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UPPER REFERENCE LIMITS FOR BIOMARKERS OF EXPOSURE TO AROMATIC DIISOCYANATES

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ABSTRACT

Objectives. The objectives of this study were to determine the levels of 2,4-, and 2,6-toluenediamine, 1,5-naphthtalenediamine and 4,4'-methylenediphenyldianiline in hydrolyzed urine and plasma for occupationally unexposed workers and to calculate upper reference limits (URLs). These analytes are biomarkers of exposure to 2,4- and 2,6-toluene diisocyanate (2,4-TDI and 2,6-TDI), 1,5-naphtalene diisocyanate (NDI) and 4,4'-methylenediphenyl diisocyanate (MDI), respectively.

Methods. The biomarker levels were determined in urinary and plasma samples obtained from 121 occupationally unexposed workers. Based on these biomarkers levels and the biomarker levels in an occupationally exposed group of workers, URLs were calculated. The method used for these calculations was based on the receiver operator characteristic curve method technique, and the URLs were set at the optimum of sum of sensitivity and specificity.

Results. URLs for the different diisocyanates were calculated to be in the range of 0.1-0.5 μg/L. Occupationally unexposed workers had detectable biomarker levels of the diisocyanates investigated. Especially abundant was the biomarkers of MDI which were found in 97% of both urinary and plasma samples. For the other biomarkers, 0-15% of the unexposed workers had detectable levels. The detected levels were mostly close to the limit of detection (LOD), but urinary levels of biomarkers of MDI up to 60 times the LOD were found. The sensitivities and specificities for classification of the workers as occupationally exposed or not, were in the range of 88-100% and 97-100%, respectively.

Conclusions. URLs were calculated that may be applicable when screening for occupational exposure. A worker with a biomarker level above the URL will be classified as occupationally exposed. Biomarkers of aromatic diisocyanates, especially biomarkers of

MDI, were present among occupationally unexposed workers, but the source and nature of the exposure is unknown.

Key Words. Reference values, biological monitoring, occupational exposure, polyurethane, receiver operator characteristic curves.

INTRODUCTION

Aromatic diisocyanates are respiratory irritants that may cause occupational asthma (Vandenplas et al 1993, Baur et al 1994, Liu and Wisnewski 2003), and are used in the manufacturing of polyurethane (PUR). The occupational exposure limits for diisocyanates in western countries, are today typically 5 ppb for an 8 h work-shift and 10-20 ppb for short-term exposure peaks. Assessment of occupational exposure to aromatic diisocyanates is usually done by air monitoring, but may also be performed by biological monitoring. Potential biomarkers of exposure to aromatic diisocyanates are protein adducts (Sabbioni et al 2001), urinary metabolites (Sepai et al 1995) and isocyanate-specific IgG antibodies (Lushniak et al 1998). Biological monitoring of exposure to aromatic diisocyanates may be performed by analysis of the hydrolytically released diisocyanate adducts/metabolites in urine or plasma. The thereby released diisocyanate-related diamines are the most commonly used biomarkers of diisocyanate exposure (Sepai et al 1995, Lind et al 1997, Kääriä et al 2001, Sennbro et al 2004a). At present, there is only one biological exposure limit applied for aromatic diisocyanates. This is the German biological tolerance value (BAT) for 4,4'-methylenediphenyl diisocyanate (MDI), whose biomarker is 4,4'-methylenedianiline (MDA). The BAT value is 10 μg/g creatinine of MDA in urine.

In biological monitoring of exposure, apart from BAT values, reference values (RVs) for the biomarkers may also be useful when evaluating biomarker levels. The RVs may be used in order to identify occupationally or environmentally exposed subjects (Aitio 1994). RVs are the measured concentrations of a biomarker in the biological fluids of a reference population, and indicate the background exposure to a given chemical (Alessio 1992). The reference population is a theoretical population, consisting of all possible individuals fulfilling defined criteria (Solberg 1987a). In practice, a representative reference sample group, consisting of an adequate number of reference individuals, is selected from the reference population (Solberg 1987a, b). The primary inclusion criteria for reference individuals in occupational toxicology studies, is that they should neither be occupationally exposed to nor be subjected to a known abnormal environmental pollution to the investigated chemical (Alessio 1992, Apostoli 1999). The RVs may differ globally as well as locally and may also differ in different subgroups according to sex, age, smoking, etc. Furthermore, the RVs may vary in time due to changes in environmental exposure (Ewers et al 1999). In preventive biological monitoring, it is useful to determine the upper reference limit (URL) (Aitio 1994). The URL is derived from the distribution of the RVs, but is not a mathematically defined entity. Usually, the URLs are set at the mean concentration plus two standard deviations, or as the 90^{th} or 95^{th} percentile (Alessio 1992). In other words, the URL corresponds to an upper boundary of the background exposure in occupationally unexposed subjects. In some cases, the URL may correspond to the limit of detection (LOD) of the analytical method. RVs or URLs for biomarkers of aromatic diisocyanates have not previously been reported. In the present study we have determined biomarkers of 2,4-toluene diisocyanate (2,4-TDI), 2,6-toluene diisocyanate (2,6-TDI), 1,5-naphtalene diisocyanate (NDI) and MDI, respectively, in hydrolyzed urine and plasma from a group of workers, occupationally unexposed to aromatic

