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Methodology to define biological reference values in the environmental and occupational fields: the contribution of the Italian Society for Reference Values (SIVR)

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Summary

Background: *Biological reference values (RVs) explore the relationships between humans and their environment and habits. RVs are fundamental in the environmental field for assessing illnesses possibly associated with environmental pollution, and also in the occupational field, especially in the absence of established biological or environmental limits.* **Objectives:** *The Italian Society for Reference Values (SIVR) determined to test criteria and procedures for the definition of RVs to be used in the environmental and occupational fields.* **Methods:** *The paper describes the SIVR methodology for defining RVs of xenobiotics and their metabolites. Aspects regarding the choice of population sample, the quality of analytical data, statistical analysis and control of variability factors are considered. The simultaneous interlaboratory circuits involved can be expected to increasingly improve the quality of the analytical data.* **Results:** *Examples of RVs produced by SIVR are presented. In particular, levels of chromium, mercury, ethylenethiourea, 3,5,6-trichloro-2-pyridinol, 2,5-hexanedione, 1-hydroxypyrene and t,t-muconic acid measured in urine and expressed in micrograms/g creatinine (μg/g creat) or micrograms/L (μg/L) are reported.* **Conclusions:** *With the proposed procedure, SIVR intends to make its activities known to the scientific community in order to increase the*

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number of laboratories involved in the definition of RVs for the Italian population. More research is needed to obtain further RVs in different biological matrices, such as hair, nails and exhaled breath. It is also necessary to update and improve the present reference values and broaden the portfolio of chemicals for which RVs are available. In the near future, SIVR intends to expand its scientific activity by using a multivariate approach for xenobiotics that may have a common origin, and to define RVs separately for children who may be exposed more than adults and be more vulnerable.

Riassunto

«La metodologia per la definizione dei Valori di Riferimento Biologici in campo ambientale e occupazionale: il contributo della Società Italiana Valori di Riferimento (SIVR)». **Introduzione:** *I valori di riferimento biologici (VR) esplorano le relazioni tra uomo, ambiente e abitudini di vita. I VR sono fondamentali in campo ambientale per valutare cluster di malattia associati con l'inquinamento, e in campo occupazionale, specialmente in assenza di valori limite biologici per gli ambienti di lavoro.* **Obiettivi:** *La Società Italiana Valori di Riferimento (SIVR) intende determinare in questo lavoro criteri e procedure per la definizione dei VR da utilizzare in campo ambientale ed occupazionale.* **Metodi:** *Il lavoro descrive la metodologia SIVR per la definizione dei VR di xenobiotici e loro metaboliti. Vengono affrontati aspetti riguardanti la scelta del campione di popolazione, la qualità del dato analitico, l'analisi statistica ed il controllo dei fattori di variabilità. La simultanea conduzione di circuiti interlaboratoriali incrementa fortemente la qualità del dato analitico.* **Risultati:** *Esempi di VR prodotti da SIVR sono presentati nel testo. Nello specifico, sono riportati cromo, mercurio, etilentiourea, 3,5,6-tricloro-2-piridinolo, 2,5-esandione, 1-idrossipirene ed acido t,t-muconico misurati nelle urine ed espressi in microgrammi/g creatinina (µg/g creat) o in microgrammi/L (µg/L).* **Conclusioni:** *Con la presente procedura, SIVR intende far conoscere la propria attività alla comunità scientifica, con l'obiettivo di incrementare il numero di laboratori applicati nella definizione di VR per la popolazione italiana. Ulteriori studi sono necessari per ottenere altri VR in differenti matrici biologiche come capello, unghia, esalato. E' inoltre necessario aggiornare e migliorare gli attuali VR con il fine di incrementare il numero di composti chimici per i quali sono disponibili VR. Nel prossimo futuro SIVR intende utilizzare un approccio multivariato per gli xenobiotici che possono avere una comune origine, ampliando la sua attività scientifica e definire separatamente i VR per i bambini che possono essere esposti più degli adulti e sono probabilmente più vulnerabili.*

Introduction

The Italian Society for Reference Values (SIVR) was founded in 1993 with the goal of carrying out cultural and scientific activities aimed at acquiring and disseminating knowledge about reference values for environmental and occupational pollutants. The main purposes were to develop technical guidelines to produce reference values for elements, compounds and their metabolites and to disseminate the results through SIVR publications and by other means. More in detail, SIVR is concerned with the theoretical foundations of reference values in environmental and biological matrices, promotion and standardization of analytical quality, pre-analytical factors influencing reference values, routine and non-conventional analytical techniques useful for determination of reference values, factors of biological variability and criteria for statistical data processing.

