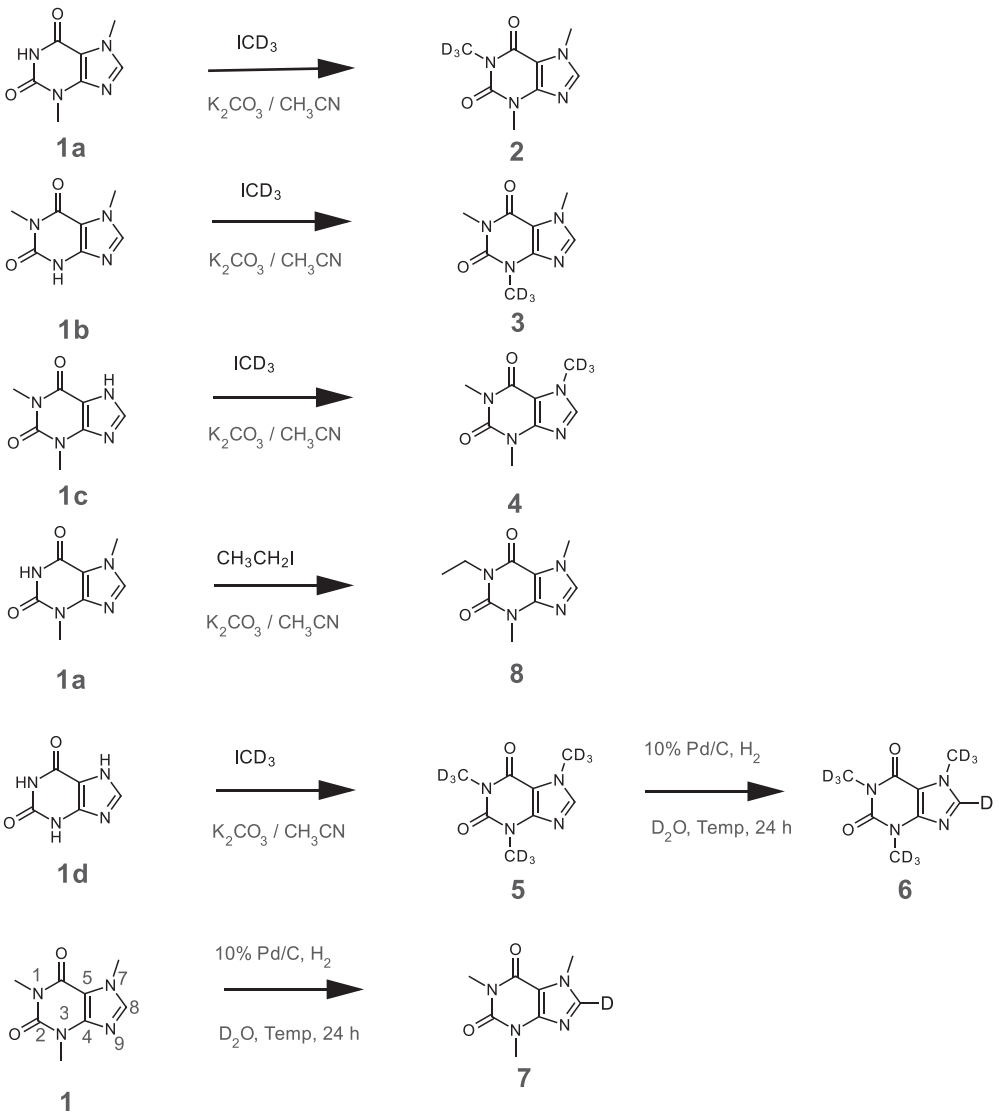

Figure 1. Chemical syntheses of caffeine isotopomers **1–7**, and N3-ethyl caffeine derivative **8**.

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EXPERIMENTAL

General

Methanol, water and caffeine (**1**) were purchased from Merck (Darmstadt, Germany) and all other solvents and reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany). Solvents and reagents in the highest state of purity were used as supplied by the vendors.

Melting points were measured on a B 545 melting point apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected. ^{1}H-NMR and proton-decoupled ^{13}C-NMR spectra (given in Tables 1 and 2, respectively) were obtained in $CDCl_3$ at 200.13 and 50.32 MHz, respectively, by means of a Bruker DPX-200 Avance instrument (Bruker Bio Spin GmbH, Rheinstetten, Germany) in 5% solution at 25°C. All shifts are given in δ ppm and are referenced to tetramethylsilane (TMS) as an internal standard (δ = 0 ppm). Coupling constants *J* are given in Hertz. Protons were assigned with the aid of COSY, and carbons with the aid of DEPT and HMQC experiments. The multiplicity symbols s and d refer to singlet and doublet, respectively.

High-performance liquid chromatography

Analysis of caffeine analogues employed a Kromasil 100-5 C 18 column (250 × 4.6 mm; CS-Chromatographie Service GmbH, Langerwehe, Germany) in a HPLC system consisting of a WellChrom K-1001 pump (Knauer, Berlin, Germany), a K-2001 UV detector (Knauer) and a manual sample injector (type 7125; Rheodyne, Bensheim, Germany) fitted with a 500 µL sample loop. Isocratic elution with water/methanol/acetic acid (30/70/0.2 v/v/v) was at a flow rate of 1 mL/min and UV monitoring at 275 nm.

For HPLC/MS studies a Kromasil 100-5 C 18 column (250 × 4.6 mm; CS-Chromatographie Service GmbH, Langerwehe, Germany) and either the eluent specified above or the respective perdeuterated eluent (D_2O/methanol-d_4/acetic acid-d_4, 30/70/0.2 (v/v/v)) at a flow rate of 0.8 mL/min was used.

