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Urinary Levels of Free 2,5-Hexanedione in Italian Subjects Non-Occupationally Exposed to n-Hexane

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Abstract: The purpose of the study is to evaluate the urinary levels of free 2,5-hexanedione (2,5-HD) in Italian subjects non-occupationally exposed to n-hexane, in order to define background values in non-occupational settings. The study was carried out on 99 subjects of the general population. The analysis of free 2,5-HD was performed by gas chromatography-mass spectrometry. Personal information about the subjects was ascertained by means of a self-administered questionnaire. The urinary levels of free 2,5-HD were in the range of <12.0–77.9 μg/L (5th–95th percentiles). The urinary excretion of the metabolite did not seem to be influenced by gender, age, smoking habit or area of residence. Statistically significant differences ($p = 0.03$) were found between the free 2,5-HD urinary levels according to the vehicular traffic intensity within the area of residence and to body mass index of subjects. The background levels of free 2,5-HD found in this study could contribute to the definition of reference values of general population non-occupationally exposed and could be useful to the toxicologists and industrial hygienists to determine whether workers have been exposed to higher levels of n-hexane than the general population.

Keywords: n-hexane; free 2,5-hexanedione; biological monitoring; non-occupationally exposed population

1. Introduction

Occupational exposure to n-hexane occurs in different industrial processes for the production of paints, glues, polyethylene and polypropylene foils, plastics, rubbers and tires where it is used as solvent [1]. Its worldwide diffusion started after the use of benzene as a solvent was banned. n-Hexane is considered a ubiquitous pollutant. It is present not only in various industrial materials [2–5] but also in home-use solvents [6] and it is emitted from furniture, paint or building/furnishing materials [7]. Experiments carried out on the emission at room temperature from particleboard showed that n-hexane was the most abundant compound among detected volatile organic compounds except formaldehyde [8]. A study carried out in three Italian cities has confirmed the continued presence of this solvent in air with average concentrations (indoor and outdoor) ranged between 8.4 and 15.2 μg/m^3 [9]. Likewise, another study carried out in two campaigns (summer and winter) in about 140 rooms of office buildings across Europe, measured indoor concentrations of n-hexane up to 9.1 and 8.6 μg/m^3, respectively [10]. n-Hexane is present also as a component of gasoline [11,12]. Since 1980, the increasing use of unleaded gasoline with a high-octane rating has led to a growing content of n-hexane in gasoline, which now stands at around 3%.

The main route of absorption is via inhalation. The metabolic pathway of the n-hexane in humans consists of a series of oxidative steps (Figure 1) which lead to the formation of several metabolites including 2,5-hexanedione (2,5-HD). This metabolite appears to be responsible for the polyneuropathy due to the exposure to n-hexane [13,14]. It is excreted through the urine after conjugation with sulfate anion or glucuronic acid or as free 2,5-HD. Total 2,5-HD was used as a biomarker for the monitoring of workers exposed to n-hexane [15,16]. It was given by the sum of the free and the 2,5-HD obtained by the conversion of other metabolites of n-hexane (4,5-dihydroxy-2-hexanone and 5-hydroxy-2-hexanone) after acid hydrolysis of the urine sample [17,18].

