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Journal of Chromatography B, 778 (2002) 237–244

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Evaluation of 2,5-hexanedione in urine of workers exposed to *n*-hexane in Brazilian shoe factories

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Abstract

Urinary 2,5-hexanedione (2,5-HD) is used as a biomarker for biological monitoring of workers exposed to *n*-hexane. The purpose of this study was to compare two types of treatment of urine samples during clean-up (with and without acidic hydrolysis) and to study the exposure situation of workers exposed to *n*-hexane during shoe manufacturing. There, various glues containing *n*-hexane are used. Quantification of 2,5-HD was carried out by gas chromatography and flame ionization detection (GC–FID). Fifty-two urine samples taken from workers of seven shoe factories were analyzed. Thirty-four persons from the administrative staff of the same factories served as controls. They were not known to be exposed to *n*-hexane. The samples treated with acidic hydrolysis showed levels (average 0.94 mg/l) ~10 times higher than samples without acidic hydrolysis (0.09 mg/l). The difference is predominantly caused by the conversion of other metabolites of *n*-hexane (e.g. 4,5-dihydroxy-2-hexanone) to 2,5-HD in the presence of acids. Our results also show, that exposure to *n*-hexane is different between various industries. Levels of 2,5-HD in urine are predominantly dependent on the type of operation (how the glue is applied on the leather during shoe manufacturing). Simple measures, e.g. using a glue handgun instead of a paintbrush significantly decreased exposure to *n*-hexane.

Keywords: Biological monitoring; 2,5-Hexanedione; Hexane

1. Introduction

n-Hexane belong to one of the most important aliphatic compounds worldwide [1,2]. In 1988 a study by FUNDACENTRO-SP (Brazil) established that ~60% of *n*-hexane is used as a solvent in various industries, ~16% as ingredient in glues, and ~14% during the manufacture of polyolefins [3]. In many industries *n*-hexane is handled in closed cycles and therefore workers are usually not exposed. However, *n*-hexane is also used in various glues. In the study by FUNDACENTRO-SP, *n*-hexane was determined to be one of the main constituents in 83 different glue brands. Therefore, exposure of workers predominantly occurs in glue industry, shoe manufacturing, and shoe repair. It is estimated that at least 500 000 workers are exposed to *n*-hexane in the glue industry. Exposure of workers is mainly by inhalation [4], while dermal uptake may only occur by direct skin–glue contact.

From a toxicological point of view, it is well known that an increase in exposure to *n*-hexane also leads to an increase of neuropathologic syndromes [5,6]. The mechanism seems to be based on an interference with neuronal axoplasmic flow [7]. In

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00036-3

the meanwhile, peripheral neuropathy caused by *n*-hexane is a recognized professional disease predominantly occurring in those countries where shoe manufacturing or shoe repair industry uses *n*-hexane as one of their basic solvents in glues [8].

Neuropathies are caused by 2,5-hexandione (2,5-HD), which is an important metabolite of *n*-hexane in humans (Fig. 1) [9–12]. 2,5-HD is excreted in urine and it is commonly used as a biomarker to assess exposure to *n*-hexane [13–17]. However, it is noteworthy that 2,5 HD is not specific for exposure to *n*-hexane. 2,5-HD is also a metabolite of 2-hexanone (methyl *n*-butyl ketone). The excretion of 2,5-HD in post-shift urine samples from workers exposed to *n*-hexane is regulated in many countries at 5 mg/g creatinine [18,19]. For this purpose, there exist a couple of different methods to analyze 2,5-HD in urine samples. The majority is based on gas chromatography and mass spectrometric detection (GC–MS) [17,20–22], while also methods based on flame ionization detection (FID) [23,24] could be traced in the literature. After derivatization using *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine GC with electron capture detection can be also used for the determination of 2,5-HD, especially after exposure to low concentrations of *n*-hexane [25]. Beside GC, separation and detection is also possible by high-performance liquid chromatography (HPLC) and UV detection [26]. In addition, several sample clean-ups are described in the literature. For instance, the urine sample can be treated by enzymatic hydrolysis followed by acidic hydrolysis [11,13, 17,21,22], by acidic hydrolysis only [16,23,27–29] or without hydrolysis at all [30]. During acidic hydrolysis 4,5-dihydroxy-2-hexanone, which is also a metabolite of *n*-hexane, is converted to 2,5-HD [31,32]. However, in most of the publications described above, sample treatment with and without acidic hydrolysis was carried out and the results were compared to each other [27,30,33–35]. The analytical methodology described in the manuscript presented here is based on a method by Perbellini et al. [13]. However, (i) GC–FID was carried out for separation and analysis of 2,5-HD instead of GC–MS; and (ii) no enzymatic hydrolysis was carried out. However, the urine samples were treated with