SIVR currently defines biological reference values (RVs) in the environmental and occupational fields as estimates of concentrations of chemical substances or their products of transformation in biological materials from non-occupationally-exposed populations (general population). Two kinds of substance are of interest: in the case of substances that play a physiological role, RVs can be considered the resultant of baseline concentrations derived from physiological-homeostatic processes alone, or

the sum of these with the part ascribed to general environmental exposure (through air, water, food and habits), characteristic of the subjects monitored. In the case of toxicants (elements or substances) extraneous to the human body (xenobiotics), which should not be present or detectable in biological matrices, the concentration levels in biological matrices of the general population are derived from the widespread dispersal and persistence of these substances in the environment. Analytical sensitivity, which has greatly improved in recent years, enables extremely low concentrations to be detected.

Reference values can therefore be used to explore the relationships between humans and their environment and habits. They can form the basis for longitudinal studies aimed at detecting changes in the environment and habits, and can be used to establish subgroups of the general population on the basis of race, age, place of residence, diet, habits, etc. The presence of xenobiotics in biological matrices does not necessarily imply a negative effect on human health, however, used in comparison with the results of biomonitoring studies, reference values give public officers responsible for protecting the environment and public health a tool for determining whether people have been exposed to higher levels of xenobiotics than are found in the general population. Biomonitoring data can also help scientists plan and conduct research on exposure and health effects (19).

In the occupational field, RVs are especially important because they represent the lowest possible limits for prevention purposes. They become fundamental for substances for which a toxicity threshold has not yet been defined (teratogens, mutagens and carcinogens) or in the absence of occupational limits (lack of available data), since for the purposes of prevention, occupational exposure involves an acceptable additional risk with respect to that associated with "normal" life. From this viewpoint, biological RVs and limit values proposed for working environments can be regarded as an integrated system of guideline values to orientate prevention in living and working environments.

The utility and necessity of RVs in the environmental and occupational fields encounters a series of problems related in particular to the inadequacy of available analytical methods, the small number of laboratories involved and the resulting difficulty of conducting and maintaining external quality programmes. Thus SIVR decided to test criteria and procedures for defining RVs. Thanks in part to the work of SIVR, in Italy RVs are now regarded as useful for assessing sources, routes of exposure, and absorption of xenobiotics in living and working environments. A number of papers have been published on this topic by SIVR (3-6, 8, 9, 12, 15, 36) but the current methodology used to define RVs has never been fully reported.

In view of the publication of new RVs for certain xenobiotics in the urine of the Italian general population, here we examine methodological aspects that led to SIVR's current procedure for defining RVs. The objective is to define criteria to control factors of technical variability, which must be brought as close as possible to the absolute limits of the method. Biological variability of the population, on the other hand, must be conserved in order to correctly express the distribution of values in the population or in the biological reference samples.

Methodology for defining biological reference values

SIVR procedure for establishing RVs

The prerequisites for assaying xenobiotics in biological matrices are the existence of accurate analytical techniques and sufficient understanding of the compound's toxicokinetics. In other words, the conditions necessary for acquiring accurate analytical data and attributing meaning to it must apply. Without these conditions it is not correct to assay the indicator or to attempt definition of a RV.

SIVR has developed a procedure that ensures standardisation, transparency and quality of RVs. There are three categories of RV, each of which involves different phases for its definition. The list of RVs is available in open access mode on the SIVR website (41): the number of reference values has increased from a few dozen in the 1990s to more than 120 in the current list. Reference values are generally revised by SIVR when new experimental data or scientific publications become available.

a) SIVR Reference Value (SIVR RV): a value produced by at least three laboratories by the SIVR procedure, the phases of which are as follows:

• Phase 1 - Identification of the project, namely the analyte or group of analytes for which it is considered useful to define a RV in biological matrices.

