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Determination of DMF modified DNA base N^4-methylcarbamoylcytosine in human urine using off-line sample clean-up, two-dimensional LC and ESI-MS/MS detection

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Abstract

A sensitive internal standard method for the analysis of a DNA-adduct of *N*,*N*-dimethylformamide (N^4-methylcarbamoylcytosine, NMC-C) in human urine has been developed. A sample pre-treatment involving an acidic hydrolysis is followed by the sample clean-up performed with solid-phase extraction (SPE) technique using a cation-exchange resin. A two-dimensional liquid chromatography is used to separate the target analyte from the matrix using first a C18 reversed phase column with incorporated hydrophilic moieties and then a C8 bonded reversed phase column for the final separation. Quantification is carried out by positive electrospray ionisation and mass spectrometry detection of the transitions from molecule ions to product ions (169 → 112 and 172 → 115) for the analyte and the labelled internal standard, respectively. The detection limit in urine reaches down to 8 ng/L (48 pmol/L). In the general population NMC-C could not be detected. In 10 out of 32 urine samples of occupationally to DMF exposed subjects NMC-C could be detected. The concentrations ranged up to 172 ng/L (1023 pmol/L) with a 95th percentile of 121 ng/L (720 pmol/L).
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Keywords: *N*,*N*-Dimethylformamide; DNA-adduct; Biochemical effect monitoring; Urine

1. Introduction

N,*N*-Dimethylformamide (DMF) is one of the most important organic solvents used in industry. Its annual worldwide production was estimated 250 000 metric tonnes in 1989 [1]. In 2002 only China had already production capacities of 146 000 metric tonnes and further plants with a total capacity of 130 000 metric tonnes were under construction [2]. DMF is primarily used in synthetic fibre industry as solvent. DMF is hepatotoxic and teratogenic (DFG, Deutsche Forschungsgemeinschaft, pregnancy group B) [3]. It was suggested to be associated with cancer incidents in the 1980s [4]. But in 1999 the IARC (International Agency for Research on Cancer) classified DMF in group 3 [5]: dimethylformamide is not classifiable as to its carcinogenicity to humans.

Especially in synthetic fibre industry workers are occupationally exposed to DMF. Ambient air monitoring is not sufficient to assess individual body burden because DMF is not only absorbed inhalatively but also percutaneously. Up to now three biomarkers have been measured to assess individual exposure of workers: the metabolite *N*-methylformamide (NMF) [6–20], the mercapturic acid *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC) [6,7,9,10,15,18,19,21,22] (both in urine) and the *N*-methylcarbamoyled-Hb-adducts [23–26]. Since Hb-adducts are considered surrogates for DNA-adducts the next step is the identification and quantification of DMF-DNA-adducts in human urine and thus to reveal the potential mutagenetic effects of DMF. The finding of AMCC and especially of *N*-methylcarbamoyled-Hb-adducts confirms that methyl isocyanate (MIC) which forms

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the same Hb-adducts is an intermediate in DMF metabolism. In vitro MIC has been demonstrated to react with DNA-bases to form N^4-methylcarbamoyl-desoxycytidine (NMC-dC), N^6-methylcarbamoyl-desoxyadenosine and N^2-methylcarbymoyl-desoxyguanosine in relative reactivities of 100:0.7:0.003 [27]. After in vitro reactions of MIC with calf-thymus DNA NMC-dC and N^6-methylcarbamoyl-desoxyadenosine could be isolated [28]. Cytidine was the most reactive substance and additionally the labelled internal standard of the adduct was easier to synthesise. Consequently the focus was on the development of an analytical method to determine DMF-DNA-adduct NMC-dC.

Since DNA-adducts are present at ultratrace levels in human urine, specific and sensitive analytical methods are crucial for the accurate identification and quantification. Traditionally ^{32}P-post-labelling and immunoaffinity assays have been used for determination of DNA-adducts. However, in the last 10 years especially methods basing on mass spectrometry and stable-isotope labelled internal standards have been proven to provide the requested performance with higher specificity and accuracy [29]. These methods generally comprise sample clean-up and chromatographic separation prior to the mass spectrometric detection. Sample clean-up and analyte enrichment, respectively, are performed off-line using SPE [30–38], immunoaffinity clean-up [39,40], liquid/liquid extraction [41] or a combination of these techniques [42–46]. Chromatographic separation is achieved with liquid chromatography (LC) [32–34,37,42–44,46–48], tandem LC [35,36] or gas chromatography (GC) [38–40]. The mass spectrometric detection either performs multiple reaction monitoring (MRM) [32–37,42–44,46–48] or selected ion monitoring (SIM) [38–40]. Besides the mass spectrometric methods, there are few other methods for DNA-adducts in urine using LC with electrochemical detection [30,31] or fluorescence detection [41] and capillary electrophoresis with laser induced fluorescence detection [44,45].

