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Quantitative determination of 5-hydroxy-*N*-methylpyrrolidone in urine for biological monitoring of *N*-methylpyrrolidone exposure

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Abstract

The aim of this work was to validate a sensitive method for quantitative analysis of 5-hydroxy-*N*-methylpyrrolidone (5-HNMP) in urine. This compound has been recommended as a marker for biological monitoring of *N*-methylpyrrolidone (NMP) exposure. Different solvents and alternative methods of extraction including liquid–liquid extraction (LLE) on Chem Elut and solid-phase extraction (SPE) on Oasis HLB columns were tested. The most efficient extraction of 5-HNMP in urine was LLE with Chem Elut columns and dichloromethane as a solvent (consistently 22% of recovery). The urinary extracts were derivatized by bis(trimethylsilyl)trifluoroacetamide and analysed by gas chromatography–mass spectrometry (GC–MS) with tetradeutered 5-HNMP as an internal standard. The detection limit of this method is 0.017 mg/l urine with an intraassay precision of 1.6–2.6%. The proposed method of extraction is simple and reproducible. Four different *m*/*z* signal ratios of TMS-5-HNMP and tetralabelled TMS-5-HNMP have been validated and could be indifferently used in case of unexpected impurities from urine matrix.
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1. Introduction

N-methyl-2-pyrrolidone (NMP, C_5H_9NO, CAS number 872-50-4) is a clear colourless liquid with a characteristic amine odour that is usually synthesised by condensation of γ-butyrolactone and methylamine. NMP is completely miscible with water and many organic solvents and is an important industrial compound that is used both as a solvent and as an intermediate in many chemical processes. Some practical applications of NMP are as a solvent for pigments, monomers, and polymers, as paint stripper, graffiti remover and spinning agent for PVC [1]. NMP replaces other toxic solvents like dichloromethane, 1,1,1-trichloroethane or chlorofluoroalkanes [1]. NMP has also medical applications as a penetration enhancer in transdermal therapy [1–3].

Experimental studies with NMP have shown low systemic toxicity (LD_{50} 2600–7000 mg/kg b.w.) but some inflammatory changes in the respiratory system have been described after repeated inhalation exposure [4]. Animal reproductive toxicity studies showed that NMP might cause developmental toxicity at doses causing mild or no maternal toxicity [5,6].

The metabolism of NMP has been studied in rats and humans. NMP is rapidly distributed in all tissues

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after intravenous, oral and dermal administration [7,8]. The highest level of radioactivity after a single i.v. administration of radiolabelled NMP was observed in liver and intestines [8]. The urinary excretion is the main elimination route of NMP metabolites. In rats, up to 80% of the administered dose is eliminated in urine within 36 h, mainly as 5-hydroxy-*N*-methylpyrrolidone (5-HNMP). The metabolites of NMP are not conjugated [7,8].

Toxicokinetics studies in human volunteers showed that within 44 h after inhalation exposure to 10, 20 or 50 mg NMP/m^3 only 2% was excreted in the urine as the parent compound [9]. In humans, Åkesson and Jönsson elucidated biotransformation steps, which include oxidation of 5-HNMP to *N*-methylsuccinimide (MSI) and further hydroxylation to 2-hydroxy-*N*-methylsuccinimide (2-MSI) [10].

The absorption rates of NMP after dermal application to volunteers [4 mg/kg undiluted or diluted with water (50:50)] were 60 or 90%, respectively. Total recovery of urinary metabolites in urine during the following 9 days was approximately 23% for undiluted NMP and only 7% for NMP diluted with water. The ratio of excreted metabolites was NMP 1%, 5-HNMP 52%, MSI 1% and 2-HMSI 46% [11].

The elimination half-life of 5-HNMP from plasma and urine is 6 and 7 h, respectively. There is a close correlation between plasma and urine levels of 5-HNMP and NMP exposure level. Therefore 5-HNMP has been recommended as a marker for biological monitoring of NMP exposure [12].