• Phase 2 - Critical examination of RVs already available in the literature for the particular biological indicator. The feasibility of the project is assessed on the basis of the knowledge acquired.

• Phase 3 - Evaluation of optimal available analytical techniques, with particular regard to limits of detection (LoD) and quantification (LoQ), considering the expected reference interval of the analyte. The equipment available in most laboratories, if appropriate, is preferable to the more sophisticated and much more expensive equipment supplied to research laboratories. For example, the usual quadrupole mass spectrometers have sufficient resolution for most routine applications. However, there are instances when this resolution is not sufficient to separate overlapping molecular or other interference. High resolution (magnetic sector) mass spectrometers allow the effect of interference to be eliminated or reduced.

• Phase 4 - Study and control of preanalytical factors.

• Phase 5 - Identification of analytical methods and activation of appropriate data quality control (inter- and intra-laboratory) in order to select labs that can take part in definition of the RV. The quantitative criteria for intra- and inter-laboratory variability and accuracy are set case by case, considering state-of-the-art measurement procedures. Expanded uncertainty values at 30% are usually associated with levels of analytes slightly above LoQ, typical of the range of concentrations of reference values. Expanded uncertainty or inter- and intra-laboratory variability and accuracy must be defined in future studies for the determination of SIVR RVs.

• Phase 6 - Identification of significant variables and preparation of a questionnaire for subjects to answer during sampling, if necessary with exclusion factors to eliminate situations of anomalous occupational or extra-occupational exposure. Some exclusion factors may be smoking of tobacco, occupational exposures and specific diseases. Some examples of variables to consider and include in the questionnaire can be: working life (occupational exposure) and para-occupational exposure, cut flowers, ornamental plants and/or pets in the home for pesticides (7, 10, 11); physical activity (major intake of airborne pollutants indoors and outdoors) for exposure to chloroform in swimming pools (13); traffic or other man-made pollution in proximity to home and work, and season of sampling for benzene (12); alternative medicines including ayurvedic, homeopathic and dietary supplements, dental or joint prostheses, bruxism (5) for metals; alcohol consumption (6, 8) for ethylenethiourea; diet and beverages for pyrethroid metabolites (25).

• Phase 7 - Identification of study population and sampling protocol. The population is defined *a priori* on the basis of geographical distribution, which should include different areas of national territory, and other aspects such as age: if the reference value concerns adults, subjects aged between 18 and 65 years are selected. The study population is defined qualitatively *a posteriori* on the basis of response to the questionnaire for aspects such as diet. Sample number should meet the standards for correct statistical analysis. The high cost of analysis and the few laboratories involved sometimes lead to analysis of a small number of samples: at least 40 samples for each variable that could significantly influence levels of the analyte in a given geopolitical area are needed to establish the extremes of the reference interval (1, 23, 42). Examples of known significant variables are tobacco smoke for benzene and its metabolites, cadmium and other metals, polycyclic aromatic hydrocarbons, and other compounds; age for copper; season of sampling for toluene. The time of sample collection must be standardized. In the case of urine, since it is difficult to collect 24-hour samples for a large number of subjects, it is preferable to collect the second morning urination (spot sample) to avoid the concentrated urine of the night, influenced by xenobiotics in the food intake of the previous day. In the case of spot samples of urine, the parameters for acceptable dilution are defined on the basis of creatinine and/or specific gravity, according to WHO criteria (creatinine 0.3-3.0 g/l, specific gravity 1.010-1.030) (45).

• Phase 8 - Statistical analysis of the data.

b) Tentative Reference Value (TRV): The TRV is the result of a preliminary phase of the definition of SIVR RVs. It is defined by specific experimentation between a small number of labs (at least two) in the SIVR circuit using preferably different methods of analysis (sample preparation and/or analytical methods). Alternatively, TRV can be values defined by a single lab of the SIVR circuit if it uses a validated analytical method and estimates the expanded uncertainty of measurement of RV concentrations. In these cases it may be indicated that the value was obtained by a single laboratory. TRV may be motivated by unavailability of a RV for the particular analyte and/or when confounding factors have not yet been identified.

