Request for Data and Information on Technologies Used To Identify Substances With the Potential to Cause Acute Systemic Toxicity. National Toxicology Program Request for Data: Federal Register Vol. 81, No. 137, p. 46696, July 18, 2016.

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Sponsoring organization: none.

Date on which this relevant information is submitted: August 29, 2016.

Submitted electronically as requested via e-mail at: niceatm@niehs.nih.gov.

1. **The response to the above request is: QSAR to replace RD50 for inhalation of volatile organic chemicals (VOCs).**

This is in response to the above request to submit data and information on approaches and/or technologies currently used to identify substances with the potential to cause acute systemic toxicity when swallowed, inhaled, or absorbed through the skin.

2. **Date:** Receipt of requested information: Deadline is September 1, 2016.

3. **Request for information: NICEATM**

As stated above in the Federal Register "NICEATM requests data and information on approaches and/or technologies currently used to identify substances with the potential to cause acute systemic toxicity. Respondents should provide information on any activities relevant to the development or validation of alternatives to *in vivo* tests currently required by regulatory agencies that assess acute oral, dermal or inhalation toxicity."

"Of specific interest are chemicals-specific data from non-animal tests for acute systemic toxicity tests, such as ethical human or animal studies or accidental human exposures."

4. **Responses to this request are voluntary. No proprietary, classified, confidential, or sensitive information should be included in responses**

The response below is in accordance with the stated request above. Furthermore, all data presented below have been published in easily available refereed scientific journals. The toxicological endpoint presented below is for a very specific and documented physiological response, "sensory irritation", used to establish a "safe level of exposure" in humans to widely used, stored and transported industrial chemicals. In fact, sensory irritation potency of widely used industrial chemicals is the basis to establish a "safe level of exposure" for at least 33% of them as presented below. This physiological response is a defense reflex reaction to prevent further possible systemic toxic effects.

Executive Summary of the Response to Data Request

This response to the data requested, is specific for the inhalation route portion of for "possible acute/systemic/long-term possible toxic effects". The data provided includes the published and discussed results available at this site:

http://www.toxicology.org/education/edu/eminent.asp

At this site, scroll down to Yves Alarie Lecture. The title of the Lecture is:

"QSARs For The Fiftieth Anniversary of The RD50"

The Alarie Lecture presents and documents the basic anatomy/physiology, chemistry, details and requirements as published, related to an animal bioassay currently using mice since 1966, to estimate "safe level of exposure for humans". This can be used by Regulatory Agencies and expanded as cited below.

The first published article regarding the use of QSAR to replace or reduce the number of mice needed in this bioassay was published in 1973 and the first correlation between the potency (RD50) in mice and "safe level of exposure to humans", TLV (Threshold Limit Values), was published in 1981. These are documented in the Alarie Lecture with all appropriate references.

At the above site, the Alarie Lecture presents and makes available all the required data regarding the relationship between the potency for sensory irritation in mice. The potency is measured in this specific animal species and is abbreviated "RD50".

Then a correlation between RD50s and the "safe level of exposure for humans", given as TLVs was established. TLVs are "Threshold Limit Values", published yearly since 1946 by the ACGIH (American Conference of Governmental Industrial Hygienists). The Occupational Safety and Health Administration (OSHA), a US Regulatory Agency established in 1970, incorporated these values by reference to become Permissible Exposure Limits (PELs). The PELs stipulated in 1970, when OSHA was established, were TLVs published by the ACGIH in 1968.

At the above site, the data are presented regarding the correlation between the sensory irritation potency in mice (RD50s) and the corresponding TLVs. This is presented for 102 volatile organic chemicals (VOCs) of industrial importance. Since the RD50 was measured in several laboratories for several of these chemicals, 184 RD50 values are presented in the Table 1. Such data, are required for the next step, establishing an *in-silico* procedure to replace experiments in mice.

At the above site, the Alarie Lecture presents the required OECD (Organization for Economic Cooperation and Development) specific principles to be followed and fulfilled regarding replacement of animal bioassay, such as the RD50 bioassay, by an *in-silico* procedure. Therefore a description of how the sensory irritation potency (RD50) of industrially widely used, stored and transported volatile organic chemicals, (VOCs), can then be estimated from computational chemistry/computational statistical analysis techniques to obtain a QSAR (Quantitative Structure Activity Relationship) is presented. Thus the animal inhalation bioassay can then be either completely eliminated or the number of animals used can be greatly reduced. Such a QSAR was published in 2015 and presented in the Alarie Lecture.