diisocyanates. These biomarkers were 2,4-toluenediamine (2,4-TDA), 2,6-toluenediamine (2,6-TDA), 1,5-diaminonaphthalene (NDA) and MDA.

The objectives of our study were to determine RVs and to calculate URLs for these urinary and plasma biomarkers.

MATERIALS AND METHODS

Study design

The study comprised a reference group consisting of workers from five different workplaces, selected in southern Sweden. The criteria for selection of these plants, apart from the geographic region, were that no isocyanates, PUR or other plastics should be handled and that no heating operations were performed. Hence, the workers at these plants were expected to be occupationally unexposed to the diisocyanates investigated in this study. The total 121 workers included 27 postmen employed at a mail distribution company, 20 meat cutters employed at a butchery, 25 assemblers of armature at an electric light fittings company, 38 employees at a supermarket and eleven operators from a dairy product plant. Further characteristics of the reference group are described in table 1.

The study also comprised an exposed group consisting of 110 occupationally exposed workers from thirteen different plants, selected in southern Sweden, previously described by Sennbro et al (2004b). The criteria for selection of the exposed plants, apart from the geographic region, were that PUR or isocyanates should be handled on daily basis by at least three employees. Furthermore, the plants with the expected highest isocyanate exposure, including different types of industrial processes, were selected. The plants were two continuous foaming plants, two flame lamination plants, seven moulding plants and two

plants with low- or non-heating processes. Each worker was classified as "exposed" to each specific diisocyanate, if quantifiable exposure was found according to personal whole-day air measurements. This was performed once for each worker, using a method with 1-(2-methoxyphenyl)piperazine impregnated filters (Health and Safety Executive, 1999).

All biological samples, except the urinary samples from 23 of the exposed workers, were collected in connection with a medical examination. The 23 urinary samples were collected at the day of air monitoring, which was carried out within two weeks from the day of the medical examination. The urinary samples were collected in polyethylene bottles as a pooled sample for each worker during the last four hours of the work shift. This sampling procedure was chosen because a previous study has shown that the measured urinary TDI biomarkers related to recent exposure have a urinary half-life of a few hours (Brorson et al 1991). The blood samples were collected at any point of time during the second half of the work shift by arm vein puncture, in Venoject® blood sampling tubes containing heparin. The less precise timing with blood sampling was possible, due to the fact that the plasma biomarkers of these diisocyanates have been shown to be protein adducts (Sennbro et al 2003), having half-lives of about three weeks (Lind et al 1997). The blood and urinary samples were stored cold, either in a refrigerator or in a cool box, until arrival to the laboratory. At the laboratory, the blood cells were separated from the plasma by centrifugation. Both the urinary and the plasma samples were then stored in polyethylene tubes at -20°C until analysis. The time from arrival at the laboratory until analysis was less than 4 months.

The exposure to different combinations of diisocyanates and the biological samples obtained from both the exposed group and the reference group are presented in table 2. The previously reported air exposure levels (Sennbro et al 2004b) and the previously partly described biomarker levels (Sennbro et al 2003) for the exposed workers are given in table 3.

The study was approved by the Ethical Committee at Lund University, Sweden, and was performed with a written informed consent of the workers.