Sample size must meet the standards already set for SIVR RVs. If factors of variability are not known, a pilot study can be performed to define data variability and hence the appropriate sample size for obtaining a TRV through statistical tests. TRV is reviewed by SIVR within 1-2 years of publication in the case of new experimental data or scientific publications.

c) Reference Value based on available scientific literature (LRV): LRV is defined through reference to literature published in the last 10 years, preferring that based on the Italian or European population, critically assessed also using the check list described below. In this case it is worthwhile indicating any time trends or significant differences due to the use of different analytical techniques.

Application to SIVR for adoption of a new proposed RV by an external lab

A lab can formally apply to SIVR for assessment of data series of an analyte or analyte profile for adoption as a new proposed RV. To do this, the lab should apply to SIVR and undertake to provide all the technical and scientific details requested in support of the data. The board of SIVR sets up a commission that examines the documentation and can if necessary request further information. At the end of this phase, the commission concisely expresses its decision, which it submits to the board, which in turn makes the final decision about acceptance of the data and its coding as SIVR RV or TRV. For applications based on the literature (LRVs), the commission expresses its decision about the completeness of the evidence produced.

Criteria used by SIVR to examine documentation

Unambiguous and transparent examination procedures and criteria for the final decision have been defined *a priori* by SIVR. These criteria are defined in two checklists used by the commission to examine data. The contents of the lists are summarised as follows:

- Laboratory Organization
  - The lab is accredited for the specific analytical determination by a recognised body, such as ACCREDIA (the Italian Accreditation Body) in Italy.
  - A lab that does not meet the above criteria may apply for assessment of its data, provided it supplies objective evidence as requested by SIVR for validation of the method.

The laboratory must provide the commission with documentation of quality, with special reference to the technical procedures used for instrument management and traceability of measurements. The laboratory must also demonstrate that it implemented a system of quality assurance of the results by participation in interlaboratory circuits or by performing other specific tests on the analyte.

- Validation of the method. The analytical method must be validated and the validation parameters defined. Availability of the following information is considered during validation:
  - field of application (in terms of matrix and concentration);
  - limits of detection (LoD) and quantification (LoQ);
  - precision (narrow and broad reproducibility);
  - recovery and its uncertainty;
  - calibration uncertainty;
  - uncertainty of the final measurement.
- Analysis of variables
  - population sample selection (selection criteria used, sufficient sample number based on stratification variables);

- collection of biological samples (information on method, type of container, control of pre-analytical factors, conservation of samples).
- Statistical analysis
  - type of distribution (normal, log-normal, other) and definition of outliers (e.g. Grubb test);
  - descriptive analysis of data (sample size, mean, standard deviation, median, geometric mean, geometric standard deviation, percentiles, minimum and maximum values);
  - evaluation of the influence of known factors and factors deduced from the questionnaire, on data variability.
- Relevant scientific publications
  - type of documentation available (peer-reviewed articles, official documents of national and international bodies, European directives, national legislation, and unpublished data if original and appropriately documented);
  - type of study (studies designed to define RVs and/or studies conducted on subjects occupationally exposed to xenobiotics, including control groups of subjects not occupationally exposed, reviews of the literature);
  - relevance and adequacy of published data and preanalytical and analytical methods used.

Statistical analysis and expression of RVs

Sample size affects statistical analysis. The recommended steps are generally as follows:

- Division into sub-samples (if applicable) on the basis of exposure to reduce data variability (urban/rural residence, smoking/non-smoking, organic/conventional nutrition).
- Description of distribution of homogeneous data (Kolmogorov-Smirnov or alternative testing for normal distribution of unaltered and log-transformed data).
- Identification and characterization of outliers.
- Specification of the amount of data below LoD/LoQ and its statistical treatment (28, 32). In the definition of RVs, SIVR tends not to use the limit of detection as lower threshold, above which analytical results can be provided in quantitative terms, but rather the limit of quantification, i.e. the smallest concentration of analyte that can be identified and measured with known precision and accuracy.
- Description of data (min-max, percentiles, index of central tendency and variability of data).
- Analysis of variance (ANOVA) and/or multiple regression analysis to define variables with a significant influence on the data (definition of the model that best fits the data).