To the best of our knowledge only one method (using capillary gas chromatography–mass spectrometry (CG–MS)) has been published for the quantitative analysis of 5-HNMP in urine [1] and plasma [13]. The authors reported a detection limit of 0.2 mg/l, an intra-day precision of 2–4% and a between-day precision of 4–21% in the range of concentrations 4–60 mg/l [1]. We were unable to purify urinary samples sufficiently to reach a detection limit of 0.2 mg/l with the extraction columns described in this method. This was partly due to the relatively high background observed on the chromatograms. Furthermore, some other applications such as in vitro experiments might also require lower limits of detection.

The aim of this work was therefore to validate a more sensitive method for quantitative analysis of 5-HNMP in urine that could also be applicable to other media. Two approaches were considered: design of a better purification method and optimisation of the parameters of the instrumental method.

In order to select the best method of urine purification and therefore increase the signal to background ratio we have tested other extraction solvents and alternative methods of extraction such as liquid–liquid extraction (LLE) on Chem Elut columns and solid-phase extraction (SPE) on Oasis HLB columns.

2. Experimental

2.1. Apparatus

A model HP6890A gas chromatograph (Hewlett-Packard GmbH, Waldbrom, Germany) with an autosampler and a mass selective detector HP 5972A (Hewlett Packard, Palo Alto, CA, USA) was used. The WCOT fused-silica column (30 m×0.25 mm) with a CP-Sil 5CB (low bleed/MS) stationary phase and film thickness 0.25 μm was from Chrompack (Middelburg, The Netherlands). A Reactivap III PIERCE (Rockford, IL, USA) was used to evaporate solvents under nitrogen flow. The vacuum unit for SPE extraction was from Supelco (Bellefonte, PA, USA).

2.2. Chemicals and reagents

Ethyl acetate (99.5%) and 2-propanol (99.8%) were from Riedel-de Haën (Seelze, Germany). Dichloromethane and other solvents (analytical purity) were from Sigma–Aldrich (Steinheim, Germany). bis(Trimethylsilyl)trifluoroacetamid, 99+% (BSTFA) was from Aldrich (Milwaukee, MI, USA). 5-hydroxy-*N*-methypyrrolidone (5-HNMP) and tetradeuterium labelled 5-HNMP ([$^2H_4$]-5-HNMP) were synthesised by Synthelec (Lund, Sweden) and their purity was estimated greater than 98% as verified by the presence of a single spot on thin-layer chromatography (TLC). The confirmation of their structure was performed by NMR-analysis. For specific liquid–liquid extraction Chem Elut columns (1 ml sample capacity) from Varian (Harbor City, CA,

USA) were used. HLB Oasis and SepPac C_8 columns (both 1 ml) for solid-phase extraction were purchased from Waters (Milford, MA, USA).

2.3. Preparation of standards

A standard solution (400 mg/l) was prepared by addition of 40 mg 5-HNMP to 100 ml of water. Calibration was performed by further dilution of this standard solution in the range 0.5–20 mg 5-HNMP/l. The internal standard solution was prepared by addition of 10 mg of [$^2H_4$]-5-HNMP to 100 ml of water. For determination of the extraction recovery, standard solutions of 5-HNMP were prepared in methanol at the desired concentrations.

2.4. Extraction and derivatization

The urinary samples were extracted with different methods, which are detailed in the Results section. To the dry extract 100 μl of BSTFA and 0.5 ml of ethyl acetate was added. The derivatization was performed overnight at room temperature.

2.5. Analysis

After extraction and derivatization, samples (1 μl) were injected in splitless mode at 250°C. The carrier gas was helium at 14.7 p.s.i. The initial column temperature was 70°C for 1 min, then the temperature was increased by 15°C/min to 250°C. The MS interface was kept at 280°C.

The selected ion monitoring (SIM) for trimethylsilyl derivative of 5-HNMP (TMS-5-HNMP) and for trimethylsilyl derivative of internal standard (TMS-[$^2H_4$]-5-HNMP) was performed at m/z 172.1, 186.1 and m/z 176.1, 190.1, respectively. The peak area ratios were used for quantitative analysis.

2.6. Storage of samples

Urinary samples were stored without any pretreatment in polyethylene bottles at the specified temperature until analysis.