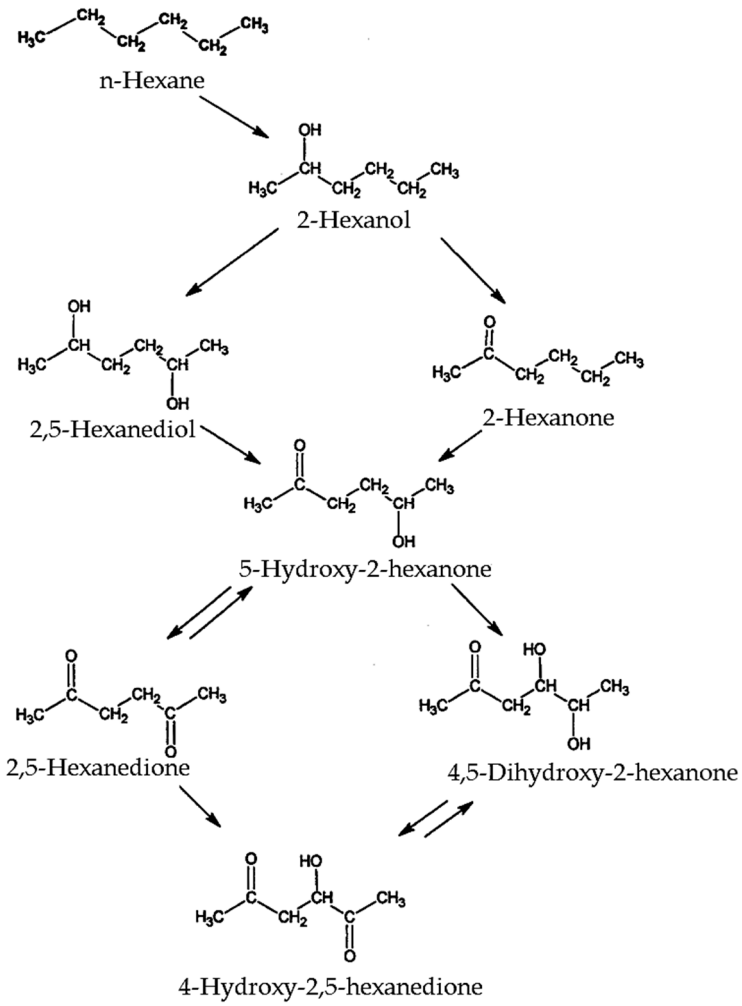

Figure 1. Metabolic pathway of n-hexane in humans.

Total 2,5-HD was not only detected in the urine of workers occupationally exposed to n-hexane, but was detected, in lower concentrations, in the urine of subjects of the general population by several studies [19–26]. The knowledge of the urinary levels of this metabolite determined in conditions of non-occupational exposure and of the confounding factors that may affect them, is essential for interpreting results of biological monitoring in the exposure assessment. In the occupational field the comparison between the data from biological monitoring in workers and the background levels in non-occupationally exposed subjects is informative about the existence of exposures greater than the general population.

The importance of background values is demonstrated by the growing number of studies carried out on the general population all over the world [27–29].

Since 2005, the Italian Society of Reference Values (SIVR) defined ranges of reference values in the urine of non-occupationally exposed subjects for many biomarkers. SIVR reported for total 2,5-HD a range (5th–95th percentiles) between 100 and 760 µg/L [30].

In the last few years, total 2,5-HD no longer appeared to be the best biomarker of exposure to n-hexane, although it appears correlated with airborne n-hexane, because of the analytical procedure to quantify it, that involves an acidic hydrolyzation, also converts non-toxic metabolites into 2,5-HD and the yield varies with the hydrolysis conditions and the laboratory performances. Free 2,5-HD seems to be more reliable than total in order to assess the exposure to n-hexane [18,23,31].

The purpose of the present study is to evaluate the urinary levels of free 2,5-HD in non-occupationally exposed subjects to n-hexane, in order to contribute to the definition of background values and to provide a useful element for the occupational exposure assessment to this solvent.

2. Materials and Methods

2.1. Study Population

The study was carried out on 99 subjects non-occupationally exposed to n-hexane. Before enrollment, each subject received information about the aim of the study and was invited to fill up a questionnaire in order to collect personal information about gender, age, body mass index (BMI), smoking habit, area of residence and vehicular traffic intensity. An identification number was assigned to each completed questionnaire. The characteristics of the studied subjects are summarized in Table 1.

Table 1. Characteristics of the studied population.