The Alarie Lecture follows the requirements and style of the Webinar Series: Alternative Approaches for Acute Inhalation Toxicity to Address Global Regulatory and Non-regulatory Data Requirements. It is co-hosted by PETA International Consortium Ltd (PISC) and the US NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), 2015, as described at: http://www.piscitd.org.uk/acute-inhalation-toxicity/

Initial Research Regarding the Appropriate Measurement in Mice for Recognition of Sensory Irritation and Establishing Potency of Airborne Chemicals as Sensory Irritants

The first article was published by Alarie (1966). Part of this article contained a review of prior articles regarding the anatomical/physiological basis of the nasal trigeminal nerve stimulation inducing the breathing inhibitory reflex, clearly a defense mechanism. This review included the first article published in 1870 and included articles comparing obligatory nose-breathing animals vs. humans. Regarding the estimation of the potency of airborne chemicals as sensory irritants, the classic linear regression analysis was used, with the "50% response, (i.e., 50% decrease in respiratory rate), with 95% confidence intervals" being the most reliable way to express potency and was abbreviated "RD50". The response, the decrease in respiratory frequency occurring in a unique and characteristic breathing pattern due to inhalation of airborne chemicals with sensory irritation capabilities, was fully-documented in this article. The estimated RD50 exposure concentration, +/- 95% confidence intervals, was then used in subsequent articles to describe the potency of a variety of airborne chemicals as sensory irritants. Also this article reported using 52 chemicals, tested under code, with the resulting observation that positive/negative responses in mice (the characteristic pause in the breathing pattern), were obtained and qualitatively correlated with positive/negative responses of sensory irritation in human volunteers exposed to approximately the same exposure concentrations of these chemicals. The list of these chemicals with the results was then published in a review by Alarie (1973a).

The second published article provided a direct demonstration, using electrical stimulation of the nasal trigeminal nerve, that this was the initial pathway for the reflex interruption of the breathing pattern in a characteristic way (Ulrich et al., 1972). This study also included trigeminal action potentials recorded during inhalation of sensory irritants and finally, demonstrated that sectioning of the trigeminal nerve abolished this reflex reaction (Ulrich et al., 1972).

With the above published data from fifty years ago, a series of updated findings are presented below to satisfy the requirements of this request. These will complement the Alarie Lecture with details and more published data than could be included in a 50 minutes lecture.

Compliance with OECD Principles for the Validation, for Regulatory Purposes, of Quantitative Structure-Activity Relationship (QSAR) Models

1) a defined endpoint of the bioassay (OECD Principle 1)

The above three references can be considered as a starting point for a "defined endpoint" but much more is available and much more is required to proceed to a QSAR capable of replacing a bioassay.

This will include the published findings for multiple chemicals of industrial importance evaluated with this RD50 bioassay using mice.

The bioassay, as originally developed, was intended to screen airborne chemicals to discover the most potent and rapid-acting (less than one minute) sensory irritants. It then became evident that the bioassay could possibly be "reversed" so that it could be used to evaluate the exposure concentration at which humans could be exposed and *not complain* of sensory irritation and possibly could be considered to be a "safe level of exposure" such as a TLV.

In order to use the bioassay for this purpose, the following is a minimum requirement:

a) Evaluate the potency (RD50) in mice of known sensory irritants in humans, particularly widely used chemicals of industrial importance for which a TLV was established to prevent sensory irritation, which has been described as upper respiratory tract irritation/eye irritation by the ACGIH.

b) From the potency (RD50) in mice, establish a potency range in humans from intolerable sensory irritation down to a no-effect level (i.e., a possible TLV).

c) Extend the exposure duration to accommodate much slower-acting chemicals and extend the recovery period as well.

d) Also use chemicals reported by humans as sensory irritants, even if no TLV is available, and establish that such can be detected by the bioassay as sensory irritants.

Such research was initiated and the selections below indicate the wide variety of chemicals used:
sulfur dioxide (Alarie et al. 1973a), sulfite aerosols (Alarie et al. 1973b), chlorine and hydrogen chloride (Barrow et al. 1977), formaldehyde and acrolein (Kane and Alarie 1977), complex mixtures generated in the Los Angeles photochemical smog evaluated in human volunteers for sensory eye irritation (Kane and Alarie 1978a) including how to interpret such results in humans with chemical reactivity (Yeung and Phillips 1973). Mixtures of formaldehyde and acrolein (Kane and Alarie 1978b), interactions of acrolein and sulfur dioxide (Kane and Alarie 1979), toluene diisocyanate (Sangha and Alarie 1979), 11 common industrial solvents (Kane et al. 1980), eight commonly used alkylbenzenes (Nielsen and Alarie 1982), etc.

A first attempt was made to estimate levels of reaction in humans, from the potency (RD50) in mice for 11 chemicals of industrial importance, regarding acceptable exposure in industry i.e., TLV levels. With this limited number of chemicals, at least a range of effects/relationships between the potency in mice, RD50s, and what would be an appropriate TLV was proposed. These were developed from findings reported in the literature on sensory irritation potency in human volunteers and/or industrial workers exposed to these chemicals, Kane et al. (1979). This effort continued with more data in a review article (Alarie et al. 1980).

With more potency results (RD50s) available, it was then proposed that 0.03 x RD50 could be appropriate for a TLV. Using the data for 26 chemicals for which both RD50 and TLV values were available, a linear regression analysis was conducted plotting the logarithm of 0.03 x RD50 vs. the corresponding logarithm of the TLV for each chemical. An excellent correlation was published in 1981 (Alarie 1981, a,b,c) showing a regression analysis with a correlation r^2 value of 0.89. Similar results were later published by Alarie and Luo (1986) with 40 chemicals for which RD50 and TLV values were available. Other important details were published, including other toxicological effects of sensory irritants used in the RD50 bioassay by Barrow et al. (1986) and also by Buckley et al. (1984).