3. Results and discussion

3.1. Quantitative analysis

In the mass spectra of TMS-5-HNMP the major fragments were m/z 186.1 (M^+-H), 172.1 (M^+-CH_3) and 98 (M^+-O-TMS). Due to high background from the urine matrix, the mass at m/z 98 present in the spectrum of TMS-5-HNMP was not useful for a quantitative purpose. For quantitative analysis, masses 186.1 and 172.1 for TMS-5-HNMP seemed to be the best choice. TMS-[$^2H_4$]-5-HNMP corresponding fragments at m/z 190.1 and 176.1 were selected for quantitative analysis of internal standard. For the next developments, quantitative analysis was therefore performed on the base of abundance ratio at different m/z values: 186.1/176.1, 186.1/190.1, 172.1/176.1 and 172.1/190.1.

3.2. Extraction tests

For the clean up of urine samples we tested two methods of extraction: liquid–liquid extraction (LLE) and solid-phase extraction (SPE).

Regarding LLE, Chem Elute columns were used to compare efficiency of extraction with different solvents: dichloromethane, cyclohexane, toluene, ethyl acetate, ethyl acetate/methanol and ethyl acetate/2-propanol, the latter mixtures in a ratio 90:10.

In order to quantitate 5-HNMP liquid–liquid extraction was carried out on Chem Elut columns. Practically, 1 ml of urine sample containing 20 mg/l of 5-HNMP was applied to the column, which was eluted six times with 4 ml of the tested solvent. The six fractions were collected separately. The volume of each collected fraction was then reduced by evaporation to dryness under nitrogen flow, at room temperature. Derivatization and analysis was performed as reported under materials and methods. The efficiency of 5-HNMP extraction using the six different solvents is shown in Fig. 1. The highest recoveries of 5-HNMP were obtained after elution with dichloromethane and the mixture of ethyl acetate/2-propanol (90:10). The less efficient extraction solvent was cyclohexane. Efficiency of extraction obtained with the mixture of ethyl acetate/methanol (90:10) was clearly less than with di-

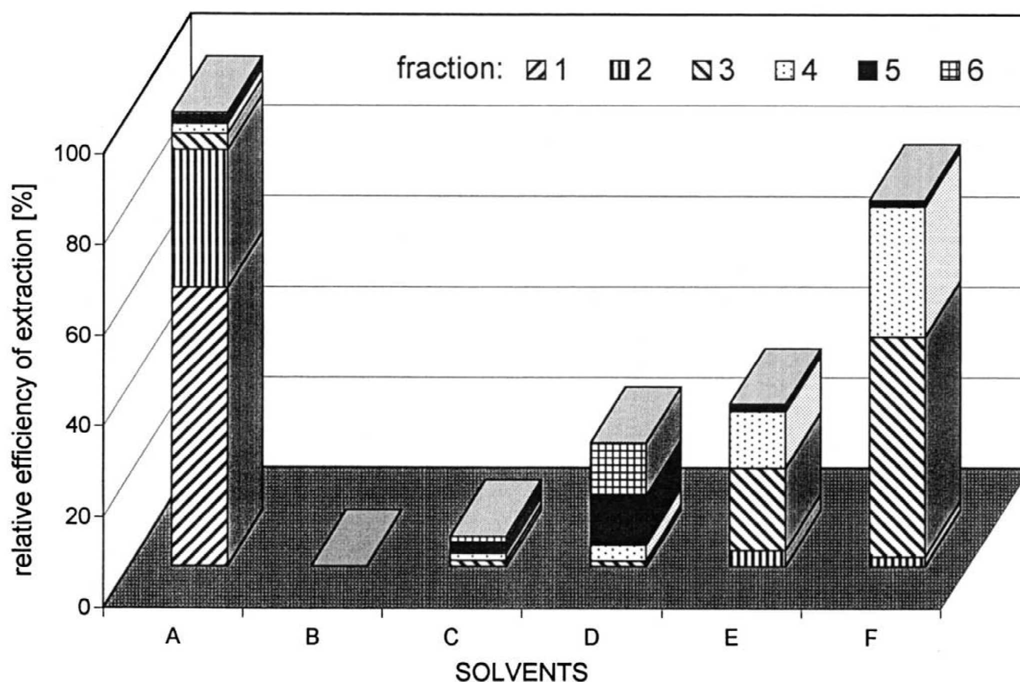

Fig. 1. Relative efficiency of 5-HNMP extraction on Chem Elut columns with different solvents (reference: solvent A). (A) dichloromethane, (B) cyclohexane, (C) toluene, (D) ethyl acetate, (E) ethyl acetate/methanol in composition 90:10, (F) ethyl acetate/2-propanol in composition 90:10. Six fractions were collected and analysed separately.