Parameter	Males	Females	Total
Number	52	47	99
Smoking habit			
No	32	33	65
<5 cigarettes/day	9	6	15
5–10 cigarettes/day	3	5	8
11–20 cigarettes/day	8	3	11
Residence			
Urban	37	37	74
Rural	15	9	24
Industrial	-	1	1
Traffic intensity			
Low	20	14	34
Medium	27	21	48
High	5	12	17
Age			
<30 years	16	8	24
30–50 years	25	30	55
>50 years	11	9	20
BMI			
<25 kg/m^2	30	35	65
25–30 kg/m^2	15	8	23
>30 kg/m^2	7	4	11

2.2. Analytical Method

Each subject provided one spot fresh urine sample (the second micturition of the morning). The samples were collected in sterile polypropylene containers without the addition of preservatives or stabilizers and stored at −20 °C until analysis, of which was performed within 2 weeks from collection. Blank samples (water) were analyzed to confirm no contamination from the containers. Free

2,5-HD urinary concentration was determined by gas chromatography coupled with mass spectroscopy (GC/MS) according to the method of Perbellini et al. with some modifications [32]. Briefly, after addition of a volume of 50 µl of a solution of cyclohexanone of 23.7 mg/L, as an internal standard, free 2,5-HD was extracted from the urine samples (5 mL) using diatomaceous earth columns and dichloromethane as solvent (volume of 8 mL, twice). In order to maintain the analyte into the solution and avoid its evaporation, p-Isopropyl-toluene (10 µL) was added to the organic solution as a *keeper* [33]. The organic solutions were evaporated to reduce their volume until about 500 µL; the concentrated extract was then transferred to autosampler vials.

One microliter of the dichloromethane solution was injected into the GC/MS system to the free 2,5-HD determination. All analyses were performed on a Perkin Elmer AutoSystem XL Gas Chromatograph coupled with a Turbo Mass Upgrade quadrupole mass spectrometer. Chromatographic separation of analytes was carried out through an Agilent Technologies PONA capillary column of the following dimensions: length 50 m, internal diameter 0.2 mm and film thickness 0.5 µm, at a constant helium flow rate of 1.4 mL/min. The GC oven was programmed from an initial temperature of 50 °C (held for 1 min) to 80 °C at 10 °C/min (held for 5 min) and to 200 °C at 20 °C/min (held for 5 min) with a transfer line temperature of 250 °C. The injection temperature was 250 °C. The MS detector was operated in EI mode (70 eV). Single ions m/z 55 and 99 Da were registered for the quantification (Dwell time 0.05 sec). The 2,5-HD and cyclohexanone retention time were 11.32 and 10.36 min, respectively.

Ten replicates of two standards in urine spiked with concentrations 48.65 µg/L and 389.2 µg/L of 2,5-HD independent of the calibration standards, were prepared (50 mL each) and 5 mL were tested the same day in order to assess intra-day precision; the remainder was divided into 5 mL aliquots, stored at −20 °C and tested on four subsequent days in order to assess the inter-day precision. For the two concentrations 48.65 µg/L and 389.2 µg/L, the intra-assay precisions were 2.98% and 2.20% and the inter-assay precisions were 3.07% and 2.06%, respectively.

The accuracy was calculated by analyzing ten aliquots of urine, containing 2,5-HD concentrations at the two levels previously described. The relative percentage error of the calibration curve was found to range between 0.69% and 2.77%.

The quantification limit, expressed as ten times the ratio between the standard deviation and the calibration plot slope, was 12.0 µg/L; the standard deviation was calculated by injections at the lowest point of the calibration curve [34].

On a smaller number of subjects (76 of 99, because urine samples from 23 subjects were not sufficient to perform the analysis) the concentration of total 2,5-HD was also analyzed, using the same analytical method previously described, preceded by hot acid hydrolysis.