Mass spectrometry

For HPLC/MS measurements the outlet of the UV detector was coupled to a mass spectrometer (Surveyor MSQ; Thermo Fisher Scientific GmbH, Dreieich, Germany) with an electrospray interface. Nebuliser gas pressure was 4 bar and desolvation temperature was 575°C. The sprayer voltage and the cone voltage were 3000 and 80–100 V, respectively. For the breakdown graph, cone voltages of 20, 40, 60, 70, 80, 90, 100 and 110 V were used. Positive ion spectra were recorded over a *m/z* range of 1–298 at a scan time of 2 s. The Xcalibur® software (version 1.4) provided with the instrument permitted scans of the chromatograms for ions of a desired *m/z* over the range of 50–250.

For analyses of the synthesized compounds the same parameters were used but the cone voltage was set to 20 V.

General procedure for the synthesis of mono-trideuteromethyl caffeine isotopomers 2–4 and N1-ethyl caffeine 8 (Fig. 1)

Deuteromethylation of 3,7-dimethylxanthine (theobromine, **1a**), 1,7-dimethylxanthine (paraxanthine, **1b**), 1,3-dimethylxanthine (theophylline, **1c**), and xanthine (**1c**) was performed according to a classical alkylation reaction described using an alkyl halide in the presence of a base.[12,14]

Table 1. ^{1}H-NMR spectral data of compounds **1–7** (δ-values in ppm)

cpd	N1-Me	N3-Me	N7-Me	H8
1	3.41 (s)	3.59 (s)	4.00 (d, $^{4}J_{H7-H8}$ = 0.6 Hz)	7.53 (d, $^{4}J_{H8-H7}$ = 0.6 Hz)
2	*absent*	3.61 (s)	4.02 (s)	7.53 (s)
3	3.43 (s)	*absent*	4.01 (d, $^{4}J_{H7-H8}$ = 0.59 Hz)	7.54 (s)
4	3.43 (s)	3.61 (s)	*absent*	7.55 (s)
5	*absent*	*absent*	*absent*	7.55 (s)
6	*absent*	*absent*	*absent*	*absent*
7	3.42 (s)	3.60 (s)	4.01 (s)	*absent*

Table 2. Proton decoupled ^{13}C-NMR spectral data of compounds **1–7** (δ-values in ppm)

cpd	N1-Me	N3-Me	N7-Me	C2	C4	C5	C6	C8
1	28.32	30.15	34.00	152.11	149.07	107.99	155.81	141.78
2	*27.26* *	30.16	34.01	152.13	149.09	108.02	155.85	141.76
3	28.34	*29.18**	34.04	152.12	149.00	108.01	155.83	141.72
4	28.35	30.21	*32.97**	152.12	149.03	108.00	155.83	141.71
5	*27.28* *	*29.19**	*32.98**	152.13	149.03	108.01	155.85	141.76
6	*27.34* *	*29.16**	*32.96**	152.12	149.06	108.00	155.84	*141.19***
7	28.34	30.17	34.00	152.13	149.07	107.97	155.84	*141.17***

*septet, $^{1}J_{C-D}$ = 21.2 Hz,
**triplet, $^{1}J_{C-D}$ = 33.6 Hz

Under the exclusion of moisture the respective xanthine was suspended in dry acetonitrile (7 mL/mmol), dry potassium carbonate (K_2CO_3, 1.2 eq.) was added, and the suspension stirred at 80°C for 30 min. After the addition of iodomethane-d_3 (CD_3I, 1.2 eq.) the mixture was refluxed for the time specified, cooled to ambient temperature, filtered through a 0.2 µ filter, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in an aqueous solution of sodium chloride (1 g) and K_2CO_3 (100 mg) in H_2O (100 mL)[12] and the aqueous solution was extracted three times with chloroform. The combined organic extracts were evaporated to dryness *in vacuo* and the obtained product recrystallized from ethanol.

1-Trideuteromethyl-3,7-dimethylxanthine, N1-d_3-caffeine, ***2***

3,7-Dimethylxanthine **1a** (532 mg, 2.94 mmol) in CH_3CN (20 mL), K_2CO_3 (490 mg, 3.53 mmol and CD_3I (511 mg, 219 µL, 3.53 mmol), reaction time 3 h, crude yield 835 mg, dry $CHCl_3$ extract yield 434 mg, recrystallized yield 303 mg (52.4%), mp 235.4°C. HPLC: k′ 2.278, purity 99.3%. MS (ESI) calc. for $C_8H_7D_3N_4O_2$, exact mass 197.10, *m/z* 198.1 $[M+H]^+$.

3-Trideuteromethyl-1,7-dimethylxanthine, N3-d_3-caffeine, ***3***

1,7-Dimethylxanthine **1b** (485 mg, 2.7 mmol) in CH_3CN (20 mL), K_2CO_3 (442 mg, 3.2 mmol) and CD_3I (463 mg, 199 µL, 3.2 mmol), reaction time 3 h, crude yield 767 mg, dry $CHCl_3$ extract yield 362 mg, recrystallized yield 268 mg (50.5%), mp 236.3°C. HPLC: k′ 2.264, purity 99.2%. MS (ESI) calc. for $C_8H_7D_3N_4O_2$, exact mass 197.10, *m/z* 198.0 $[M+H]^+$.

7-Trideuteromethyl-1,3-dimethylxanthine, N7-d_3-caffeine, ***4***

1,3-Dimethylxanthine **1c** (563 mg, 3.1 mmol) in CH_3CN (20 mL), K_2CO_3 (514 mg, 3.7 mmol) and CD_3I (540 mg, 232 µL, 3.7 mmol), reaction time 5.5 h, crude yield 913 mg, dry $CHCl_3$-extract yield 466 mg, recrystallized yield 376 mg (61.6%), mp 236.4°C. HPLC: k′ 2.256, purity 98.4%. MS (ESI) calc. for $C_8H_7D_3N_4O_2$, exact mass 197.10, *m/z* 198.1 $[M+H]^+$.