Determination of biomarker levels

The biomarker levels in the urinary and plasma samples were quantified according to a method previously described by Sennbro et al (2003). In principle, the biological samples were hydrolyzed for 24 h in 0.3 M NaOH in order to release 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4'-MDA, which are biomarkers of exposure to 2,4-TDI, 2,6-TDI, NDI and MDI, respectively. The diamines were extracted with toluene and after derivatisation with pentafluoropropionic acid anhydride, the derivatives were quantified with gas chromatography and mass spectrometry. As internal standard, tri-deuterated 2,4-TDA was used for all four analytes. In both urine and plasma, the LOD was 0.1 µg/L for 2,4-TDA, 2,6-TDA and NDA and 0.05 µg/L for MDA. The limit of quantification (LOQ) in both urine and plasma was 0.6 µg/L for NDA and 0.5 µg/L for the other analytes. Each biological sample was analyzed two times and the average value was reported. In the calculations of URLs (see below), the used biomarker levels were the ones as If the difference between two samples was >0.2 µg/L below the LOQ or >20% above the LOQ, the sample was analyzed once more and the average of the two closest values was then reported. In each analytical batch, chemical blanks were analyzed in order to screen for possible contaminations. The biomarkers in urinary and plasma samples are referred to by use of the prefixes U- and P, respectively. The precision of the method was determined to be 7-19%.

Calculation of upper reference limits

The statistical approach to calculate the URLs was based on the receiver operator characteristic (ROC) curve method (Altman 1991). For each possible value of the URL, the sensitivity and specificity in this material was calculated. The sensitivity equals the probability of being truly classified as occupationally exposed by the URL, *i.e.* the number of exposed workers that have levels of biomarkers above the URL, divided by the total number of exposed workers. The specificity equals the probability of being truly classified as occupationally unexposed by the URL, *i.e.* the number of reference workers with biomarker levels at or below the URL, divided by the total number of reference workers. The URL was set at the biomarker level, at which the sum of sensitivity and specificity was maximal, *i.e.* where the probability of misclassification of workers as occupationally exposed or unexposed was minimal. This is exemplified in figure 1 for U-2,4-TDA. When there was no overlap in biomarker levels for the two groups, the URL was set at the highest biomarker level found in the reference group. Also, the more "regular" reference limits set at the 95^{th} percentile of the biomarker levels in the reference group were calculated.

Statistics

The Spearman's rank coefficient (r_s) was used to evaluate the correlation between variables, and the independent-samples t-test was used to compare the biomarker levels in groups dichotomized according to the characteristics parameters in table 1 (for age; the groups were separated by the median).

RESULTS

The biomarker levels for the reference group and the URLs with the calculated sensitivity and specificity are described in table 4.

For the biomarkers of 2,4-TDI, 2,6-TDI and NDI, there were few or no individuals among the reference group with detectable levels. None of these biomarkers were significantly affected by sex, age, usage of tobacco, atopy or regular use of medical drugs.

For the biomarkers of MDI, the great majority (97%) of the subjects in the reference group had detectable levels. Four of the referents, all women, had U-MDA above the LOQ in the range of 0.6-3 µg/L. The corresponding levels of P-MDA for these referents were in the range of 0.1-0.3 µg/L. There was no correlation between individual P-MDA and U-MDA (r_s = 0.05, p = 0.6) for the whole reference group. Neither P-MDA or U-MDA were significantly affected by sex, usage of tobacco, atopy or regular use of medical drugs. P-MDA was negatively correlated to age (r_s = -0.24, p = 0.01). U-MDA was not significantly affected by age.

DISCUSSION

In our study, we have determined the levels of biomarkers for aromatic diisocyanates in urine and plasma for a group of occupationally unexposed workers. Based on those reference values and the biomarker levels among an exposed group of workers, we have calculated upper reference limits, using the ROC curve technique. These URLs may be used when screening for occupational exposure, where a worker with a biomarker level above the URL will be classified as occupationally exposed.

When using biomarkers in exposure assessment, it is important to be able to interpret the levels. This may be performed by comparison with RVs and URLs, as presented in the present paper. The concept and the theory of RVs was originally defined in the disciplines of clinical chemistry and haematology (Solberg 1987a). In these disciplines, the analytes are essential and endogenous and thus, subjected to homeostasis. In consequence, the RVs are often given as the central 95^{th} percentile (Solberg 1987b), i.e. a reference interval limited by two reference limits. As the RVs in clinical chemistry and haematology are used for medical conclusions, for example early recognition of a disease, documentation of the health status of the reference individuals is of major importance when establishing the RVs. The implementation of RVs in occupational toxicology has met some problems. Most occupational toxins are exogenous and purely toxic which means that the biomarkers in occupational toxicology should not be present in the human body. Thus, in occupational toxicology URLs rather than reference intervals are of more interest. In occupational toxicology, the reference group should be similar to the exposed group, except for the specific occupational exposure (Aitio 1994). This means that the exposure situation rather than the health status is of significance for the RVs.