Finally, SIVR provides the interval of concentrations truncated arbitrarily at the 5th and 95th percentiles, central indices (geometric mean and median) of the population examined and any sub-samples based on highly significant factors (smoking, gender, season, etc.). In line with IUPAC (29) and NHANES standards (19, 32), these parameters provide a realistic indication of the interval of concentrations of experimental values, the distribution of which often proves asymmetrical and log-normal-like.

The RVs also contain a list of variability factors that may affect single parameters: an example is smoking for analytes such as cadmium, benzene and its metabolites and polycyclic aromatic hydrocarbons and their metabolites.

For all the analytes, the specific commission designed by the SIVR draws up a sheet of information on data sources (experiment or literature), the participating lab/s, any annotations considered significant to identify the data (zone and method of sampling, analytical technique, etc.), the statistical distribution of the data and variability factors found to be significant by statistical analysis. In the sheets, the RVs are identified as SIVR RV, TRV and LRV according to the procedure used to define them, described above. The sheets are available in open access mode on the SIVR website (41).

EXAMPLES OF REFERENCE VALUES PRODUCED BY SIVR

The reference values determined by SIVR using the procedure described above are summarized in Table 1, where the variables that significantly influenced the values and the analytical technique used are reported. The concentrations of some analytes measured in urine are expressed in micrograms/g creatinine (μg/g creat) so that they can be compared

with those of other authors (14) and with Biological Exposure Indices (2).

Some of the analytes in table 1 are classified as carcinogenic to humans by IARC (International Agency for Research on Cancer) or are metabolites of carcinogenic compounds for which exposure must be kept to a technically feasible minimum. For other selected compounds, such as pesticides, it was important to define levels of exposure of the general population in Italy, not only for the purpose of monitoring workers (e.g. to determine whether individual and collective measures to protect workers are effective), but also to identify groups at risk in certain areas (intensive agriculture; areas close to incinerators and industrial plants).

The studies cited in table 1 concluded that, with respect to previous studies, the modified RVs of xenobiotics (metals) in the urine of the general population may largely be attributed to: improvement in analytical instrumentation and methods; more accurate definition of reference groups (selection of individuals to examine on the basis of standardised criteria in a questionnaire on personal habits, lifestyle, occupational or non-occupational exposure and medical history); strict control of pre-analytical factors; and definition of factors of variability. Other aspects highlighted concerned proper statistical analysis of the data and identification of determinants related to diet as a main source of intake for compounds such as pesticides.

Table 1 - SIVR reference values for several xenobiotics in urine: the values were obtained by specific studies with rigorous data quality control protocols, conducted in SIVR circuit laboratories.

Analyte	Units	5th - 95th percentile	GM (or as otherwise specified)	Analytical technique	Factors of variability	References
chromium	µg/L	0.05-0.24	0.08	ET-AAS	A, R, S, ANT	3
mercury total	µg/g creat	0.12-5.02	0.79 (median)	FI/ICP-MS	D, M, A, G, R, PRD, ANT	5
ethylenethiourea	µg/g creat	<0.5-5.0	1.0	HPLC-UV	D, B, R, S	6, 8
3,5,6-trichloro-2-pyridinol	µg/L	<0.5-8.0	2.0	HRGC/MS	D, B, R	9
2,5-hexanedione (total)	mg/L	<0.10 – 0.76	0.23 (mean)	GC/FID	G	15
1-hydroxypyrene	µg/g creat	0.03-0.50 NSm 0.03-0.3 Sm 0.05-0.7	0.015 NSm 0.1 Sm 0.2	HPLC-FLD	S, D, R	36
t,t-muconic acid	µg/g creat	15.2-163.1 NSm 14.4-143.1 Sm 18.0-236.9	47.5 NSm 40.6 Sm 69.6	HPLC-UV	G, R, D, S, B	12