3,7-Dimethyl-1-ethyl-xanthine, N1-ethyl-caffeine, ***8***

3,7-Dimethylxanthine **1a** (400 mg, 2.2 mmol) in CH_3CN (20 mL), K_2CO_3 (400 mg, 2.9 mmol) and C_2H_5I (530 mg, 274 µL, 3.4 mmol), reaction time 15 h, dry $CHCl_3$-extract yield 130 mg, recrystallised yield 15 mg (3.2%), mp 164.7°C. HPLC: purity 98.32%. ^{1}H-NMR ($CDCl_3$): 1.25–1.32 (t, 3H, $N^3CH_2CH_3$), 3.60 (s, 3H, N^7CH_3), 4.02 (s, 3H, N^7CH_3), 4.06–4.17 (m, 2H, $N^1CH_2CH_4$), 7.52 (s, 1H, C^8H). MS (ESI) calc. for $C_9H_{12}N_4O_2$, exact mass 208.1 *m/z* 209.02 $[M+H]^+$.

1,3,7-Tri-trideuteromethylxanthine, caffeine-d_9, ***5***

To a stirred suspension of xanthine **1d** (517 mg, 3.83 mmol) in CH_3CN (25 mL) was added K_2CO_3 (700 mg, 5.07 mmol) and the mixture was stirred at 70°C for 1.5 h. After the addition of CD_3I (1.5 g, 10.3 mmol) the mixture was refluxed for 4 h and an additional amount of CD_3I (1.5 g, 10.3 mmol) was added. After 4 h the temperature was set to 40°C and stirring was continued for 12 h. The mixture was filtered through a 0.2 µ filter and the filtrate was evaporated to dryness *in vacuo*. The solid residue (889 mg) was dissolved in a solution of sodium chloride (1 g) and K_2CO_3 (100 mg) in H_2O (100 mL), and the solution was extracted three times with chloroform. Evaporation of the combined organic extracts to dryness gave a solid residue (243 mg) which was recrystallized from ethanol to furnish 155 mg (20%) of product, mp 236.1°C. HPLC: k′ 2.22, purity 99.05%. MS (ESI) calc. for $C_8HD_9N_4O_2$, exact mass 203.14, *m/z* 204.16 $[M+H]^+$.

General procedure for the deuteration of carbon-8 in caffeines via palladium-catalyzed H–D exchange reaction[15,16]

8-Deutero-1,3,7-tri-trideuteromethylxanthine, caffeine-d_{10}, ***6***

At ambient temperature caffeine-d_9 **5** (240 mg, 1.17 mmol) was dissolved in D_2O (15 mL) and 10% Pd/C (24 mg) was added. A gentle hydrogen flow was bubbled for 60 s through the solution. The flask was tightly stoppered and the contents stirred at 80°C for 70 h. The reaction mixture was filtered through a 0.2 µ filter and the filtrate evaporated to dryness *in vacuo* to yield 218 mg (90.8%) of product, mp 237.8°C. HPLC: k′ 2.18, purity 99.5%.

8-Deutero-1,3,7-trimethylxanthine, caffeine-d_1, ***7***

At ambient temperature 10% Pd/C (26 mg) was added to a stirred solution of caffeine **1** (264 mg, 1.36 mmol) in D_2O (15 mL). At that temperature a gentle flow of hydrogen was bubbled through the solution for 60 s. The flask was tightly stoppered and the contents stirred at 90°C for 1.5 h. After cooling to ambient temperature the reaction mixture was filtered through a 0.2 µ filter and the filtrate was evaporated to dryness *in vacuo* giving 241 mg (86.9%) of analytically pure product as colourless crystals, mp 236.2°C. HPLC: k′ 2.27, purity 98.7%. MS (ESI) calc. for $C_8H_9D_1N_4O_2$, exact mass 195.09, *m/z* 196.09 $[M+H]^+$. MS (ESI) calc. for $C_8D_{10}N_4O_2$, exact mass 204.17, *m/z* 205.18 $[M+H]^+$.

NMR spectra of compounds 1–7

The ^{1}H and ^{13}C NMR spectra of compounds **2** to **7** were identical to an authentic standard of unlabeled caffeine **1**, differing only as italicized due to the incorporation of deuterium. The ^{1}H-NMR and proton decoupled ^{13}C-NMR spectral data of compounds **1–7** are given in Tables 1 and 2, respectively.

Computer calculations

An in-house developed computer program (Listing 1) written in Python[17] (version 2.6). Python runs on Windows, Linux/Unix, Mac OS X, and has been ported to the Java and. NET virtual machines. It is free to use, even for commercial products, because of its open source license. The script calculated possible compositions of fragment ions $[F+1]^+$ of given masses taking into account only the elements in the molecular formula of caffeine. The number of 'hits' per fragment are summarized in Table 3. If a composition of elements agrees with the mass of a calculated fragment (F_c) the 'double-bond equivalents' (DBE) parameter was computed.

RESULTS AND DISCUSSION

The use of isotopomers for the structural analysis of fragments obtained in mass spectrometry has been established for a long time.[18] Thus, specifically for this