NMC-dC and NMC-C are not suitable for fluorescence or electrochemical detection. Furthermore, they could not be analysed by GC–MS because they were not stable under derivatisation conditions. Therefore, an analytical method using LC–MS/MS was developed. For the first time this newly developed method made it possible to determine NMC-C in urine samples of DMF-exposed workers.

2. Experimental

2.1. Chemicals and standards

Acetonitrile (LiChrosolv), methanol (LiChrosolv, HPLC-grade and SupraSolv GC-grade), formic acid (98–100%, p.a.), hydrochloric acid (25%, p.a.) and ammoniac solution (32%, extra pure) were purchased from Merck (Darmstadt, Germany). Water was deionised by a Milli-Q treatment system (Millipore, Bedford, USA). N^4-(*N*-Methylcarbamoyl)desoxycytidine (NMC-dC) was synthesised by the Biochemical Institute for Environmental Carcinogens (Grosshansdorf, Germany). Isotope labelled (2-^{13}C, 1,3-^{15}N) cytosine was purchased from Promochem (Wesel, Germany).

N^4-(*N*-Methylcarbamoyl)desoxycytosine (NMC-C) and labelled NMC-C* (2-^{13}C, 1,3-^{15}N) was synthesised in house from cytosine (isotope labelled cytosine) and methyl isocyanate in adaptation to the synthesis of Tamura et al. [28]. Briefly, 50 mg cytosine was dissolved in 35 mL DMF, 30 μL of MIC were added and the reaction mixture was left at 18 °C for 3 days. Then the solvent was evaporated under vacuum, the residue was dissolved in methanol and purified using preparative LC. The products were characterised by mass spectrometry and ^{1}H NMR. Stock solutions of NMC-C (10 mg/L), isotope labelled NMC-C* (10 mg/L) and NMC-dC (100 mg/L) were prepared in methanol, dilutions were made in water. Stock solutions were stored at −18 °C, diluted solutions at 4 °C.

2.2. Standard preparation

For urinary calibration standards pooled urine was collected. The urine was frozen, thawed and subsequently filtered through a 45 μm nitrocellulose membrane (Millipore, Beford, USA). This procedure was repeated two times. Afterwards 20 mL aliquots of the pooled urine were spiked with a working solution of NMC-dC (20 μg/L) yielding seven calibration standards ranging from 0 to 500 ng/L (0–1761 pmol/L). An aqueous calibration was prepared likewise. Furthermore, two control materials in urine 100 ng/L (352 pmol/L) and 250 ng/L (880 pmol/L) in urine were prepared. Standards and control materials were frozen at −18 °C until further use. They were processed like all other samples.

2.3. Sample preparation

Urine samples were thawed. To a 20 mL aliquote of the urine sample 20 μL of an internal standard solution (labelled NMC-C 300 ng/L, 1754 pmol/L) was added. For acidic hydrolysis the urine samples were adjusted to pH 1 with 25% HCl. The samples were then incubated at 70 °C for 1 h and left for cooling afterwards. For sample clean-up solid phase extraction was performed using Waters Oasis MCX extraction cartridges (6 mL, 150 mg, Waters, Massachusetts, Ireland). The sulfonyl moieties of these cation exchange cartridges can retain NMC-C as it is positively charged at pH 1. The cartridges were washed using acetonitrile, methanol, water (8 mL each) and equilibrated with 8 mL 0.1 M HCl. The hydrolysed cooled down samples were passed through the cartridges followed by 3 mL of 0.1 M HCl. Then the cartridges were dried applying vacuum. After washing with 3 mL methanol for further reduction of the urinary matrix the cartridges were dried again. Finally, the analyte was eluted with 3 mL ammoniac methanol solution prepared from 98 mL of methanol and 2 mL of 32% ammonia. During all steps the solutions trickled slowly. The eluate was evaporated to