chloromethane or the mixture ethyl acetate/2-propanol (90:10). In the method described by Åkesson et al. [1] an ethyl acetate/methanol mixture has been proposed in the ratio 80:20 but such ratio was not applicable to Chem Elut columns for which a maximum percentage of 10% water miscible solvent is allowed. Unfortunately Åkesson et al. did not mention in their paper the gain obtained in term of recovery from the mixture ethyl acetate/methanol (90:10) to the finally proposed 80:20 ratio. Furthermore, consistent with Åkesson's data, extraction of 5-HNMP from urine with ethyl acetate alone was less effective than with ethyl acetate/methanol mixture (Fig. 1). Finally, it should be stressed that dichloromethane was the only solvent for which the majority of the substance was collected in the first fraction of 4 ml.

In light of these preliminary results, the extraction recovery of 5-HNMP was then calculated for the most effective solvents for the LLE procedure in the range 1–20 mg/l. Given the results presented in Fig. 1, only four fractions for dichloromethane and five fractions for ethylacetate/2-propanol (90:10) extraction were collected. The recovery for dichloromethane extraction of 5-HNMP from urine is shown in Table 1. The extraction of 5-HNMP from urine with ethyl acetate/2-propanol mixture was less effective (data not shown). The parameters of quantitative analysis of 5-HNMP in urine after Chem Elut/dichloromethane extraction for four different m/z ratios are presented in Table 2 and illustrated in chromatograms on Fig. 2. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the International Conference on Harmonization [14] with nine different urines spiked with 5-HNMP at levels approximating the LOD (0.01, 0.005, 0.0025 mg/l). Dichloromethane extracts gave lower background than extracts of ethyl acetate/2-propanol.

Regarding SPE extraction of 5-HMNP from urine we tested the same two solvents, dichloromethane and the mixture of ethyl acetate/2-propanol (90:10). The SPE method tested was based on the use of HLB columns. These columns contain a patented copoly-

Table 1
Recovery of 5-HNMP extraction from urine with dichloromethane on ChemElut columns (LLE)

Concentration of 5-HNMP	m/z Ratio used for quantitative analysis			
	186/176	186/190	172/176	172/190
5 mg/l	22.1% (n=10) SD 0.9	22.0% (n=10) SD 1.0	22.6% (n=10) SD 0.9	22.5% (n=10) SD 0.9
10 mg/l	22.4% (n=10) SD 1.1	22.4% (n=10) SD 1.1	22.4% (n=10) SD 1.1	22.3% (n=10) SD 1.0

Table 2
Parameters of quantitative analysis of 5-HNMP in urine after ChemElut/dichloromethane extraction

Parameters	m/z Ratio for quantitative analysis			
	186/176	186/190	172/176	172/190
Detection limit [mg/l]	0.021	0.017	0.034	0.031
Quantification limit [mg/l]	0.069	0.058	0.115	0.102
Slope [l/mg]	0.189	0.284	0.274	0.422
Intercept	0.002	0.007	0.024	0.041
r^2	0.999	0.999	0.999	0.999
Interassay variation (%)	2.6	2.1	2.2	1.6