2.3. Statistical Analysis

Statistical analysis was carried out using the StatsDirect statistical software. A non-normal distribution of the variable was observed by the Shapiro–Wilk test. Thus, the non-parametric test was applied to compare the differences between groups (Mann–Whitney U-test). Correlations between variables were assessed by linear regression analysis. In all tests, a p-value lower than 0.05 (two-tailed) was considered statistically significant. If the concentration of free 2,5-HD was lower than the limit of quantification (LOQ), half of the LOQ was used for statistical analysis as a balance point between the solutions that underestimate (equal to 0) or overestimate (equal to LOQ) the true value.

3. Results

The study population aged 39.6 ± 12.8 years (range: 17–67 years), was composed of 52 males (52.5%) and 47 females (47.5%). The percentage of smokers was 34.3%, 41% of whom were females and 59% males. Moreover, almost 75% of the subjects lived in urban areas, 54% of which lived in areas characterized by medium intensity of vehicular traffic and 20% in high traffic density areas. Regarding weight, 65.7% of subjects were normal weight (BMI < 25 kg/m^2), 23.2% were overweight

(25 < BMI < 30 kg/m^2) and 11.1% were obese (BMI > 30 kg/m^2). The characteristics of subjects were comparable to those of the Italian population in the same range of age [35].

The levels of free 2,5-HD in the 99 samples were in the range of <12.0–154.9 µg/L and had a non-normal distribution (Shapiro–Wilk test). A total of 39.4% of data (39 subjects) were below the quantification limit of 12 µg/L (Figure 2).

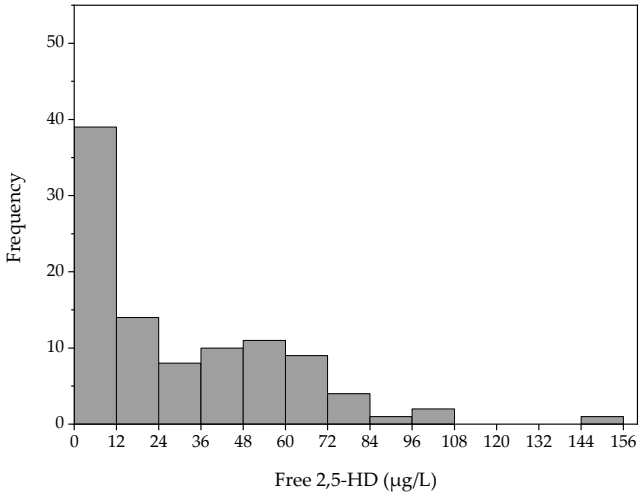

Figure 2. Distribution of the free 2,5-hexanedione (2,5-HD) levels in the studied subjects (µg/L).

Table 2 reports the description in terms of percentiles, geometric mean, arithmetic mean and variability of data considered in totality as well as stratified by gender, class of age, smoking habit, residence and traffic intensity, BMI, expressed in µg/L.

Table 2. Urinary levels of free 2,5-HD in all samples and differentiated by gender, class of age, smoking habit, area of residence, traffic intensity and BMI.