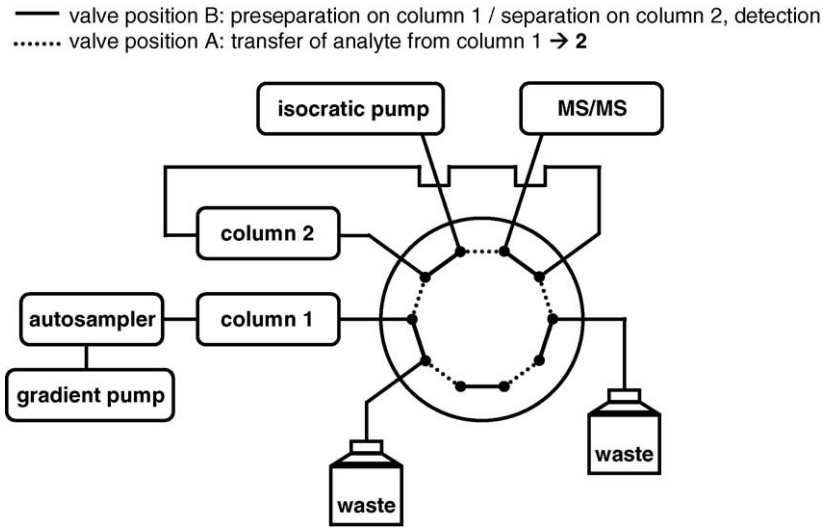

Fig. 1. Two-dimensional HPLC system with 10 port valve (also possible with six port valve), gradient pump eluent: 0.02% HCOOH/MeOH, 90/10, v/v; isocratic pump 0.02% HCOOH/MeOH, 70/30, v/v.

dryness under a gentle stream of N_2 and the residue was redissolved in 200 μL water.

2.4. Liquid chromatography

The HPLC system consisted of a Hewlett-Packard HP 1100 Series HPLC apparatus (auto sampler, quaternary pump, vacuum degasser) and an additional isocratic Merck-Hitachi L6000A pump from Merck (Darmstadt, Germany). Two analytical columns were used: column 1 was a silica based C18 column with polar endcapping Synergi Hydro-RP 80A, 250 mm × 4.6 mm, 4 μm particle size (Phenomenex, Aschaffenburg, Germany) and column 2 was a silica based C8 column LiChrospher 60 RP-Select B 250 mm × 4.6 mm, 5 μm particle size (Merck, Darmstadt, Germany). An illustration of the chromatographic system is given in Fig. 1 and the details of chromatographic procedure are presented in Table 1. Sample injection, chromatographic separation, cleanup and regeneration on column 1 were performed with the quaternary pump. The isocratic pump was used for the chromatographic separation on column 2 and to keep a constant flow of mobile phase going into the mass spectrometer. The injection volume was 50 μL. On column 1 a pre-separation of matrix and analyte was achieved using 0.02% formic acid and methanol (90/10, v/v) at a flow rate of 0.5 mL/min while on column 2 the isocratic pump delivered an eluent consisting of 0.02% formic acid and methanol (70/30, v/v) at a flow rate 0.3 mL/min. To transfer the analyte fraction (15.8–19.2 min) from column 1 onto column 2 a 10 port valve on the API 2000 Sciex MS/MS (PE Biosystems, Langen, Germany) switched from position B into position A. In position A, the eluent of the quaternary pump goes via columns 1 and 2 into the waste while the isocratic pump delivers the eluent directly into the mass spectrometer. After

Table 1
Analytical procedure for HPLC system, valve position, eluent composition on column 1, events on column 1 and 2

Time [min]	Valve	0.02% HCOOH / MeOH	Column 1	Column 2
0.1	B	90/10	Sample injection, separation	Rinsing 0.02% HCOOH / MeOH 70/30
15.8	A		Transfer of analyte: column 1 → 2; isocratic pump directly into MS	
19.2	B	0/100	Rinsing	Separation + detection 0.02% HCOOH / MeOH 70/30
20				
21				
26				
27		90/10	Equilibration	
35				

the transfer of the analyte zone the valve switched back and the final separation was achieved on column 2. Meanwhile column 1 was rinsed applying 100% methanol and then reconditioned with the initial eluent. All steps are controlled by Analyst 1.1 Software from Perkin-Elmer except the isocratic pump.