When calculating the URLs in our study, we used biomarker level data for both unexposed as well as exposed workers. This gave the opportunity to calculate URLs for the

biomarkers with control over both its specificity and sensitivity. The ROC curve technique, usually used for methods in clinical screening, was adopted for this purpose. In the matter of exposure assessment, the ROC curve technique has been used sparsely (Myers et al 2003, Morton et al 2004), and has previously not been used to calculate URLs. The benefit of the ROC curve method in our case, was that the URLs attained a higher specificity (97-100%) without considerable loss of sensitivity, as compared to the regular methods (95%). Of course, in other cases, the overlap of biomarker levels between the groups may be larger and then one has to consider whether to optimize the specificity or the sensitivity. The calculated specificity and sensitivity will evidently depend on the range of exposure to which workers are exposed. In our study, we believe that the exposure levels for those exposed to TDI were relevant for determination of the URLs, since the exposure ranged down close to the limit of quantification of the air monitoring method. The workers exposed to NDI and MDI were few in number; hence the URLs for the corresponding biomarkers are less adequate. During the progress of the publishing of this paper, yet another plant using MDI with ten exposed workers has been studied by our staff. The personal 8 h TWA air exposure to MDI for the workers was low (0.03-0.3 μg/m^3). The U-MDA and P-MDA for the ten workers, was in the range of 0.5-5 μg/L and 0.3-1 μg/L, respectively. If these workers were included in the calculations, the URLs (as presented in table 4) would be slightly lower for both U-MDA (approx 0.45 μg/L) and P-MDA (0.3 μg/L). Further, the specificity would be 95% and 96%, and the sensitivity would be 100% and 89%, for U-MDA and P-MDA , respectively. We find the approach by using the ROC curve method attractive for calculation of URLs and recommend it for other types of exposure, as well.

Our results show that levels of biomarkers are present in occupationally unexposed workers. The frequency of detectable biomarkers of MDI was 97% in both urine and plasma, while in the range of 0-15% for the biomarkers of the other aromatic diisocyanates. The levels

of biomarkers in the exposed group were considerably higher and the calculated URLs for classification of workers as occupationally exposed or not, had sensitivity and specificity of 88-100% and 97-100%, respectively.

When comparing biomarker levels of aromatic diisocyanates in different studies, it is important that the same analytical procedures have been used. Especially the conditions of hydrolysis are of importance for these biomarkers; we used alkaline hydrolysis since this optimizes the recovery of released adducts as compared to acid hydrolysis. Brunmark et al (1995), using alkaline hydrolysis conditions, previously observed that all five healthy volunteers in their study had low levels (<0.2 μg/L) of MDA in hydrolyzed urine and plasma prior to epicutaneous exposure. On the other hand, Dalene et al (1996) found that out of totally 30 workers exposed to thermal degradation products of MDI, only 18 and four workers, respectively, had detectable levels of MDA in hydrolyzed plasma and urine. However, in this study, acid hydrolysis was used in the sample preparation.

In the exposed group, a strong significant correlation was previously observed between biomarker levels in urine and plasma (Sennbro et al 2003). Such a correlation was not observed for the biomarkers of MDI in the group of occupationally unexposed workers in the present study. The only available parameter that influenced the MDI biomarker levels was age, which was negatively correlated to P-MDA. That correlation was weak and is hard to explain and might simply be random.

Still, the finding of the high frequency and also in some cases high levels of biomarkers of MDI is interesting and could be due to an unknown background exposure. When comparing the U-MDA levels in our reference group with the applied BAT value for MDI exposure, the levels are low, and should not be a significant risk factor. However, as these biomarkers are not specific to MDI exposure, the levels found could also originate from exposure to MDA per se. If this is the case, it is important to identify and quantify this source

of exposure, since MDA is a known carcinogen to animals and possible to humans (IARC 1986). Various sources of MDI/MDA are possible and need to be elucidated. As MDI is used in glues and packaging materials, for example in the food industry (Damant et al 1995), there is a possibility that food and beverages may be contaminated by MDA, originating from hydrolyzed unreacted MDI or degraded MDI-based plastics. Also, as MDA is absorbed through the human skin (Brunmark et al 1995), skin contact with such MDA-containing MDI-based plastic, may also be a source of exposure. Furthermore, different PUR products, such as flexible foams and adhesives have been shown to emit unreacted MDI (Wirts et al 2003 , Krone et al 2003), which is yet a potential source of exposure.