Footnotes:
GM geometric mean
ET-AAS: Electrothermal atomization-atomic absorption spectrometry
HRGC-MS: High resolution gas chromatography-mass spectrometry
FI/ICP-MS: Flow injection/inductively coupled plasma mass spectrometry
GC/FID: Gas chromatography/flame ionization detector
HPLC-UV: High-performance liquid chromatography ultraviolet detection
HPLC-FLD: High-performance liquid chromatography fluorometric detection
A: age; D: diet; G: gender; R: residence; S: tobacco smoking; ANT: human activities; M: medicines including ayurvedic, homeopathic and other alternative medicines, dietary supplements; PRD: dental prostheses; S: smoking; B: beverages
NSm: non-smokers; Sm: smokers

Discussion

The procedure allowed SIVR to define RVs valid in Italy for the purpose of contributing to the assessment of exposure in living and working environments. Most of the RVs in table 1 were produced in the first decade of 2000. In some cases they have limitations related to sample size and the analytical techniques used. For example, urinary chromium is currently detected by ICP-MS, a more sensitive technique that replaced AAS. On the other hand, although not representative of the entire Italian population, the data on pesticides (ethylenethiourea, 3,5,6-trichloro-2-pyridinol) is similar to that of most recent studies that use different analytical techniques (19, 17). The RV of 1-hydroxypyrene produced in the 1990s should be revised and reassessed, because it could be lower since the ban on smoking in public places. Similarly, the RVs for 2,5-hexanedione need revision due to the nonspecific analytical technique used to determine them, as well as to make them comparable with biological limit values for the free metabolite (2). Thus SIVR is currently reviewing the list of RVs, adding the year of sample collection and the method of analysis.

Nevertheless, there are various methodological problems in defining RVs depending on the particular xenobiotic. Pre-analytical factors (sampling method, transport, conservation and preparation of samples), analytical factors (instruments, analytical method, quality control) and statistical analysis of the data must be all considered and designed for proper definition of RVs. An important technical parameter is the accuracy of analytical data, which is particularly critical if we consider that determination of low concentrations of xenobiotics in any matrix often requires extraction, purification and concentration procedures and in some cases derivatisation followed by analysis using highly specific and sensitive instruments. To determine the accuracy of analytical data in the absence of official methods of analysis and reference materials in the matrix at concentrations sufficient for RVs, participation in interlaboratory circuits is especially important. In the light of the above and the fact that the UNI ENV 13005-2000 (43) standard introduced expanded uncertainty as a parameter characterising the validity of analytical data, measurement uncertainty must also be estimated when defining RVs for xenobiotics. However, with the publication of the UNI EN ISO/IEC 17025:2005 standard (27), measurement uncertainty began to be considered a hallmark of inadequate testing methods. If uncertainty is known, it is possible to objectively verify whether certain limits and tolerances have been observed, detect more or less significant differences and above all compare results obtained in different places and at different times.

The great relevance and importance of a proper evaluation and definition of RVs is documented by the growing number of human biomonitoring studies carried out on the general population in Europe and USA (1, 16, 18, 20, 21, 24, 26, 30, 34, 35, 37, 38, 40, 46). In these countries the reference values were obtained by national public agencies funded by specific grants, and not by a scientific society, as in our case. Sometimes very large groups of population were analysed, as in the case of the US National Health and Nutrition Examination Survey (NHANES). Furthermore, in these studies specific pediatric RVs were defined and different biological matrices (such as hair, milk, deciduous teeth etc.) were used.