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Listing 1

```
#!/usr/bin/env python

#user input
FragmentMass   =110  # ms - fragment
HydrogenExtra  =0    # additional allowed hydrogen atoms

# constants for caffeine
Carbon = 8; Hydrogen = 10; Nitrogen = 4
Oxygen = 2; Dezi = 1.00

# molecular mass of parent compound
MolecularMass  = Carbon*12 + Hydrogen + Nitrogen*14 + Oxygen*16

while True:
    FragmentMass = input ('Mass of fragment(<return> = end): ')
    if FragmentMass <= 0:
        break

    #program-variables
    NumSolutions = 0       # number of solutions
    Parameter=0            # double bond equivalents
    Possibilities=0        # number of possible outcomes
    IonType='M'            # M=normal molecule, R=radical

    # control variables of atoms in fragment
    Ci = 0; Hi = 0; Ni = 0; Oi = 0; Mass = 0

    for Ci in range (0,Carbon+1):
        for Hi in range (0, Hydrogen + HydrogenExtra +1):
            for Oi in range (0, Oxygen +1):
                for Ni in range (0, Nitrogen +1):
                    Possibilities=Possibilities+1
                    if  (Ci * 12 + Ni * 14 + Oi*16 + Hi +1) == FragmentMass:
                        NumSolutions = NumSolutions + 1;
                        #Calculation: Degree of unsaturation
                        Parameter = (Ni * 15) + (Ci * 14) + (Oi * 16) +2 - (FragmentMass-1)
                        if ((Parameter % 2) == 0) and (Parameter/2 >= 0):
                            IonType = 'M'
                        else:
                            IonType = 'R';
                        print 'IonType: %s / C: %d H: %d N: %d O: %d //Parameter: %.1f' %\
                              (IonType, Ci,Hi, Ni,Oi, Parameter/2.)
    #Output
    print  '************************************************'
    print  'Molecular Mass: %d' % (MolecularMass)
    print  '[M+H]+: %d' % (MolecularMass + 1)
    print  'ESI - fragment found [M+H]+: %d' % FragmentMass
    print  '************************************************'
    print 'Possibilities: %d' %(Possibilities)
    print 'NumSolutions: %d' %(NumSolutions)
    print '************************************************'
    print

#end
```

purpose, ^{15}N-isotopomers of caffeine have already been synthesized for the structural elucidation of its fragments obtained in EI-MS experiments.[19]

For the investigation of the fragmentation chemistry of xanthine derivatives in the ESI-MS mode, in addition to other unlabeled xanthines, a deuterated isotopomer of caffeine has been described and examined.[12] For fragmentation experiments the authors of this work have used a collision cell; however, such a cell is not available in small and simple mass spectrometers. Commonly used machines for routine MS provide the possibility to obtain fragments exclusively by increasing the cone voltage to achieve a so-called 'cone voltage collision-induced dissociation' (CID).

Some comparative studies show that many similarities exist between CID fragments and those which are formed in a collision chamber, particularly when comparing fragmentations of pure protonated molecules.[20] Spectra obtained from preliminary CID fragmentation experiments with caffeine revealed a very close similarity to a published spectrum whose fragments had been generated by means of a collision

Table 3. Approach for the determination of the molecular formulae of fragments **a–g** using information from Table 1 and the Python script. For structures of **a–g**, see Fig. 8

		Step 1	Step 2		Step 3			
Fragment	*m/z*	Number of hits	Number of H in fragment	Number of solutions with steps 1 and 2	Number of N	Number of solutions with step 3	Elemental composition	Type $F=[M+H]^+$ $R^{\cdot}=[F^{\cdot}+H]^+$
a	195	***1***	10	-	-	-	$C_8H_{10}N_4O_2$	F
b	181	***2***	8	***1***	$N \geq 3$	***1***	$C_7H_8N_4O_2$	F
c	138	***9***	7	***2***	$N \geq 3$	***1***	$C_6H_7N_3O$	F
d	123	***13***	4	***2***	$N \geq 3$	***1***	$C_5H_4N_3O$	$R^{\cdot}$
e	110	***12***	7	***2***	$N \geq 2$	***1***	$C_5H_7N_3$	F
f	83	***14***	6	***2***	$N \geq 2$	***1***	$C_4H_6N_2$	F
g	69	***12***	4	***2***	$N \geq 1$	***1***	$C_3H_4N_2$	F

chamber.[12] Intrigued by this study, it was our aim to gain a deeper insight into the pure CID fragmentation chemistry of xanthine derivatives. Using unlabelled caffeine as a model substance we have additionally synthesized a number of deuterated caffeine isotopomers and one caffeine derivative bearing an ethyl instead of a methyl substituent at position 3 of the xanthine scaffold.

Syntheses

Several synthetic routes for the preparation of deuterated caffeine derivatives have been described in the literature.[12,14,21,22]

The syntheses of the 1-, 3-, and 7-mono-trideuteromethyl isotopomers **2**, **3**, and **4**, the 1,3,7-tris-trideuteromethyl isotopomer **5** of caffeine and the N-3-ethyl derivative **7** (Fig. 1) were carried out using modified literature procedures. In order to obtain the C-8 deuterated caffeine isotopomer **7**, various described synthetic routes were investigated.[23,24]

It appeared, however, that high reaction temperatures and long reaction times, prerequisite conditions for a sufficiently high degree of C-H/C-D isotopic exchange, resulted in poor deuterium incorporation and led to products of low purity. Moreover, uncatalysed reactions in D_2O at 100°C for more than 10 h did not afford the target compound **9** in a satisfactory product purity.[25]

Finally, a recently published isotopic exchange method was adopted, namely the heterogeneous Pd-catalysed reaction of a solution of caffeine in a D_2O medium previously saturated with hydrogen.[15] The Pd-catalysed exchange method was also used to obtain the perdeuterated caffeine isotopomer **6** from compound **5**.

All resulting products were analysed by ^{1}H-NMR, ^{13}C-NMR, ESI-MS and HPLC and contained no or maximal 2% measurable impurities as determined by HPLC.

ESI–MS experiments

General experiments

Breakdown graphs. Figure 2 shows a typical ESI mass spectrum of caffeine **1** which was obtained at a cone voltage of 80 V. The fragmentation pattern is consistent with that of earlier published spectral data.[9,12] Considerations about the formation, the composition, and the chemical structures of the obtained fragments are the subject of the following discussion (*vide infra*).

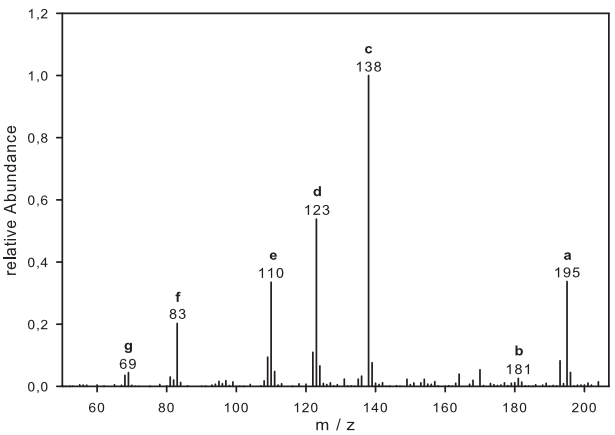

Figure 2. ESI-CID ion spectrum of caffeine **1** at a cone voltage of 90 V.