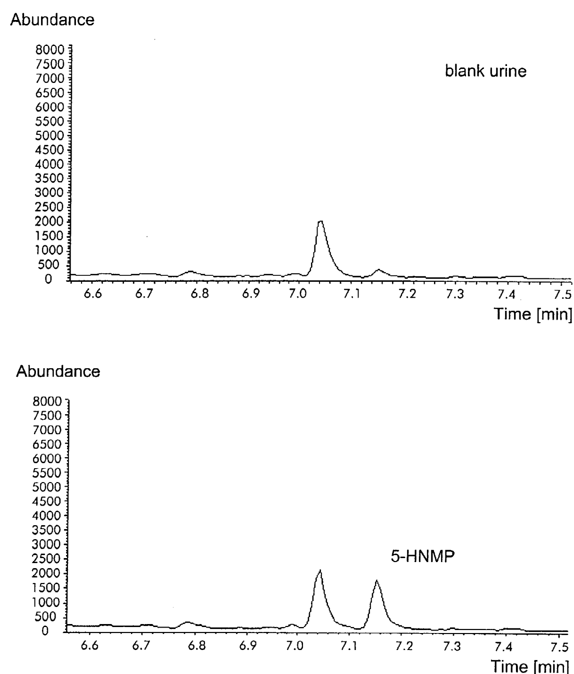

Fig. 2. Chromatograms of urinary samples after LLE extraction with dichloromethane at the m/z 172.1. Blank urine and urine spiked with 0.2 mg 5-HNMP/l.

mer as a sorbent, designed to have a hydrophilic–lipophilic balance (HLB) and are expected to give reproducible results even if the cartridge runs dry. HLB columns were first washed with 1 ml of methanol and equilibrated with 2 ml of water. Then 1 ml of urine sample containing 5-HNMP was applied. Columns were washed with 1 ml of water and dried with vacuum during 15 min, and then eluted with 1.5 ml of dichloromethane or 1.5 ml of ethyl acetate/2-propanol (90:10) mixture. Extracts were dried under nitrogen stream at room temperature. Samples were then derivatized and analysed as mentioned previously. The recovery of 5-HNMP extraction using SPE columns and dichloromethane or ethyl acetate/2-propanol elution was not better to LLE extraction (data not shown).

3.3. Stability tests

The stability was evaluated following two different protocols and using two different kinds of urinary samples: either urine spiked with 5-HNMP at the concentration 5 mg/l or urine samples of workers occupationally exposed to NMP and containing