2.5. Mass spectrometry

A Sciex API 2000 LC/MS/MS system equipped with a turbo ion spray interface was used for MS/MS detection in multiple reaction monitoring (MRM) mode. The ion source conditions were an ion spray voltage of 5500 V (positive mode) and a temperature of 450 °C. Nitrogen was used as nebuliser gas, auxiliary gas and curtain gas with 65, 40 and 50 psi, respectively. The collision gas (nitrogen) for the MS/MS mode at quadrupole 2 was set at 4 (instrument units). Resolution was set at "unit" for Q1 and at "low" for Q3. Settling time and pause between mass ranges were both 5 ms. Scan time for each transition was 150 ms. The transition for NMC-C and the internal standard (NMC-C*) were *m/z* 169 → 112 and 172 → 115, respectively. The molecule ion and the main fragment ions of NMC-C are shown in Fig. 2. Declustering (DP), focusing (FP), entrance (EP) potential were set at 21 V, 330 V and −8 V, respectively. Collision energy (CE) was 27 V.

Continuous flow injections of standard solutions for the analytes had been performed to establish the MS–MS operating conditions with the syringe pump system of the API 2000.

2.6. Validation and calibration

Standards and control materials were processed as described in Section 2.3. For calibration the quotient of the peak areas of NMC-C and NMC-C* was plotted as a function of the concentrations. The linear calibration curves showed correlation coefficients better than 0.99 over the concentration range from 0 to 1761 pmol/L. All calculations were performed by Analyst 1.1 Software. Precision and accuracy was investigated. Two control materials in urine

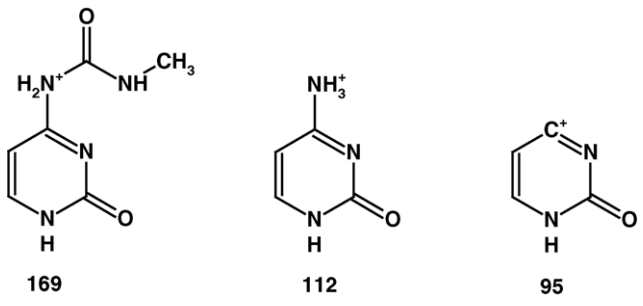

Fig. 2. Molecular structures to the masses chosen for MS/MS detections: 169: protonated N^4-methylcarbamoylcytosine (M = 168 g/mol); 112: protonated cytosine (M = 111 g/mol); and 95: cytosine–NH_3.

100 ng/L (352 pmol/L) and 250 ng/L (880 pmol/L) were analysed for within-series imprecision eight times in a row and for between-day imprecision on eight different days. To investigate the robustness of the method in dependence of different matrices 10 individual urines (creatinine content from 0.28 to 2.22 g/L) were analysed unspiked and spiked with 100 ng/L (352 pmol/L) each.

2.7. Recovery and sensitivity studies

To estimate the losses in sample preparation and the effect of quenching in ESI a comparison of processed standards and non-processed standard was carried out. Therefore, an aqueous standard containing 30 μg/L IS NMC-C* was prepared and analysed without sample preparation. This aqueous standard, a processed aqueous and a processed urine standard were measured according to 1.4 and 1.5 with ESI ionisation, but also with APCI ionisation. The APCI measurement was carried out according to the ESI measurement except for the ion source and the isocratic eluent consisting of 20 mM ammonium formiate, pH 4 and MeOH (70/30, v/v).

2.8. Confirmation of analyte structure

For structural confirmation of the analyte fractions were collected and analysed with another two-dimensional chromatographic separation with MS/MS detection using other stationary phases. Three samples of exposed workers which had revealed a NMC-C content were chosen. Each sample was injected three times and the fractions of the analyte (29–30.5 min) were collected in a HPLC-vial. The fractions were evaporated to dryness under a gentle stream of nitrogen and reconstituted in 200 μL H_2O. Fifty microlitres of the solution were injected onto another LC–MS/MS system. The same apparatus as described above was employed but the chromatographic conditions and stationary phases were changed. The first analytical column was a RP12 bonded silica column with trimethylsilyl endcapping Synergy Max-RP 80A, 150 mm × 4.6 mm, 4 μm particle size (Phenomenex, Aschaffenburg, Germany) and the second was a silica-based phenyl-hexyl phase column Luna Phenyl-Hexyl, 150 mm × 4.6 mm, 3 μm particle size (Phenomenex, Aschaffenburg, Germany). On the first column, 0.02% formic acid and methanol (90/10, v/v) at a flow rate of 0.4 mL/min were used as eluent and, on the second column, 0.02% formic acid and methanol (70/30, v/v) at a flow rate 0.3 mL/min. The analyte fraction (11–12.6 min) was transferred from the first column onto the second. Mass spectrometry detection was performed as described above, but additionally qualifier transitions were monitored. These transition were *m/z* 169 → 95 and 172 → 98 for NMC-C and the internal standard (NMC-C*) respectively. Declustering (DP), focusing (FP) and entrance (EP) potential were set at 31 V, 360 V and −9.5 V, respectively. Collision energy (CE) was 37 V.