	Sample Size	Geometric Mean ± GSD (µg/L)	Mean (µg/L)	Selected Percentile (µg/L)				
				5th	25th	50th	75th	95th
Total	99	19.1 ± 2.8	31.4	<12.0	<12.0	20.3	54.3	77.9
Gender								
Males	52	17.9 ± 2.7	31.8	<12.0	<12.0	20.6	58.2	85.5
Females	47	18.1 ± 3.0	31.0	<12.0	<12.0	20.3	52.2	72.2
Age								
<30 years	24	19.8 ± 2.7	30.3	<12.0	<12.0	21.5	49.4	79.7
30–50 years	54	19.8 ± 2.9	32.1	<12.0	<12.0	23.9	53.1	74.2
>50 years	21	17.0 ± 3.1	30.9	<12.0	<12.0	14.3	65.0	77.4
Smoking habit								
No	65	17.1 ± 2.7	27.0	<12.0	<12.0	15.9	47.4	69.0
Smokers	34	23.8 ± 3.1	39.9	<12.0	<12.0	33.0	59.9	97.4
<5 cigarettes/day	15	24.9 ± 2.7	35.5	<12.0	<12.0	37.9	57.9	66.2
5–10 cigarettes/day	8	16.3 ± 3.1	27.5	<12.0	<12.0	14.4	42.4	71.7
11–20 cigarettes/day	11	29.5 ± 3.8	54.9	<12.0	<12.0	57.9	84.6	128.0
Residence								
Industrial	1	-	-	<12.0		-		
Urban	74	17.7 ± 2.9	29.9	<12.0	<12.0	16.7	52.4	75.9
Rural	24	25.7 ± 2.7	37.3	<12.0	<12.0	31.4	58.5	81.0
Traffic intensity								
Low	34	26.3 ± 2.8	39.9	<12.0	<12.0	36.4 [a]	60.5	89.3
Medium	48	15.6 ± 2.8	27.1	<12.0	<12.0	13.3	40.3	76.6
High	17	17.9 ± 2.6	26.9	<12.0	<12.0	15.9	47.4	68.4
BMI								
<25 kg/m^2	65	16.9 ± 2.8	27.8	<12.0	<12.0	16.0 [b]	47.0	68.3
25–30 kg/m^2	23	27.6 ± 2.9	41.9	<12.0	<12.0	39.4	85.0	93.6
>30 kg/m^2	11	18.4 ± 2.9	31.3	<12.0	<12.0	15.9	44.3	90.7

[a] $p = 0.03$, Low traffic intensity vs medium traffic intensity (Mann–Whitney test); [b] $p = 0.03$, BMI < 25 kg/m^2 vs 25 < BMI < 30 kg/m^2 (Mann–Whitney test).

The 5th–95th percentile range of data was <12.0–77.9 μg/L. No differences were found in relation to the gender or class of age, although the 2,5-HD excretion was lower in the subjects over 50 years old (see geometric mean or median). Higher levels of free 2,5-HD levels were observed in smokers (median 33.0 μg/L) than in non-smokers (median 15.9 μg/L) or in subjects who lived in rural areas (median 31.4 μg/L) than those found in urine of subjects who lived in urban areas (median 16.7 μg/L), but the differences, even in these cases, were not statistically significant ($p = 0.14$, Mann–Whitney test).

Urinary levels of free 2,5-HD were also evaluated according to the intensity of vehicular traffic in the area of residence. A statistically significant difference ($p = 0.03$, Mann–Whitney test) was found between the free 2,5-HD urinary excretion in subjects who lived in lowintensity vehicular traffic areas (median 36.4 μg/L) in comparison of those who lived in medium intensity vehicular traffic areas (median 13.3 μg/L). A statistically significant difference ($p = 0.03$) was also found between subjects with a BMI < 25 kg/m^2 (median 16.0 kg/m^2) and subjects with a BMI within the range 25–30 kg/m^2 (median 39.4 kg/m^2).

Total 2,5-HD levels analyzed in 76 subjects, were in the range 61.1–402.8 μg/L (5th–95th percentiles), with a mean value equal to 177.9 μg/L (geometric mean ± GSD: 151.2 ± 1.8 μg/L; median 151.5 μg/L), comparable to the range of reference values adopted by SIVR of <100–760 μg/L (5th–95th percentiles) [30].

A statistically significant correlation (r = 0.56; $p < 0.0001$) was found between total 2,5-HD and free 2,5-HD levels above the LOQ (Figure 3). According to the correlation equation, free 2,5-HD represented about 18% of the total.