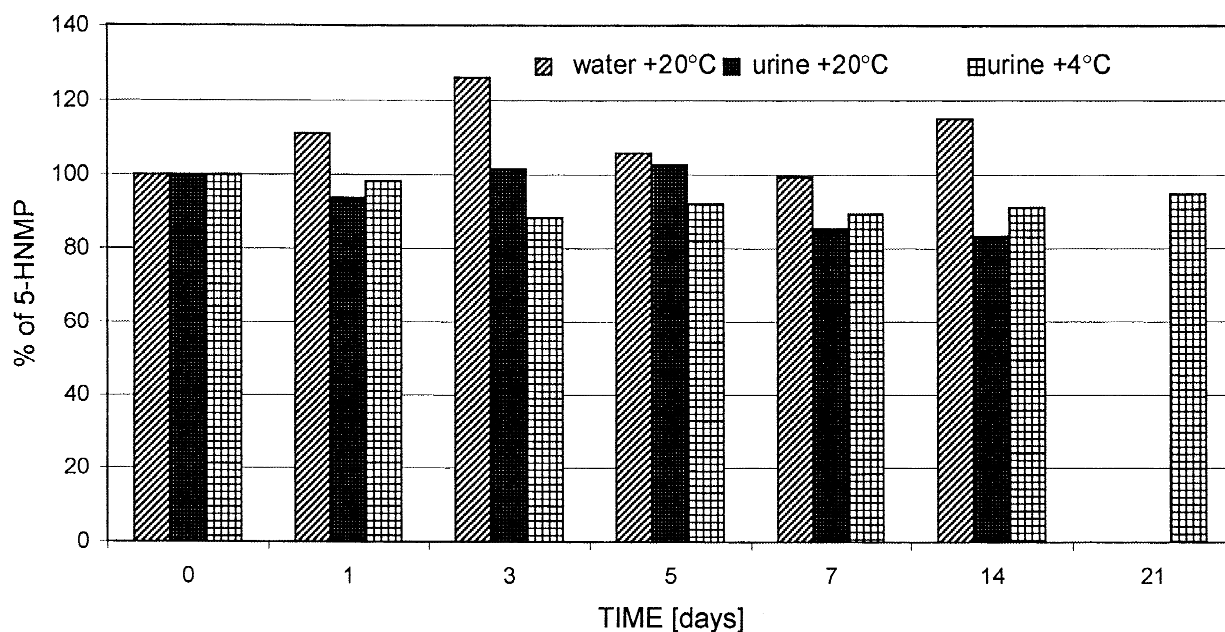

Fig. 3. Stability of 5-HNMP in urine and water. Urine spiked with 5 mg 5-HNMP/l, water with 5 mg 5-HNMP/l.

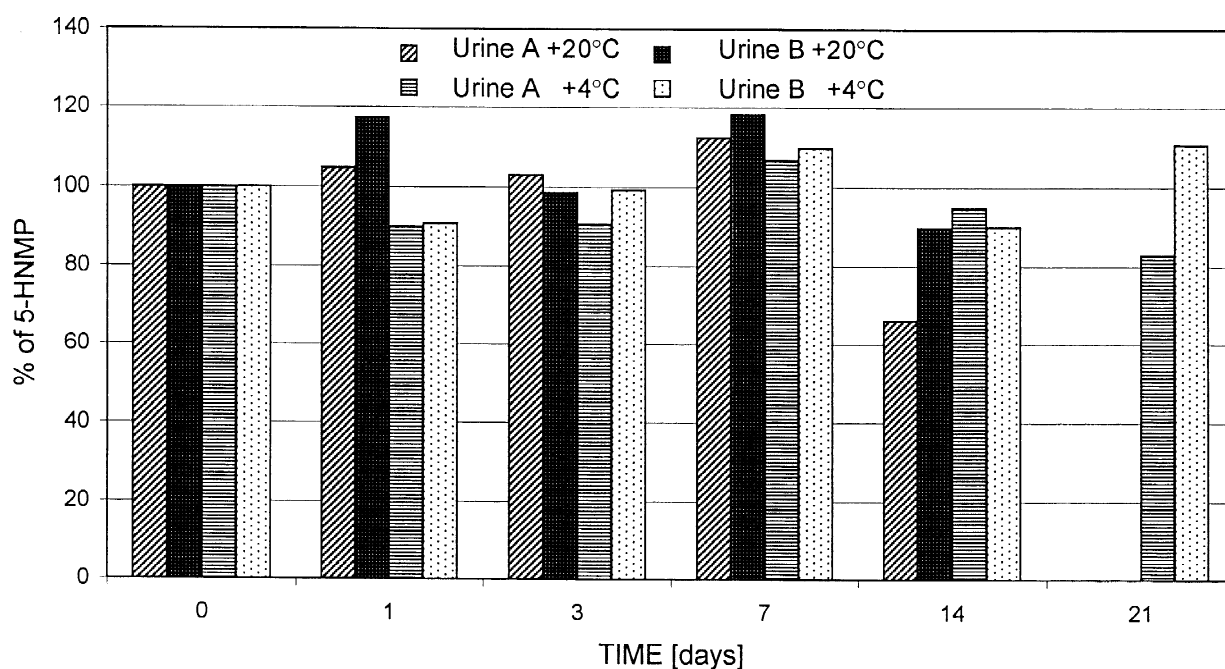

Fig. 4. Urine of occupationally exposed workers – (A) containing 1.55 mg 5-HNMP/l, (B) containing 7.14 mg 5-HNMP/l.

approximately 1.55 mg/l and 7.14 mg/l of 5-HNMP as measured by our selected protocol (Chem Elut/dichloromethane). Samples were stored at room temperature or kept refrigerated (+4°C). The stability was assessed over up to 2 weeks at room temperature, and 3 weeks at +4°C.

Urine samples were stable at least during 2 weeks in the refrigerator but only during 1 week at room temperature (Figs. 3 and 4).