2.9. Study subjects

Two groups of persons were investigated. One comprised male workers occupationally exposed to DMF ($n = 32$). The employees worked in polyacrylic fibre industry and carried out different working tasks (fibre spinning, fibre crimping, etc.). All of them had been employed longer than one year. Urine samples were collected in 1997 and stored at −18 °C. The control collective ($n = 24$; 11 male, 13 female) was taken out of the general population without any history of occupational exposure to DMF. The urine samples were collected in 2002 and stored at −18 °C. Urinary creatinine concentrations were determined according to Larsen [49].

3. Results and discussion

3.1. General considerations

DNA-adducts of DMF had never been measured before. The DNA-adducts already measured by modern methods in the last decade were either DNA-adducts originating from oxidative stress (oxidised products like 8-OH-dG [30–33] and 8-oxo-dA [34,47], etheno-DNA-adducts [35–38]), alkylating agents in cigarette smoke [39,40], PAH [43–45], or aflatoxin [41,42,46]. All these DNA-adducts were found to be present in urine samples in ng/L level or even lower. So, the main requirement of the method besides its specificity was a sensitivity down to ng/L level, especially as it was not clear if and in which concentrations NMC-dC or NMC-C would occur. Regarding this aspect NMC-dC was hydrolysed to NMC-C in order to attain a lower detection limit by determining the sum of both parameters.

3.2. Sample preparation

For the hydrolysis of NMC-dC to NMC-C different possibilities including different acids, buffers and enzymes were tested. Hydrolysis with enzymes (which required buffer systems), buffers and weak acids showed decreased recoveries compared to HCl hydrolysis, especially regarding the combination with the subsequent analyte enrichment. Thus, hydrolysis with HCl was proven to be the most appropriate despite the partial hydrolysis of the methylcarbamoyl group, which amounted ca. 20% for both NMC-dC and NMC-C under the optimised conditions.

Many novel HPLC techniques for biological fluids analysis employ on-line enrichment of analytes using restricted access materials (RAM) [50]. NMC-C could not be retained on the tested RAM-phases (C4, C8, C18). Therefore, a more laborious off-line enrichment was required and a SPE method was successfully developed. From the tested extraction materials only materials with polar groups could retain NMC-C. Among these a cation exchange material showed to achieve the best analyte enrichment up to 100% and separation from interfering matrix components. After the hydrolysis at pH 1 NMC-C should be quantitatively in ionic form due to the partial protonation of the amino groups. Thus, the positively charged molecule of NMC-C can be quantitatively retained by a divinylbenzene–*N*-vinylpyrrolidone copolymer based cation exchange cartridge with sulfonyl moieties. At the end of the SPE procedure the analyte was eluted from the resin. The eluate was evaporated to dryness in reconstituted in 200 μL resulting in an enrichment factor 100 (20 mL → 200 μL).

3.3. LC–MS

Two-dimensional LC was employed to achieve a sufficient separation between matrix components and analyte. Combining two different columns, Synergi Hydro-RP column and LiChrospher RP-Select the chromatographic separation can be improved. Synergy Hydro-RP has a polar endcapping which enables retention of polar compounds like NMC-C via polar interactions, hydrogen bonding or electrostatic interactions. LiChrospher RP-Select is a silica based C8 column. By optimising the chromatographic parameters it was possible to separate matrix components interfering with the quantifier transitions *m/z* 169 → 112 and 172 → 115 for NMC-C and the internal standard NMC-C*. Whereas, the transitions *m/z* 169 → 95 and 172 → 98 which were intended to be used as confirmative qualifiers were hardly intensive in the investigated concentration range and additionally disturbed by matrix components. For the quantification of the analyte the above column combination was preferred to the one used in the analyte confirmation due to a lower level of interferences even though a longer transfer time of about 3.4 min has to be accepted. APCI was tested first as ionisation source, but it provided less sensitivity compared to ESI and the noise was higher and more uneven so that integration of peaks was difficult.

Thus, ESI was used as ion source for the MS/MS detection. The compound-specific mass spectrometer parameters were optimised for each compound automatically by the Quantitative Optimisation Wizard of the Sciex Analyst™ software. The source specific parameters were optimised manually for the LC conditions used during analysis depending on flow rate and eluent composition.