In conclusion, we have calculated upper reference limits which may be applicable when screening for occupational exposure. The detected biomarker levels of diisocyanates in occupationally unexposed workers may originate from an unknown source of exposure to the diisocyanates or to their corresponding diamines.

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TABLES

Table 1. The characteristics of the reference group. The parameters were partly determined by a questionnaire filled in by the workers themselves and partly during an interview by a physician.

Characteristics	Men n=59 (49%)	Women n=62 (51%)	All n=121 (100%)
Age			
median (range)	41 (21 - 59)	43 (20 - 63)	42 (20 - 63)
Tobacco users [1]			
n (%)	31 (53)	27 (44)	58 (48)
Atopy [2]			
n (%)	16 (27)	15 (24)	31 (26)
Regular medication [3]			
n (%)	20 (34)	30 (48)	50 (41)

[1] Smokers and snuff-takers.

[2] According to a screening test for allergy (Phadiatope).

[3] Persons taking medical drugs regularly on prescription, including contraceptives.

Table 2. A description of the number of workers exposed to different combination of diisocyanates and the corresponding obtained urinary (U) and plasma (P) samples for the exposed and unexposed group. TDI = toluene diisocyanate; NDI = 1,5-naphthalene diisocyanate; MDI = 4,4'-methylenediphenyl diisocyanate.

Air exposure	Both U and P	Only U	Only P	Total
2,4- and 2,6-TDI	71	16	1	88
Only 2,6-TDI	2	-	-	2
2,6-TDI and MDI	3	-	-	3
Only NDI	6	6	-	12
NDI and MDI	3	-	-	3
Only MDI	2	-	-	2
All exposed	87	22	1	110
All unexposed	117	3	1	121

Table 3. A description of the air exposure levels to diisocyanates and of biomarkers levels in urine (U-) and plasma (P-) for the occupationally exposed workers, used in the calculations of upper reference limits. TDI = toluene diisocyanate; NDI = 1,5-naphthalene diisocyanate; MDI = 4,4'-methylenediphenyl diisocyanate.

Exposure	Air level [a]			Biomarker	Biomarker level [b]		
	n	Median ($\mu g/m^3$)	Range ($\mu g/m^3$)		n	Median ($\mu g/L$)	Range ($\mu g/L$)
2,4-TDI	88	1.4	0.02 – 15	U-2,4-TDA	87	4.7	0.2 – 76
				P-2,4-TDA	72	6.8	0.2 – 31
2,6-TDI	93	1.3	0.02 – 24	U-2,6-TDA	92	4.9	<0.1 – 43
				P-2,6-TDA	77	6	<0.1 – 62
NDI	15	3.3	0.16 – 15	U-NDA	15	7.4	0.7 – 81
				P-NDA	9	20	4.7 – 59
MDI	8	0.6	0.02 – 7.8	U-MDA	8	5.2	0.5 – 78
				P-MDA	8	4.7	0.2 – 74

[a] The air levels have in part previously been reported in Sennbro et al (2004b).

[b] The biomarker levels have in part previously been reported in Sennbro et al (2003).

Table 4. Levels of biomarkers in urinary samples (n=120) and plasma samples (n=118) from occupationally unexposed workers and upper reference limits (URLs) with calculated specificity and sensitivity. U = urinary samples, P = plasma samples, TDA = toluenediamine; NDA = 1,5-diaminonaphthalene; MDI = 4,4'-methylenedianiline; LOD = limit of detection.