3.4. Application for biomonitoring purposes

The method described above for the determination of 5-HNMP in urine is suitable for assessing occupational and/or environmental exposure to NMP. Regarding occupational exposure, Fig. 5 illustrates the urinary concentrations of 5-HNMP obtained during different intervals after respiratory exposure to a mixture containing 32% of NMP in a painting industry. The highest concentration was observed in the fraction between 6 and 16 h after the end of exposure, which seems consistent with the data of Åkesson and Jönsson in volunteers exposed to NMP [12]. The level measured on Monday before work (0.5 mg/l) was probably a residue from the exposure of the preceding week. To be able to measure such residual concentration an optimisation of the detection limit of the analytical method appeared of crucial importance.

Furthermore, as indicated in the introduction, Jönsson and co-worker [11] have recently reported lower urinary excretion of metabolites (7%) after dermal exposure to NMP diluted with water (50:50) compared with non diluted NMP (approximately 22%). Combining such situations with low levels of exposure to NMP justifies the need to use highly sensitive methods to design reliable biomonitoring programmes.

4. Conclusions

After testing different methods and solvents of extraction, we finally selected liquid–liquid extraction on Chem Elut columns with dichloromethane as an eluent. The low background of urine extracts allows the quantification of quite low amount of

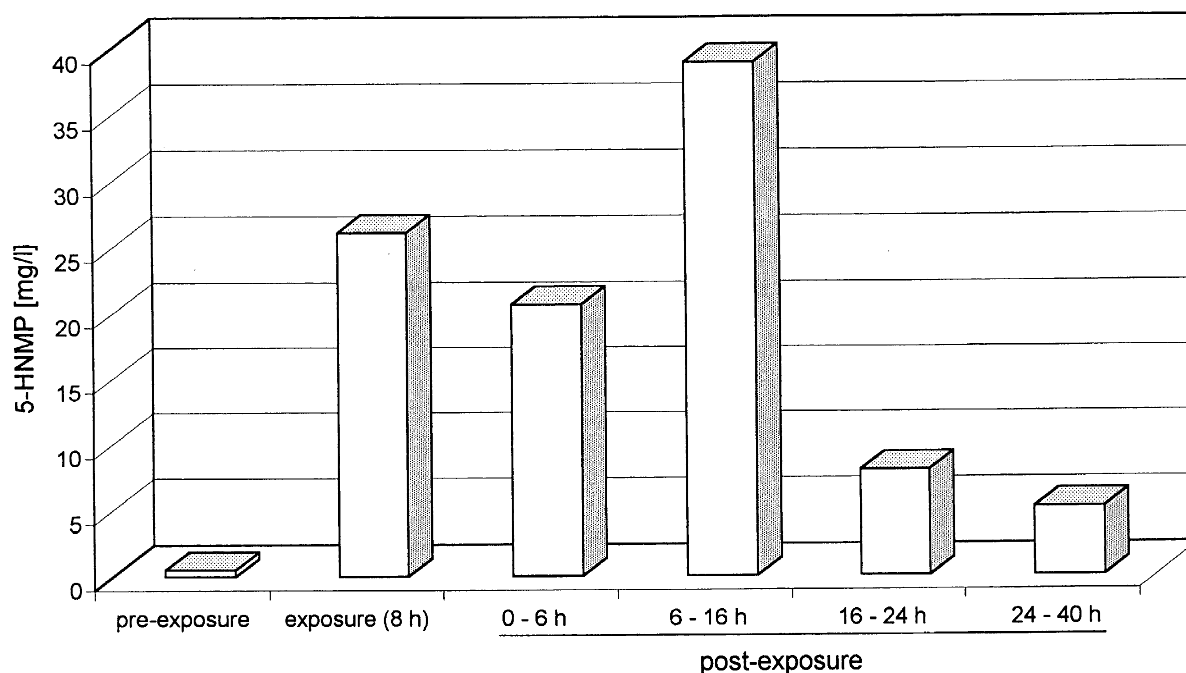

Fig. 5. 5-HNMP in different urine fractions of a worker exposed to NMP.

5-HNMP in urine samples (Table 2). The proposed method of extraction is simple and reproducible. Four different m/z signal ratios of TMS-5-HNMP and tetralabelled TMS-5-HNMP have been validated and can be indifferently used in case of unexpected impurities from urine matrix.

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