3.4. Calibration graphs

Urinary and aqueous standards were used for calibration. Without relating to the internal standard the slope of the urinary calibration curve was about half of the aqueous calibration curve. This was probably due to the decrease of sensitivity in urinary matrix by quenching effects from coeluting substances. Since the internal standard was implemented these effects could be counterbalanced so that both calibration curves aqueous and urinary showed similar slopes (0.0029 and 0.0027) with excellent correlations coefficients (0.9999 and 0.9996). In urinary blanks no traces of NMC-C could be detected. The calibration range (0–500 ng/L;

Table 2
Imprecision and accuracy of the determination NMC-C in human urine

	Range (mean) (ng/L)	Imprecision (%)	Accuracy relative recovery (%)
Intra-day $n = 8$			
$Q1_{low}$ (61.4 ng/L)	60–68 (63)	5	98–111 (103)
$Q2_{high}$ (153.2 ng/L)	153–164 (159)	2	98–105 (104)
Inter-day $n = 8$			
$Q1_{low}$ (61.4 ng/L)	57–66 (62)	5	93–107 (100)
$Q2_{high}$ (153.2 ng/L)	150–167 (157)	4	98–109 (102)
Inter individual $n = 10$, robustness in dependence of matrices (creatinine 0.5–2.22 g/L)			
C (61.4 ng/L)	47–68 (60)	9	77–111 (98)

0–1761 pmol/L NMC-dC) was adapted to preliminary half quantitative measurements of samples of exposed workers. Furthermore, it was in good agreement with the concentration ranges of other DNA-adducts measured (e.g. etheno-adducts) [35,37,38,51]. For routine measurements the urine calibration was used.

3.5. Reliability of the method

3.5.1. Precision and accuracy

Precision and accuracy were checked with special recovery experiments. To assess within-series and between-day imprecision the two control materials in urine were measured one day in a row and on eight different days. Commonly the control materials ($Q1_{low}$, $Q2_{high}$) have concentrations about 10 times and 100 times higher than the limit of detection. In this case these concentrations would not have been appropriate because they would have been much higher than expected concentrations in native samples. Therefore, concentrations of 100 ng/L (352 pmol/L) and 250 ng/L (880 pmol/L) NMC-dC were chosen for $Q1_{low}$ and $Q2_{high}$, respectively. For $Q1_{low}$, the within-series and between-day imprecision were both 5%, for $Q2_{high}$ they were 2% and 4%. The robustness of the method in dependence of different urine matrices was assessed by relative recovery experiments in 10 urine specimens. These were chosen in order to reflect a composition as different as possible. As indicator served the creatinine content ranging from 0.28 to 2.22 g/L. The relative recovery experiment was conducted by analysing the urine specimens unspiked and spiked with 100 ng/L (352 pmol/L) NMC-dC. The relative recoveries ranged from 77% to 110% with a mean of 98%. Further data for the precision and accuracy experiments including range, mean, standard deviation and accuracy are presented in Table 2. These figures demonstrate the excellent precision, reproducibility and robustness of the method.

3.6. Detection limit and quantification limit

The limit of detection (LOD = 3 × S/N) was estimated using the lowest urine standard and amounted 8 ng/L (48 pmol/L) NMC-C. It can be 10 fold higher in other urine specimen due to a loss of sensitivity by individual quenching effects of coeluting components. These quenching effects are noticeable when internal standard also shows quenching due to the special matrix. The limit of quantification (LOQ = 6 × S/N) is 16 ng/L (96 pmol/L).

3.7. Recovery and sensitivity studies

Processed and non-processed standards were measured with ESI and APCI in order to assess sample loss and quenching effects. The concentration of IS NMC-C* was 30 μg/L in all vials (processed and non-processed standards), so that the peak areas could be directly compared. In APCI the molecules are ionised in the gas phase while ESI transfers ions from solution into gas phase. In APCI ions from solvent, buffer and matrix support ionisation of the analyte, whereas in ESI they compete with the analyte for ionisation. This competition for ionisation results in a loss of sensitivity for the analyte, so called quenching. Hence, measurements were performed by APCI to assess the effect of quenching in ESI and to deduce thereby the sample loss during preparation. In APCI the peak area of IS NMC-C* of the processed aqueous and the processed urine standard were 76% and 75% of the non-processed aqueous standard (for details see Table 3). This data implies that the sample loss in preparation is about 25%. In ESI the peak area of IS NMC-C* decreases to 70% in the processed aqueous sample and to 36% in the processed urine sample compared to the non-processed standard. Since in APCI the peak areas are stable the significant decline in ESI demonstrates the strong effect of quenching. Sample loss in preparation can be estimated to 25%, while sensitivity loss in ESI amounts approximately 50%.