The definition of reference values requires large-scale analytical campaigns. The choice of the matrix on which to conduct the analysis should be a good compromise between the biological significance of the assay and the feasibility of the levy. Blood is often considered the elective matrix because it is in direct contact and equilibrium with all organs, but sampling involves an invasive procedure with ethical and practical problems, that can be an impediment when monitoring vulnerable populations (children). The use of non-invasive matrices has advantages, such as the possibility of repeating the sampling at different times, a better study participation rate and lower costs. Among the matrices that can be sampled non-invasively, urine is often the first choice, because it permits routine collection and determination of metabolites, as well as being applicable to non-persistent chemicals. The only drawback is the need to correct for dilution. Other widely used matrices are exhaled breath that allows direct assessment of exposure to volatile chemicals, finger/

toe nails and hair that can provide a historical overview of exposure, influenced, however, by external contamination (22).

Sources of exposure for the general population are extremely variable and depend on the xenobiotic considered. For some xenobiotics it is necessary to consider the contribution derived from indoor and outdoor sources of overall individual exposure (12), whereas for others the contributions of diet and habits, such as smoking, are crucial (19). Thus for defining biological RVs the choice of a representative population sample must necessarily consider exposure sources linked to culture, religion and residence, as well as differences associated with race, gender and age, which may influence toxicokinetic aspects (33). The time and place of definition of RVs become part of the RV itself. Inclusion/exclusion factors may be used to reduce the size of the population sample to analyse or to obtain information targeted at certain population groups for variables that could significantly affect the data (9). These variables can be investigated, standardising the manner of data collection through questionnaires prepared specifically for single xenobiotics.

The modes of environmental dissemination of individual xenobiotics may lead to a different exposure for adults and children. If a substance is dispersed in the air mainly as vapor, exposure of adults and children should be similar, whereas children are usually more exposed to airborne particulate: depending on their age, children may spend much of their time on the floor, where they may come into contact with dust and soil. A substantial quantity of contaminated matter may be ingested through fingers and other objects placed in the mouth (non-nutritional ingestion caused by hand-to-mouth and object-to-mouth behaviour). Studies reported by U.S. Environmental Protection Agency investigators (44) estimate that children have a 12-times greater health risk than adults associated with ingestion of dust and soil. Another important aspect to consider is the route of exposure: in the case of largely dietary intake, children are potentially exposed more than adults because they consume more water, milk, fruit juice and fresh food in relation to body weight (31). In conclusion, major differences in susceptibility to exposure to xenobiotics between adults and children are determined by diet, socio-behavioral habits, physiology and body shape.

Conclusions

Many aspects must be carefully weighed in order to establish RVs of real significance; hence the need for appropriate and transparent methodology concerning sources of exposure, definition of reference population, and all issues related to sampling and analysis.

In the past, the absence of sufficiently sensitive and specific methods made it impossible to fix RVs for many elements and compounds of high toxicological interest. The increasing use of multi-element techniques that ensure determination of a wide spectrum of analytes in the biological sample has enabled new SIVR RV projects. This activity has been continuing for a number of years and has produced a SIVR method with known uncertainty for the determination of elements in urine by ICP-MS (to be completed and published), and the RVs for a broad spectrum of elements in urine of the Italian general population with assessment of intra- and inter-individual variability (to be completed and published). Production of these values followed a preliminary phase of intercalibration between different laboratories in the SIVR circuit with experimental comparisons to evaluate the precision and accuracy of the proposed method. Performed on real matrices, these intercalibrations enabled quantification of measurement uncertainty over a wide range of concentrations, including those of RVs. The RVs produced by SIVR were only based on data provided by laboratories that participated positively in the intercalibration circuit and obtained acceptable uncertainty values. RV quality is therefore guaranteed by experimental activity and rigorous quality control by different laboratories using the same analytical procedure.

The SIVR production of RVs has not yet concerned biological matrices such as hair, finger/toe nails and exhaled breath. In the near future, SIVR intends to broaden its scientific activity by focusing on these and other aspects, including a multivariate approach for xenobiotics that may have a common origin. SIVR also deems it necessary to define RVs

separately for children (19, 39), because children are not "little adults", may be exposed more than adults and are probably more vulnerable. It is also necessary to update and improve the present reference values and enlarge the portfolio of chemicals for which RVs are available.

In conclusion, with the proposed procedure, SIVR intends to make its activities known to the scientific community in order to increase the number of laboratories involved in the definition of RVs for the Italian population.

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