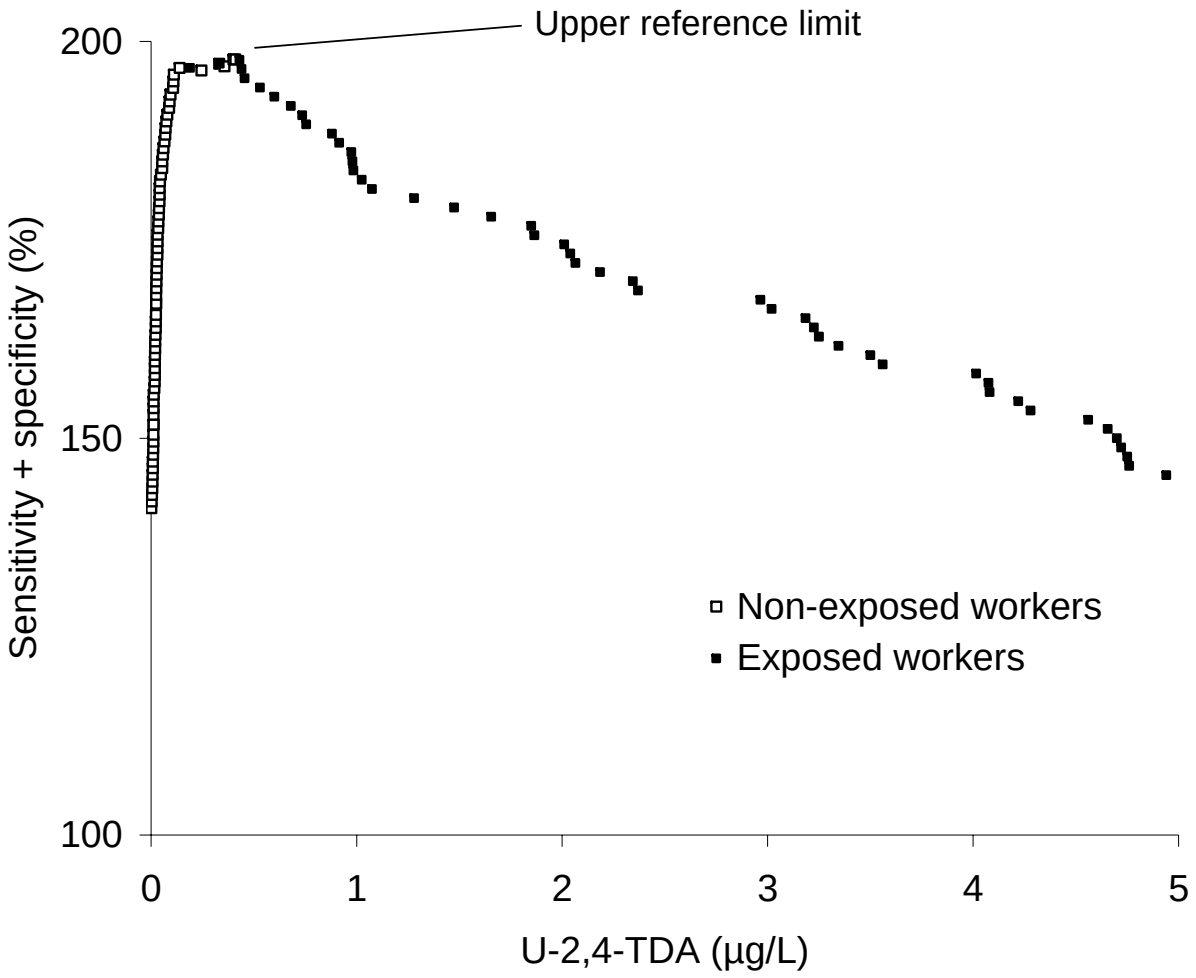
Biomarker	Range (µg/L)	Median (µg/L)	95th perc. (µg/L)	Frequency [a] (%)	URL (µg/L)	Sensitivity (%)	Specificity (%)
U-2,4-TDA	<0.1 – 0.4	<0.1	0.1	7	0.4	94	100
P-2,4-TDA	<0.1 – 0.1	<0.1	0.1	2	0.1	100	100
U-2,6-TDA	<0.1 – 0.2	<0.1	0.1	15	0.2	97	100
P-2,6-TDA	<0.1 – 0.1	<0.1	0.1	2	0.2	99	100
U-NDA	<0.1 – 0.2	<0.1	<0.1	3	0.2	100	100
P-NDA	<0.1	<0.1	<0.1	0	0.1	100	100
U-MDA	<0.05 – 3	0.2	0.4	97	0.5	100	97
P-MDA	<0.05 – 0.4	0.2	0.3	97	0.4	88	100

[a] Frequency of samples with biomarkers levels ≥ LOD. The LOD was 0.05 µg/L for U-MDA and P-MDA and 0.1 µg/L for the other analytes.

FIGURE LEGEND

Figure 1. Statistical approach for the determination of upper reference limits, exemplified by the urinary biomarkers of 2,4-TDI. Each dot indicates the level of 2,4-TDA in a hydrolyzed urinary sample (U-2,4-TDA) for either occupationally unexposed (open squares) or occupationally exposed (filled squares) workers. Note that the data is only shown for biomarker levels in the range of 0-5 µg/L and that the x-axis intercepts the y-axis at 100%.

FIGURE 1.


Upper reference limit
200
150
100
Sensitivity + specificity (%)
0
1
2
3
4
5
U-2,4-TDA (µg/L)
Non-exposed workers
Exposed workers