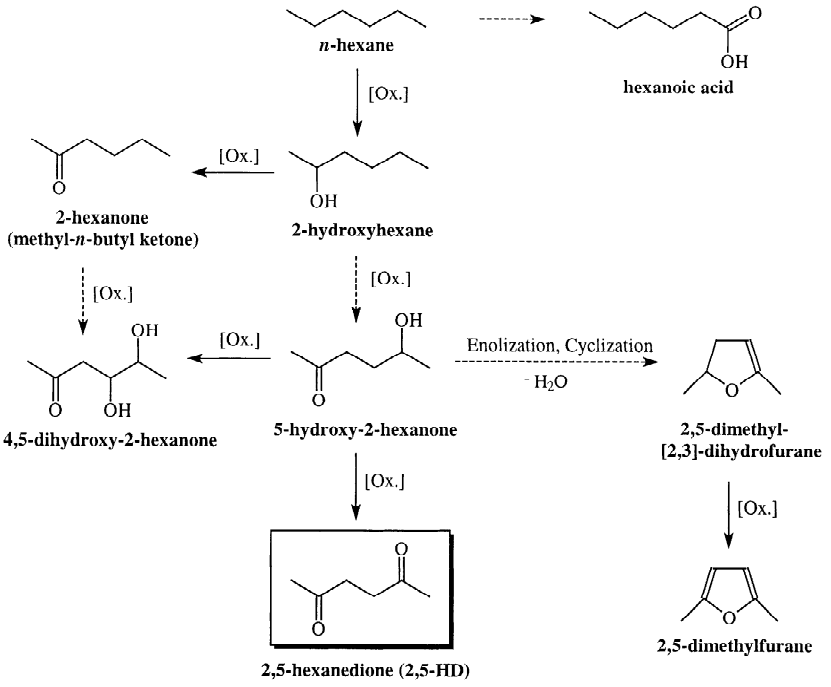

Fig. 1. Simplified scheme for the biotransformation of *n*-hexane in-vivo. Other metabolites (not shown here) are pentanoic acid, γ-valerolactone, and norleucine (2-aminohexanoic acid).

and without acidic hydrolysis prior extraction of 2,5-HD with dichloromethane. The techniques were compared and applied to a collective of urine samples derived from workers employed in the shoe manufacturing and shoe repair industry. Overall, the methods presented in the current manuscript represent a typical example for biological monitoring of occupational exposure to *n*-hexane.

2. Experimental

2.1. Chemicals

2,5-HD, and cyclohexanone (internal standard) were purchased from Merck, Darmstadt, Germany. All other compounds used for analysis can be regularly found a laboratory.

2.2. Apparatus

The concentrations of urinary 2,5-HD were determined using a HP 6890 gas chromatograph with flame ionization detection (GC–FID). The signals were integrated using a HP 3395 integrator. For the separation of 2,5-HD a capillary column HP-1 (crosslinked methyl siloxane, length 15 m, I.D. 530 mm, film thickness 1.5 μm) was used. The detector and injector temperatures of the GC were 250 and 200 °C. The starting temperature of the GC oven was 50 °C for 2 min. Then, the temperature raised to 100 °C with 5 °C/min and was held for 2 min. Finally, the temperature raised to 180 °C with 20 °C/min. Helium was used as a carrier gas at a flow-rate of 4.2 ml/min. Then, 1 μl of the sample was injected splitless.

2.3. Sample preparation

Subsequently, 1 g of NaCl, 60 μl of cyclohexanone (as internal stadard), and 2 ml of dichloromethane were added to 5 ml of a urine sample. The mixture was shaken for 90 s and then centrifuged (~2300 *g*) for 15 min. The organic fraction was analysed by GC–FID as described above. Another aliquot (5 ml) of the same urine sample was submitted to acidic hydrolysis prior to extraction with dichloromethane. The hydrolysis was performed by adding 300 μl of HCl to the urine sample and heating for 30 min at ~100 °C.