Table 3
Recovery and influence of quenching of the analytical method, signal intensity of IS NMC-C* in processed and non-processed standards in ESI and APCI measurements

Area 172/115 (cps)	APCI	ESI
H_2O standard	159000 (100%)	333000 (100%)
Processed samples		
H_2O standard	121000 (76%)	234000 (70%)
Urine standard	120000 (75%)	121000 (36%)

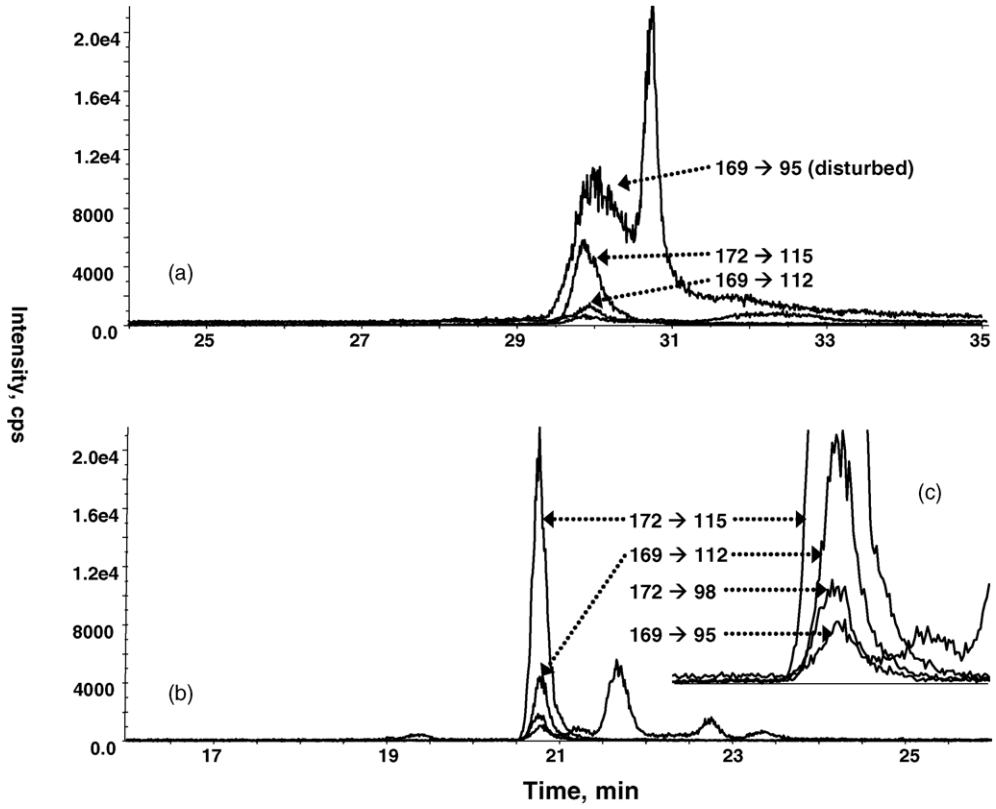

Fig. 3. Chromatogram of a processed urine sample after the first set of two analytical columns (a), after the second set of two analytical columns (b), enlargement of the peak after the second set of columns (c), transitions used for the confirmation of the structure: NMC-C 169 → 112 and 95; IS NMC-C* 172 → 115 and 98.

3.8. Confirmation of analyte structure

The analyte NMC-C was identified by its retention time and one transition (169 → 112) on the two-dimensional LC system with MS/MS detection. For further confirmation of the analyte fractions of three positive samples from exposed workers were collected after the first LC/LC separation and then reanalysed on a second LC/LC–MS/MS system. Three fractions were collected, united and reconstituted in 200 μL water to improve sensitivity. The columns for the second system were chosen in order to vary the active surfaces and to have four different separation mechanisms. The first set of columns consisted of a hydrophilic C18 and a C8 column (see Section 3.3); the second set of a silica based C12 column and a column with phenyl-hexyl functionalities. The phenyl-hexyl column is known to increase retention of polar, aromatic compounds due to π-interactions as well as reversals in analyte elution order compared to traditional RP columns. As expected these additional LC columns improved the separation of analyte and matrix components and thereby reduced quenching and raised the signal intensity. Consequently it was possible to monitor the transitions *m/z* 169 → 95 and 172 → 98 (for NMC-C and IS, respectively), which could not be measured before because of low signal intensity and interferences. In all of the three tested positive samples the analyte NMC-C could be confirmed by the identical retention time using two different sets of columns and the two transitions *m/z* 169 → 112 and 169 → 95 (for the IS NMC-C*: *m/z* 172 → 115 and 172 → 98). A representative example is presented in Fig. 3.