Breakdown graphs are established tools for the interpretation of CID fragmentation processes.[26,27] They display the percentage of a certain fragment population as a function of the cone voltage and allow the interpretation of the genesis and formation of the respective fragments. For caffeine no fragments could be observed up to 40 V but raising the cone voltage to about 70 V led to fragmentation. A further increase of the cone voltage to 80 V caused the formation of all fragments (Fig. 2); the respective breakdown graph of caffeine is shown in Fig. 3.

Ion species

In order to obtain information about the nature and identity of the individual ion species formed in the fragmentation experiments, a perdeuterated eluent with a deuteration grade of 99.98% was used. In the past, deuterated eluents have been used in mass spectrometric experiments to detect labile protons such as hydrogen atoms bound to heteroatoms, for example in amino alcohols.[28,29]

Although there are no labile hydrogens in the parent compound **1**, this technique was used in the present study in order to obtain more detailed information about the nature of the detected ions. As shown in Fig. 4, using perdeuterated eluents, the *m/z* values of fragments **a**, **c**, **d**, **e**, **f**, and **g** increased by one *m/z* value compared to those (Fig. 2)

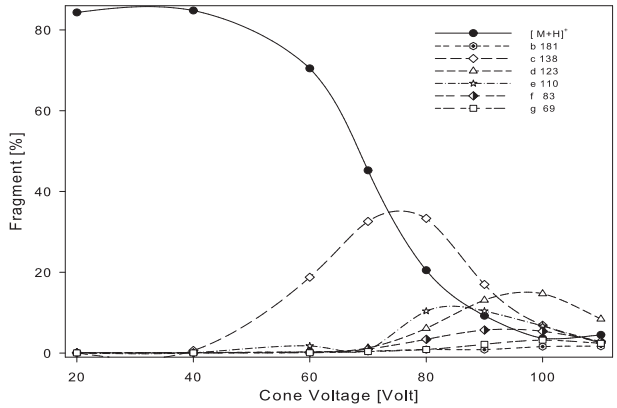

Figure 3. Breakdown graph of caffeine **1**.

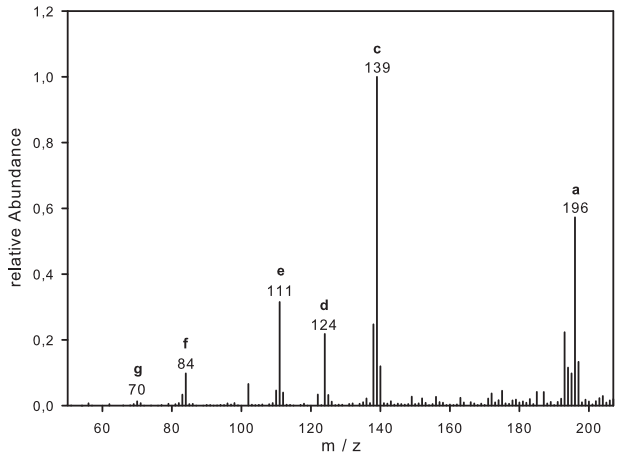

Figure 4. ESI-CID ion spectrum of caffeine **1** at a cone voltage of 90 V using a deuterated eluent system.

obtained using 'normal' hydrogen-containing eluents. This finding suggests that the charge of these fragments (F) results from addition of a deuteron and that these species can formally be written as protonated molecules such as $[F+D]^+$.

Number of hydrogen atoms

To obtain further information about the chemical nature of the observed fragments, a caffeine isotopomer was synthesized in which all hydrogen atoms are substituted by deuterium atoms (caffeine-d_{10}, **6**). The difference between the *m/z* values of the perdeuterated analogue **6** (Fig. 5) and the *m/z* values of the corresponding fragments of 'normal' caffeine **1** (Fig. 2) furnishes the number of hydrogen atoms in a given fragment. In Fig. 5 these values are tagged with a delta.

Remaining nitrogen atoms

Further fragmentation analysis of the synthesized isotopomers gives evidence about the remaining nitrogen atoms depending on the methyl groups detectable in a fragment. This information can easily be obtained, since the three possible monotrideuteromethyl derivatives **2**, **3**, and **4** (Fig. 1) had been synthesized for the present work. The results of these experiments are summarized in Table 4. The delta values are the differences between the *m/z* values of deuterated fragments and those of the corresponding values of native caffeine **1**

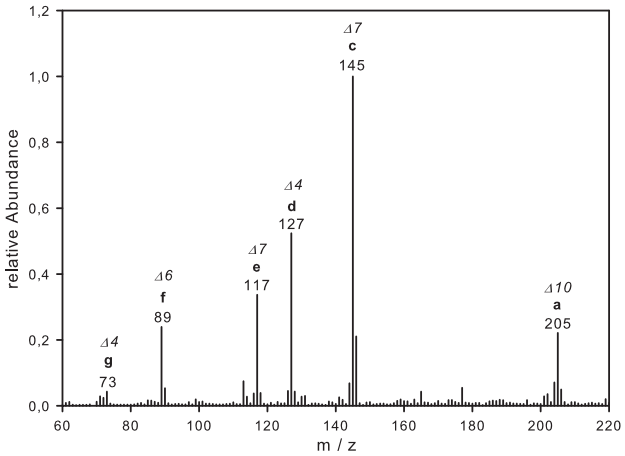

Figure 5. ESI-CID ion spectrum of caffeine-d_{10} **6** at a cone voltage of 90 V using a normal eluent system. Delta values indicate the differences in *m/z* values in comparison to those in Fig. 1.