2.4. Calibration and quality control

Stock solutions of 2,5-HD (100.0 mg/l) and cyclohexanone (100.0 mg/l) were prepared in water and stored at 4 °C. From the stock solution of 2,5-HD, various standard solutions in a concentration range between 0.1 and 20 mg/l were prepared in urine. The standard solutions were used to study the linearity of the calibration curve. Moreover, calibration standards (0.1–10.0 mg/l) were included in every analytical run.

The limit of detection for 2,5-HD was determined from a signal-to-noise ratio of 3:1 in urine samples, while a signal-to-noise ratio of 6:1 was used as the limit of quantification.

A stability test of urine samples fortified with 2,5-HD was carried out over a time period of 11 weeks at various temperatures and different defrost cycles.

Internal quality control was carried out by the determination of the within-series imprecision, the between-day imprecision, and the determination of the recovery at three different concentrations (0.1, 5.0, and 10.0 mg/l). For the determination of the imprecision, samples from each concentration were analyzed six times, while for the determination of the recovery samples from each concentration were analyzed five times.

External quality control was carried by the participation at the FIOH (Finnish Institute of Occupational Health) interlaboratory quality assurance program. Certified reference material (2,5-HD in urine) was analyzed three times.

2.5. Urine collection and subjects

Post-shift urine samples (Friday) were collected from workers exposed to *n*-hexane by direct glue contact in seven shoe industries ($n=52$). Thirteen persons were male, 39 female, while six persons were smokers, 46 non-smokers. The duration of employment was in the range between 1 and 96 months. Control samples ($n=34$) were obtained from persons working in administrative sections of the same industries. Twelve persons were male, 22

female. The great majority was non-smokers ($n=32$). The duration of employment in the control group ranged between 1 and 132 months. All persons were not exposed to cyclohexanone, which was used as internal standard during the analysis. The urine samples were stored at −20 °C until the analysis was carried out. All persons were informed about the aims of the study and signed a declaration of consent to participate on this project. The protocol was submitted and approved by the Committee of Ethics in Research of the Faculty of Pharmaceutical Sciences, University of São Paulo.

The type of exposure to *n*-hexane differs among the industries depending on the safety factors involved in using the glue. The industries (and therefore the workers) were classified in three operation types:

1. industries, in which the glue was applied with a paintbrush to the leather ($n=14$),
2. industries, in which the glue was applied with a glue handgun ($n=27$), and
3. industries where the glue was applied to the leather using a fume hood ($n=11$).

2.6. Statistical analysis

A Wilcoxon test was carried out to compare both methods, with and without urine hydrolysis. A Kruskall–Wallis test was performed to compare the urinary levels of 2,5-HD in different types of industries as mentioned above. If the concentration of 2,5-HD was lower than the limit of detection (LOD: 0.05 mg/l), half the LOD was used for statistical analysis.

3. Results and discussion

The calibration curve for 2,5-HD was linear in the range between 0.1 and 20.0 mg/l. For practical reasons, calibration material in the range between 0.1 and 10.0 mg/l was included in each analytical series only. The criteria for acceptance of an analytical run was a correlation coefficient >0.98. If the calibration curve revealed a correlation coefficient <0.98, the clean-up of the samples from this series was repeated.

The limit of detection (LOD) and quantification (LOQ) of the method were determined to be 0.05 and 0.1 mg/l, respectively.

The average results for the within-series imprecision for all three concentrations (0.1, 5.0, and 10.0 mg/l) were determined to be 5.4%, while the average between-day imprecision was calculated to be 7.0%. The average recovery for 2,5-HD was found to be 101.7%. The external FIOH quality control program revealed inaccuracy of the developed method of 9.0%. Overall, the method was proven to be suitable for occupational health surveillance of *n*-hexane exposed workers and revealed a LOD far below the BEI [18], which is currently recommended for 2,5-HD after exposure to *n*-hexane (5 mg/g creatinine).