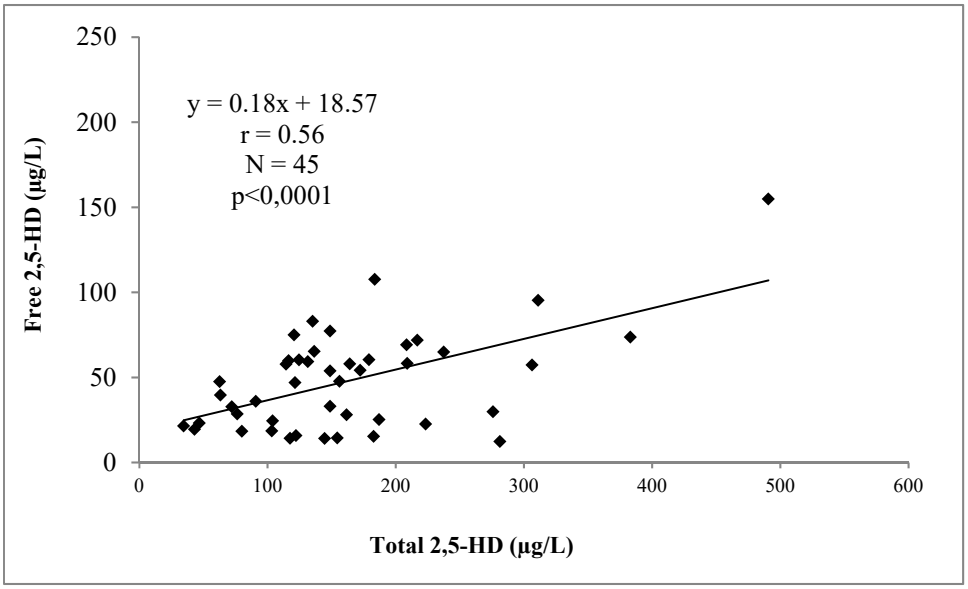

Figure 3. Correlation between the levels of free and total 2,5-HD.

4. Discussion

The use of free 2,5-HD as a biomarker to assess the occupational exposure to n-hexane was preferred, by some authors, over the total 2,5-HD, after acidic hydrolysis of urine during the quantitative analysis. This latter analytical phase, which coverts other nontoxic metabolites in 2,5-HD in addition to the fact that the toxic effects have been linked to the free 2,5-HD itself [36,37], appears to make free 2,5-HD a better biomarker of exposure and neurotoxic risk.

Some organizations proposed free 2,5-HD as a biomarker of occupational exposure to n-hexane and established limit values for this metabolite. The Japan Society of Occupational Health (JSOH), adopted a biological limit value of 0.3 mg/g creatinine [38]. Since 2003, the American Conference of Governmental Industrial Hygienists (ACGIH), instead, adopted a biological exposure index (BEI) equal to 0.4 mg/L [39] that was changed to 0.5 mg/L, on the basis of more recent studies,

in 2019 [40]. On the contrary with what was established by JSOH, ACGIH reported that no correction for creatinine concentration was recommended because it does not give a significant improvement in the exposure–excretion relationship [31]. The JSOH and ACGIH limit values could be considered substantially superimposable if a mean value of creatinine of 1.69 g/L is taken into account [41].

In occupational medicine, the comparison of biological monitoring data in the exposure assessment with the occupational limit value is essential to evaluate the probability of occurrence of health effects and the need of a reduction of exposure, as the comparison with the background level of a biomarker, could be very useful to health professionals to assess the exposure that is likely to be occupational and not from the general environment. Levels at or above the levels of biomarker in the fluids of the general population will have a high probability of resulting from occupational exposure.

The aim of the present study is to contribute to the definition of the background urinary levels of free 2,5-HD in the Italian general population. Many studies report the levels of total 2,5-HD in the urine of non-occupationally exposed subjects quantified after acidic hydrolysis of urine [23,26,42,43] or they quantified free 2,5-HD in urine of occupationally exposed subjects [22,44,45] but the studies that analyzed the levels of free 2,5-HD in general population are very few [21,46]. Moreover they were carried out on a small number of subjects or the analytical method used had a very high limit of detection. In these studies the levels of total 2,5-HD in urine from non-occupationally exposed subjects varied from about 100 µg/L to 1 mg/L [42]; instead, a detectable amount of free 2,5-HD was not found [21,46]. Free 2,5-HD represents a small percentage of the total. In a study where 2,5-HD was measured in the urine of workers in the shoe industry, the urinary levels of free metabolite were in the range of 30 to 5590 µg/L with a mean value equal to 790 µg/L and represented the 14.2% of the total [45].