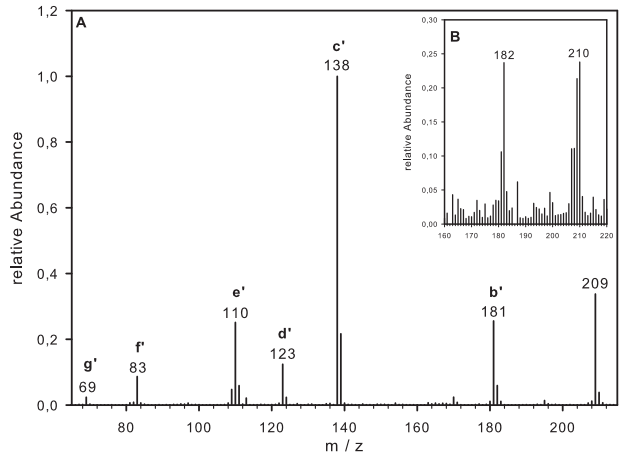

Figure 6. ESI-CID ion spectra of N3-ethyl caffeine **8** at a cone voltage of 80 V using (A) a normal eluent system and (B) a deuterated eluent system.

(Fig. 2). It can be supposed that a nitrogen atom is still present in a fragment if the corresponding (bound) methyl group can be detected. The minimum number of nitrogen atoms in a given fragment should therefore at least equal the number of methyl groups still present in the molecule.

Molecular formulae

With this information about the individual fragments (summarized in Table 3) it is possible to calculate the molecular formula of a particular fragment and to clearly determine whether this species is a radical or a saturated protonated molecule.

For this purpose, a simple Python (2.7) script was written. Python has the advantages of being open-source, is available for all relevant computer platforms and forces a human-readable source code. The execution speed of the interpreter language is sufficiently fast on modern personal computers and has already been used in the past for mass spectrum analysis.[30]

Table 4. Peaks of fragments resulting from ESI CID spectra of caffeine isotopomeres. Delta values indicate the difference between *m/z* values of one given fragment and the *m/z* value of the corresponding caffeine peak in column 1. For structures of compounds **1–7**, see Fig. 1

	Caffeine (1)	N1,3,7-tris-deuteromethyl (5)	N1d3 (2)	N3d3 (3)	N7d3 (4)	C8d1 (7)	D10 (6)	Caffeine with perdeuterated eluent
a	**195**	204	198	198	198	196	205	196
		Δ 9	Δ 3	Δ 3	Δ 3	Δ 1	Δ 10	Δ 1
b	**181**	187 (vw)	184 (vw)	181 (vw))	181 (vw)	182 (vw)	n.d.	n.d.
		Δ 6	Δ 3	Δ 0	Δ 0	Δ 1		
c	**138**	144	138	141	141	139	145	139
		Δ 6	Δ 0	Δ 3	Δ 3	Δ 1	Δ 7	Δ 1
d	**123**	126	123	123 (126)$^{15\%}$	126 (123)$^{15\%}$	124	127	124
		Δ 3	Δ 0	Δ 0 (Δ 3)	Δ 3 (Δ 0)	Δ 1	Δ 4	Δ 1
e	**110**	116	110	113	113	111	117	111
		Δ 6	Δ 0	Δ 3	Δ 3	Δ 1	Δ 7	Δ 1
f	**83**	89	83	86	86	83	89	84
		Δ 6	Δ 0	Δ 3	Δ 3	Δ 0	Δ 6	Δ 1
g	**69**	72	69	72	69	70	73	70
		Δ 3	Δ 0	Δ 3	Δ 0	Δ 1	Δ 4	Δ 1

In a nested loop construction starting with the maximum number of available atoms of each element all possible compilations of molecular compositions are calculated. The results are summarized in Table 3 (step 1) which shows the number of hits for each fragment **a–g**.

The next step to reduce this number is to consider the number of hydrogen atoms in the fragment, which is obtained by comparing the spectra of unlabelled caffeine **1** and caffeine-d_{10} **6**. These results are listed in Table 4.

Taking into account the minimum number of available nitrogen atoms for a given fragment, it can be deduced that only one possible elemental composition exists for the respective fragment (Table 3, step 3).

Double-bond equivalents

Finally, further consideration of possible 'double-bond equivalents' is helpful for the decision whether a given fragment is an unsaturated radical or a saturated protonated molecule. This parameter is also calculated by the Python script.

The total number of rings and sites of unsaturation can be calculated from any elemental composition by using the following formula[31]:

$$D = 1 + 0.5 \cdot \sum_{i}^{i_{max}} N_i \cdot (V_i - 2)$$

where D is the unsaturation, i_{max} is the total number of different elements in the composition, N_i the number of atoms of element i, and V_i is the valence of atom i.

The conclusions that can be drawn from the experiments of the present work compared to the ESI-CID fragmentation processes of caffeine are summarized in Fig. 8. Although the chemical structures of the fragments are only postulated, both their molecular formulae and the origin of the atoms from the parent caffeine molecule are strongly supported by the presented arguments.

Fragmentation mechanisms

Fragment ***b***

Although fragment **b** does not provide an unambiguous signal (Fig. 2), it is most likely formed by a demethylation reaction followed by subsequent addition of hydrogen $[M–CH_3+2H]^+$. In order to verify this hypothesis, compound **8**, an N1-ethylated homologue of caffeine, was synthesised and used for further MS investigations.

In contrast to peak **b** in the caffeine spectrum (Fig. 2) the corresponding peak **b′** in the mass spectrum of **8** at *m/z* 181 (Fig. 6(A)) appears as a prominent peak. In the presence of a deuterated eluent, peak **b′** of homologue **8** appears at *m/z* 182 (Fig. 6(B)). This indicates that the fragmentation process of the ethyl group takes place via an ethyl/hydrogen exchange reaction and that addition of a positively charged deuteron (D^+), absorbed from the eluent, is responsible for the positive charge of the resulting ion.