The stability test for spiked urine samples showed that the levels of 2,5-HD were stable for 48 h at room temperature, while 2,5-HD concentrations did not change after 7 or 11 weeks when the urine samples were kept at 4 or −20 °C. Levels of 2,5-HD were also unchanged after three defrost cycles.

The results for the determination of 2,5-HD in urine samples of the workers are shown in Fig. 2. Although the BEI value is given in mg 2,5-HD/g creatinine, our results are presented in mg/l, since there is no particular reason to normalize for creatinine. Fig. 3 shows the GC–FID chromatograms

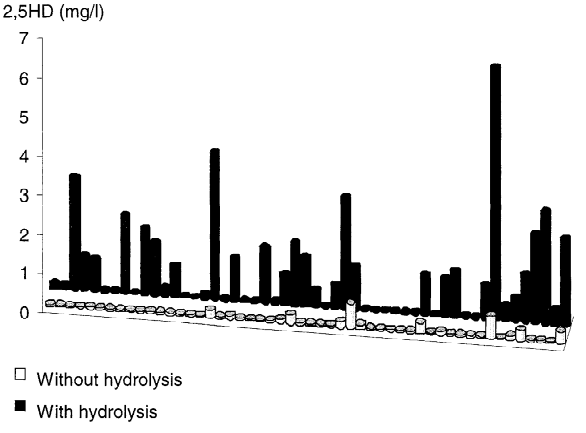

Fig. 2. Concentration of 2,5-HD (mg/l) found in the 52 samples of workers exposed to glue solvents in shoe industries (without and with hydrolysis).

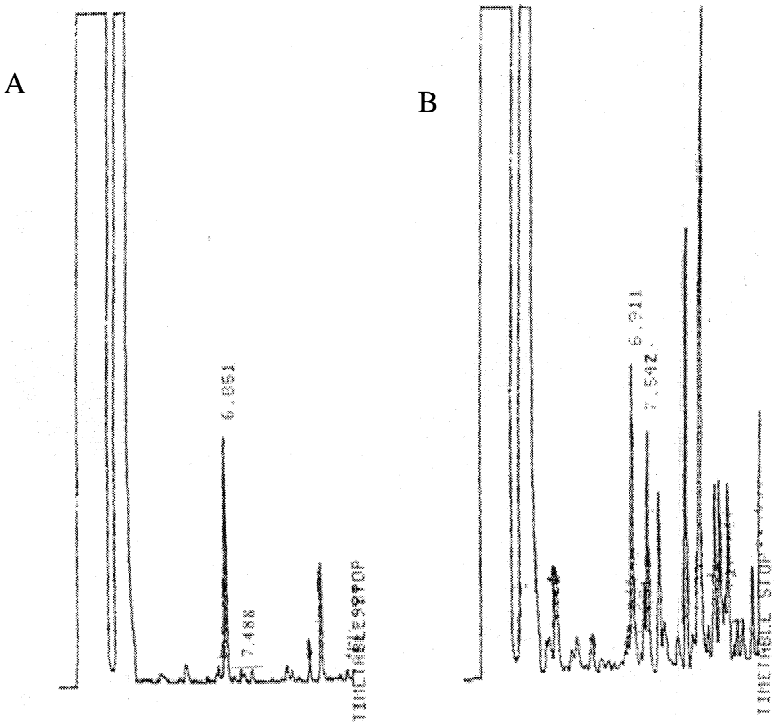

Fig. 3. Analytical chromatogram of a urine sample, without (A) and with hydrolysis (B) from a worker exposed to *n*-hexane by direct contact with glue. Retention times: cyclohexanone (internal standard) 6.8±0.2 min; 2,5-HD 7.5±0.2 min.

from one worker exposed to *n*-hexane. The concentrations of 2,5-HD in the urine samples of exposed workers determined by acidic hydrolysis were ~10-times higher than those determined without acidic hydrolysis. An average concentration of 0.94 and 0.09 mg/l was determined, respectively. The difference can be explained by the fact that during hydrolysis of urine sample, other metabolites of *n*-hexane, e.g. 5-hydroxy-2-hexanone or 2,5-dimethylfurane, can be converted to 2,5-HD. Therefore, results obtained for 2,5-HD after acidic hydrolysis accounts for the total exposure to *n*-hexane, while results obtained without hydrolysis are more specific for the toxic metabolite 2,5-HD itself. Overall, the results are in accordance with others described in literature [15,16,34–36]. Detailed information on the results (descriptive statistical analysis) for the exposed workers can be found in Table 1. Fig. 4 and Table 2 shows the average concentrations of 2,5-HD in urine samples of exposed workers dependent on the three industry types. As can be seen from Fig. 4, exposure to *n*-hexane is clearly dependent on the operation type, how the glue is handled. Exposure to *n*-hexane was found to be highest in the group of workers, who applied glue on the leather by using a paintbrush (group 1), while