3.9. Results of biological monitoring

Ten from the 32 urine samples of DMF-exposed workers contained NMC-C. As expected none of the 24 samples from the control collective out of the general population contained NMC-C. The results of DMF-exposed workers are comprised in Table 4. A chromatogram of a sample from a DMF-exposed worker containing 111 ng/L NMC-C is presented in Fig. 4. In one sample of a DMF-exposed worker the quantifier transition 169 → 112 was interfered, but in all other samples a clear separation of matrix components has been achieved. The content of NMC-C in urine of DMF-exposed workers ranged from 0 to 172 ng/L (0–1023 pmol/L). Mean was 25 ng/L (149 pmol/L). NMC-C was measured for the first time in human urine, so no comparisons can be made. Generally judged, the concentration of NMC-C in human urine lay

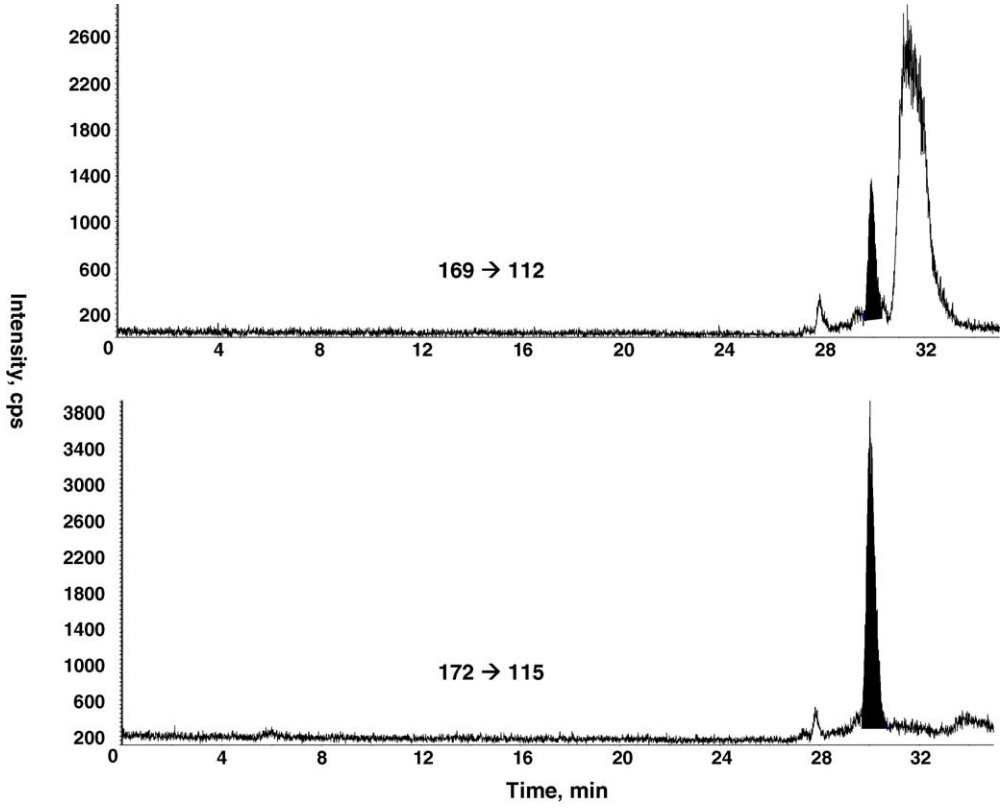

Fig. 4. Complete chromatogram of the processed urine sample A15 obtained with the presented method, transitions: NMC-C 169 → 112, IS NMC-C* 172 → 115, concentration of NMC-C 111 ng/L.

Table 4
Results of biomonitoring of NMC-C in urine samples of workers occupationally exposed to DMF

All samples $n = 31$	Concentration NMC	
	ng/L	pmol/L
Mean	25	151
Range	<LOD – 172	<LOD – 1023
Median	<LOD	<LOD
Positive samples $n = 10$		
A1	84	500
A2	49	291
A3	32	190
A4	39	232
A5	111	660
A6	31	184
A7	172	1023
A8	39	232
A9	102	607
A10	130	773

in the same order of magnitude of other DNA-adducts found in urine until now.

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