The observed *m/z* value of 182 raises the question on the origin of the hydrogen atom since exclusive homolytic cleavage of the ethyl group and subsequent addition of a positive deuteron would lead to a fragment with an *m/z* value of 181. Obviously, addition of a hydrogen atom or radical to the initially formed desethyl radical must occur after the homolytic cleavage of the ethyl group. However, the addition of a small charged particle, e.g. a proton, would decrease the *m/z* value to 180. The source of the hydrogen atom is not the deuterated eluent, because these experiments exclusively led to a fragment having an *m/z* value of 182. The above findings and considerations lead to the conclusion that the parent molecule **8** itself acts as a hydrogen donor during the fragmentation.

As shown in Fig. 7, an increase in the cone voltage leads to a relatively increased appearance of ions of the type $[M+H]^{+1}$ – n (n = 1 … 4). Assuming that the molecules were completely isolated in a vacuum, the hydrogen transfer could only take place by an *intramolecular* process that would give rise to a *m/z* value of 180 ($[M+H-H-CH_2CH_3]^{+1}$).

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Therefore, it seems to be reasonable to postulate that the hydrogen transfer occurs in an *intermolecular* fashion, but this transfer is unlikely to take place between spatially separated and hence isolated structures. This type of fragments will hereinafter be referred to as 'wet fragments', because the particle transfer must take place in a matrix.

Fragment ***c***

This fragment arises directly from the parent substance **a** by a neutral loss of methyl isocyanate (CH_3NCO). In the literature, the formation of this fragment is explained by a retro-Diels-Alder reaction and the resulting molecule is consequently presented as an open-chain structure.[10,12,19] Taking into account the calculated empirical formula $C_6H_7N_3O$ (Table 3) and the calculated number of 'double-bond equivalents' a cyclic β-lactam structure for fragment **c** seems to be feasible.[9] Except for fragment **b** all fragments observed directly or indirectly arise from fragment **c**.

The first fragmentation reaction (**a** to **c**) leads to the elimination of the N1 substituent of the xanthine core; the same is true for more complex substituted xanthines.

Fragment ***d***

All fragment peaks found are isomerically pure except peak **d** in Table 4 (*m/z* 123) which is a mixture of two isomers. This peak splits into two different fractions which are visible in the spectra of the isotopomers N3-d_3-caffeine, **3**, and N7-d_3-caffeine, **7**. Obviously, these fragments differ in the position of one methyl group.

The results clearly show (Table 3) that only peak **d** with *m/z* 123 is a protonated odd radical, which can be observed at cone voltages higher than 70 V and therefore it is an example for an exception of the 'Even-Electron Rule'.[32] Looking at the postulated fragmentation pathway for caffeine (Fig. 8) it becomes clear that the radical species **d** could arise either from the fragment **b** or fragment **c**. During the formation of fragment **d** no addition of hydrogen is observable as is the case in the reactions leading to the fragment with *m/z* 182 in Fig. 6(B) (*vide supra*). Obviously, these radical-forming reactions take place when a molecule has already travelled a longer distance and is spatially isolated so that there are actually no more solvent molecules which can act as a hydrogen transfer matrix. In the present work these fragments are referred to as 'dry fragments'.

Fragment ***e***

During the formation of all other fragments, as shown by **c** in Fig. 8, ESI fragmentation processes may be explained by bond scission of carbon–heteroatom bonds; only the reaction of fragment **c** to form fragment **e** involves a carbon–carbon bond scission with the elimination of carbon monoxide. One possible explanation for this exception is the existence of a very unstable synthon.

Indeed it is known from the literature that the parent gamma-delta unsaturated β-lactam of fragment **c**, namely *N*-methylazetinone **cb**, has an extremely limited thermal stability.[33,34] Those authors claim that lactam **cb** exists in two tautomeric forms, in the cyclic β-lactam form **ca** and in the (preferred) open-chain imino ketene form **cb**. The relief of ring strain together with a low activation energy associated with such a transformation are sufficient to account for the conversion of **ca** into **cb**.[34]

The structure of fragment **e** shown in Fig. 8 is consistent with the results shown in Table 3. Fragment **e** contains the two methyl groups stemming from nitrogens N3 and N7 and the hydrogen atom from C8 of the original molecule **a**.

Fragments ***f*** *and* ***g***

Fragments **f** and **g** can result from the decay of the reactive fragment **e** after neutral loss of HCN (**f**) or methyl cyanide (**g**), respectively. It is clearly shown in Table 4 that in fragment **f** the N1 and N7 methyl groups of parent compound **a** are retained, whereas, in fragment **g**, only the N3 methyl group of parent compound **a** is conserved. The structure in curly brackets in Fig. 8 shows a little thicker drawn the origin from the parent compound.The most probable structural embedding of these two fragments into the parent molecule **a** is represented in the structures shown in curly brackets in Fig. 8.

The characterized fragments obtained using a simple CID fragmentation protocol nicely correspond to fragments described earlier in the literature.[9–13,35] Although more

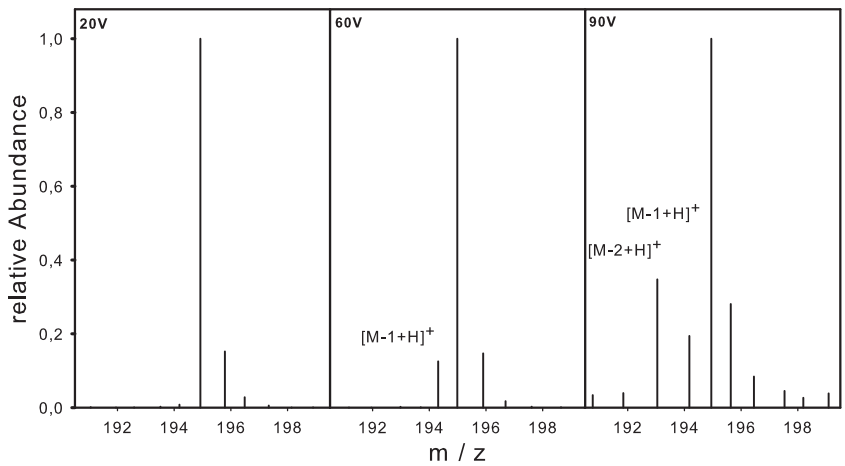

Figure 7. Details of ESI-CID spectra of caffeine **1** recorded at different cone voltages: (A) at 20 V, (B) at 60 V, and (C) at 90 V.