Table 1
Descriptive statistical analysis for the comparison of the methods without and with hydrolysis of the urine sample for determination of the 2,5-HD (mg/l)

Method	Average	Median	SD	Minimum	Maximum	PS	*n*
Without hydrolysis	0.09	0.025	0.14	0.025	0.61	16	52
With hydrolysis	0.94	0.62	1.19	0.025	6.41	39	52

SD, standard deviation; PS, positive samples; *n*, total number of analyzed samples.

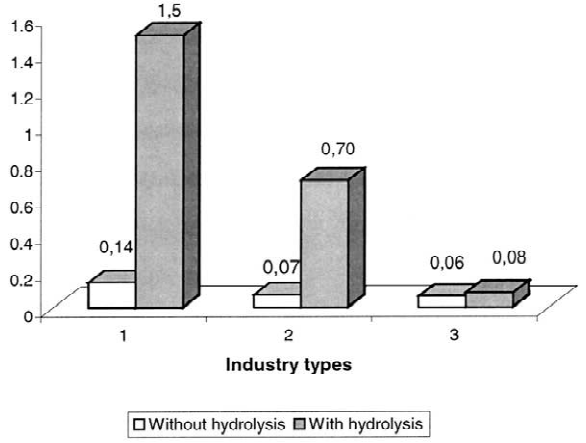

Fig. 4. Average concentrations of 2,5-HD (mg/l), in urine samples from workers exposed to glue solvents according to the operation type in various industries.

exposure was lowest in workers, who used a fume hood during their work (group 3). Total exposure to *n*-hexane is better assessed using an acidic hydrolysis during clean-up of the samples. The result represents the sum of free *n*-hexane and those metabolites, which are converted to *n*-hexane during hydrolysis. For acidic hydrolysis a 10-fold decrease was observed in the body burden between workers from group 2 (glue handgun) and group 3 (hood). A 20-fold decrease was even observed between workers of group 1 (glue paintbrush) and group 3 (hood). The results point to a considerable uptake of *n*-hexane via the ambient air. However, the levels are predominantly influenced by non-specific metabolites of *n*-hexane, which can be clearly seen by the fact, that the overall decrease of free 2,5-HD (which can be assessed by analysis without acidic hydrolysis of the urine samples) between group 1 and group 3 is only ~2-fold. The observed effect can be also seen, if the ratio ''total 2,5-HD''/''free 2,5-HD'' for the three groups is studied. The ratio can be determined to be 10.7/10.0/1.3 in groups 1, 2 and 3, respectively. With regard to the toxic metabolite 2,5-HD, the predominant measure of reducing exposure was the use of a glue handgun instead of a glue paintbrush (~2-fold decrease) and not necessarily the use of a hood, because no differences could be observed for the levels of free 2,5-HD between workers of group 2 and 3. It is necessary to mention, that the results for free 2,5-HD were also close to the LOD of the method and therefore influenced by the imprecision of the analysis at such low concentrations. Beside exposure via the ambient air, dermal absorption was also discussed by Cardona et al. [14]. They found increased levels of 2,5-HD in workers who does not wear protective gloves and concluded that cutaneous exposure seems to be an important factor during exposure to *n*-hexane. Although our results cannot totally exclude dermal absorption of *n*-hexane, the predominant route of uptake seems to be via air. Our results are in accordance with the newest findings of the DFG in Germany, who re-evaluated the route of uptake for *n*-hexane in their recently published MAK- and BAT values [19]. *n*-Hexane did loose its skin notification ''H'' and the DFG concluded that dermal absorption does not really contribute to the toxicity of *n*-hexane.