A lower ratio between free and total 2,5-HD was found by Perbellini et al. [23] and Bavazzano et al. [24] equal to 8% and 10%, respectively. A ratio equal to 10% was reported, as well, by JSOH that established a biological limit value of 0.3 mg/g creatinine for free 2,5-HD and 3 mg/g creatinine for total 2,5-HD [38]. In another study, the amount of free 2,5-HD was about one-fifth of the total 2,5-HD, although the percentage of free to total 2,5-HD varied from 0.4% to 28% in relation to the individual variability [47]. This large variability could be due to the different n-hexane metabolic rates or different factors that can affect its excretion such as ethnicity, gender, cigarette smoke or age. Some authors reported a less active form of cytochrome P450 2E1 (CYP 2E1) in the Asian population compared to the Caucasian population [48] or reported that CYP 2E1 levels decrease with age [49].

In our study, free 2,5-HD was quantified in 61.6% of urine of studied subjects and it represented 18% of the total 2,5-HD.

No difference was found in the urinary excretion of free 2,5-HD according to gender in contrast to what was found by some authors for the total 2,5-HD [26,43]. The lack of this difference could be due to the fact that the influence of metabolic rate is more, or only, evident on the fraction of metabolite that derives from the acidic hydrolysis (about 80–90% of total 2,5-HD) than the fraction of free 2,5-HD. According to our data it seems unnecessary to define a different range of free 2,5-HD in the urine of the general population in relation to the gender.

A higher urinary excretion of free 2,5-HD was found in smokers than non-smokers and inverse relation to age, although the difference was not statistically significant. The influence of these two factors was also found on the excretion of total 2,5-HD in a study carried out on 8235 subjects of the Chinese general population, where the results showed how the total 2,5-HD urinary levels statistically increase in relation to the smoking habit and statistically decrease with the increasing of age [43].

Unexpectedly, a statistically significant difference was instead found between the urinary levels of metabolite in relation to vehicular traffic intensity within area of residence of the subjects. The levels were higher in the urine of subjects exposed to low vehicular traffic intensity than those exposed to medium vehicular traffic intensity ($p = 0.03$, Mann–Whitney test). This result could be justified considering the data reported in literature, regarding the effect of inhibition of n-hexane biotransformation due to the co-exposure of toluene or xylene caused by urban traffic [50,51]. However, this result needs to be better

confirmed for accuracy as the traffic intensity in the living area was acquired by a self-administered questionnaire, and subject to the information provided by the studied subjects herein.

Interestingly, a statistically significant difference was also found in relation to BMI. Free 2,5-HD levels were higher in the urine of overweight subjects than the normal weight ($p = 0.03$). This result could be related to the high distribution in the adipose tissue, delaying the excretion of the solvent and bring about its accumulation [52]. However, this significant difference was not found between obese and normal subjects, probably in relation to the small number of subjects (only 11 subjects with BMI higher than 30 kg/m^2), although in these subjects the values tend to be higher. This result must be confirmed in a larger number of samples.

In conclusion, the background levels of free 2,5-HD reported in the present study in the range <12.0–77.9 µg/L, representing 5th–95th percentiles of data could contribute in the definition of reference values of general population non-occupationally exposed and be useful to the toxicologists and industrial hygienists to determine whether workers have been exposed to higher levels of n-hexane than general population. The background levels represent the lower limit that should be considered for the adoption of prevention measures in the workplace.

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