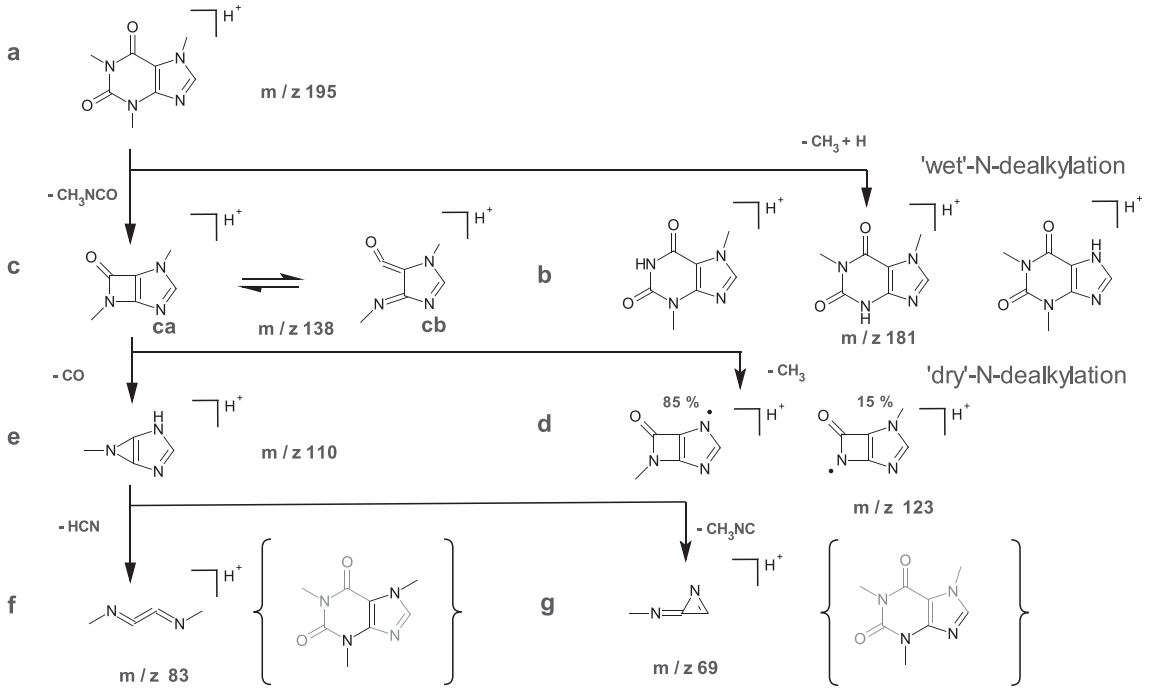

Figure 8. Proposed fragmentation pathway of the protonated caffeine molecule after CID.

sophisticated experimental MS paradigms have been used, the resulting information is very similar to that obtained in the present study.

CONCLUSIONS

The present study clearly reveals that the positive charge found on every caffeine fragment (F), formed by CID during ESI-MS investigations in the positive ion mode, originates from proton additions and results in the formation of species such as $[F+H]^+$ and $[R^{\cdot}+H]^+$. This could unambiguously be shown by experiments using perdeuterated eluents.

Additionally, the fragmentation spectrum of perdeuterated caffeine allows to precisely specify the exact number of hydrogen atoms per fragment ion equalling the *m/z* value differences between the *m/z* values of the deuterated fragments and the *m/z* values of the corresponding peaks in fragmentation spectra obtained using hydrogenated ('normal') caffeine.

With regard to the number of hydrogen atoms in a given fragment in combination with a computer-aided method for the calculation of the elemental composition and following the rule 'fragment parameter is even' (*vide supra*), both the ion type of the fragment ($[F+H]^+$ or $[R^{\cdot}+H]^+$) and its molecular formula can unequivocally be defined.

Caffeine fragments which have been identified as radical species most likely result from homolytic bond cleavage and are exceptions to the 'Even-Electron Rule'.

The formation of all fragments except one may be explained by carbon–heteroatom bond scission. Only the formation of one fragment involves a carbon–carbon bond scission, most likely due to the existence of a very unstable species.

A closer examination of the formed species suggests that two fragment types can be distinguished from N-dealkylation reactions. They are apparently generated depending on the chemical environment: mechanistically the first type is in accordance with a homolytic bond cleavage followed by the addition of a hydrogen atom so as to form a neutral molecule. Since the first step in the fragmentation is a homolytic bond scission the addition of hydrogen is formally that of a hydrogen radical. It is likely that this hydrogen transfer takes place in a (partially) solvated state ('wet' fragments) but it is implausible that these hydrogen atoms stem from the perdeuterated eluent. Consequently it is most likely that the hydrogen atoms originate from the parent compound caffeine. In the second fragment type no addition of hydrogen takes place after bond scission and accordingly these fragments are radicals which arise from originally unsolvated molecules ('dry' fragments).

Through knowledge of the molecular formulae of the fragments combined with conclusions drawn from some well-known fragmentation reactions for alkylxanthines (e.g. N-dealkylations), the deduced chemical structures presented in this work seem to be reasonable. Knowledge of the described CID fragmentation reactions allows the assembly of fragmentation patterns which may prove to be very useful for the structural elucidation of xanthine metabolites in future studies.

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