Table 2
Average concentrations of 2,5-HD (mg/l) in urine samples from workers exposed to glue solvents according to the operation type in various industries

Operation type	*n*	Without hydrolysis	With hydrolysis
1	14	0.14	1.50
2	27	0.07	0.70
3	11	0.06	0.08

n, number of samples analyzed.

As expected, different results were observed for the control group not exposed to *n*-hexane. It was not possible to detect any 2,5-HD in urine samples with acidic hydrolysis. Even after acidic hydrolysis, 2,5-HD could be detected in only one urine sample from control individuals. Our results are in contrast to others reported in the literature. Various authors found concentrations for 2,5-HD in urine from non-exposed persons in the range between 0.12 and 0.78 mg/l [14,22,30,36]. The conversion of certain ketones in the diet was discussed as a possible contributing factor for 2,5-HD in urine. Unfortunately, aspects such as diet were not considered in the study presented here and can therefore not be thoroughly discussed. Sample chromatograms from one control subject (with and without acidic hydrolysis) are shown in Fig. 5.

In summary, we presented two methods for the determination of 2,5-HD in urine samples, which are suitable for biological monitoring. The first method is based on an acidic hydrolysis of the urine sample.

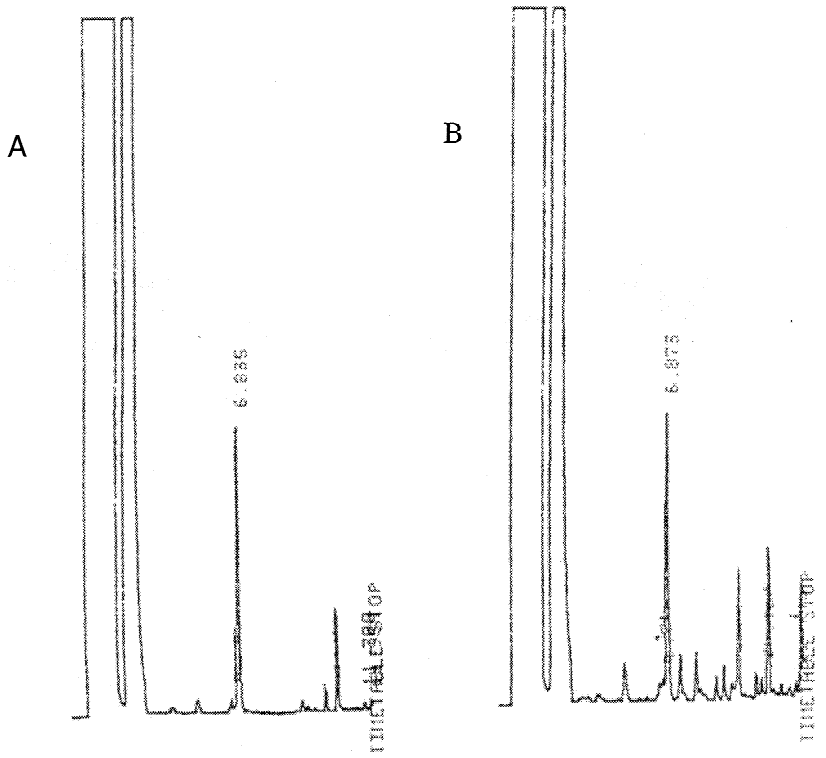

Fig. 5. Analytical chromatogram of a urine sample, without (A) and with (B) hydrolysis from a control subject not exposed to *n*-hexane. Retention times: ciclohexanone (internal standard) 6.8±0.2; 2,5-HD 7.5±0.2 min.

Using this method the ''total'' exposure to *n*-hexane is determined, since during acidic hydrolysis other metabolites of *n*-hexane are converted to 2,5-HD. Therefore, it may be the method of choice to prevent any toxic effects after exposure to *n*-hexane, although the method overestimates 2,5-HD in urine. However, it is known that only 2,5-HD itself contributes to the toxicity of *n*-hexane. Moreover, 2,5-HD may be also formed from ketones in the diet. Therefore, from a toxicological point of view, it is preferable to analyze 2,5-HD in urine samples only, which can be done by omitting acidic hydrolysis during clean-up. A sample clean-up without acidic hydrolysis would also help to establish causal relationships between 2,5-HD in urine and toxic effects of *n*-hexane.

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