Also at this time, there was a sufficient number of chemicals of no or low reactivity used as solvents and chemicals of high reactivity toward nucleophilic groups such as SH or NH_2 groups in proteins (Alarie 1973b) and (Tarantino and Sass 1974) to propose a model for a receptor protein (Nielsen and Alarie 1982). A review

published by Nielsen (1991), greatly expanded the nonreactive vs. reactive mechanisms concept for specific groups of inhaled chemicals and also introduced the specificity of receptor interaction outside of reactive/nonreactive chemicals.

In order to proceed to a general QSAR, it is desirable that the defined endpoint be tested using a wide variety of chemicals having low to high potency. Chemicals were also evaluated in mice using the aerosol form. These included known lachrymators in humans: capsaicin and 12 related chemicals (Alarie and Keller 1973, Alarie, 1990), nicotine(Wakisaka et al. 1990), *cis-4*-cyclohexylmethylcyclo-hexylamine and eight related chemicals and a series of five related diimines (Alarie 1990), 27 chemicals containing the [>C=C<] groups (Alarie, 1973b) and two known tear gases: 1-chloracetophenone and dibenz(b,f,)-1,4 oxazepine (Kumar and Sachan, 1998) as well as nanoparticles (Leppanen et al. 2015a, 2015b).

Over the years, the RD50 bioassay was used by scientists in laboratories throughout the world. The data were collected and published by Schaper (1993). The "Schaper database" contains 244 chemicals evaluated in the vapor (or gas) phase as well as in the aerosol phase and also contains a large number of mixtures. This permitted an update of the correlation using 0.03 x RD50 vs TLV for 89 chemicals. Perhaps more importantly, the Schaper database became the primary resource toward establishing structure-activity relationships (SAR) followed by quantitative structure activity relationships (QSAR). Submitted below will be the primary attempt at such, which included references to similar approaches but using smaller databases.

2) Establishing SAR/QSAR

a) Alarie, Nielsen, Andonian-Haftvan and Abraham (1995).

The Schaper database contains a total of 145 VOCs, 53 can be classified as nonreactive (nrVOCs), 89 classified as reactive (rVOCs) and three were unclassified. The above article provided the results for nrVOCs, demonstrating that the potency of such chemicals can be estimated using physicochemical descriptors.

b) Alarie, Schaper, Nielsen and Abraham (1998b).

In this article, all the VOCs in the Schaper database were used and it was clearly demonstrated that the potency of rVOCs cannot be estimated using physicochemical descriptors. However, we were able to estimate the excess potency due to chemical reactivity using related pairs of nrVOCs and rVOCs. We were also able to classify the rVOCs in five separate reaction mechanisms.

c) Alarie, Nielsen and Abraham (1998a).

In this article, we further explained the Ferguson (1939) principle or rule that we had used to separate nrVOCs from rVOCs. We also attempted to present a framework to use physicochemical descriptors and to add chemical reactivity descriptors to estimate the potency of rVOCs.

d) Luan, Weiping, Xiaoyun, Zhang, Liu, Hu, and Fan (2006).

This article used the nrVOCs and rVOCs database and proceeded, using the classic QSAR approach of integrating molecular descriptors, using training and test sets with statistical analysis to estimate RD50s. Six descriptors were retained for each class of chemicals. The authors discussed in detail the OECD principles of transparency and mechanistic interpretability for their study. As expected, due to the higher complexity of the reactive mechanisms, better description ability was found for the nrVOCs than for rVOCs. This is the first classic QSAR approach for RD50 using modern computational chemistry with statistical analysis.

e) Gupta, Basant and Singh (2015).

This article used the nrVOCs and rVOCs in the database as a single set and proceeded, using the classic QSAR approach as above. However, they used different procedures to obtain descriptors and different statistical procedures. Their article stipulated clearly that they are aware of the OECD principles and their Figure 1 presents a diagram showing the QSAR modeling steps and respective OECD principles. A total of only eight descriptors was required, four of them to reliably define the reactive and non-reactive categories, and five of them for regression analysis for potency. Clearly the results are excellent and their conclusion “The proposed QSARs outperformed the previous studies and can be used as the tools

for the sensory irritation potency evaluations of VOCs for regulatory purpose" is warranted.

Updating the Schaper Database For Preparation and Testing The Results of QSARs

Following the publication of Gupta et al. 2015, an update of the Schaper (1993) database is warranted since new chemicals have been evaluated since then and also some TLV values were reduced. Also, in some cases, the basis for a chemical's TLV was changed from sensory irritation to another endpoint, as published by the ACGIH in 2015.

Therefore, it is important to establish that the correlation between 0.03 x RD50 and TLV values is still appropriate. TLV values are really what we want to obtain for new chemicals, from the QSAR using RD50 values. If the relationship, judged by the r^2 value, is still appropriate, then calculating a TLV from the QSAR estimated RD50 value is warranted.

Table 1 [*2015 Update of the 1993 Schaper database of RD50 and their TLV values*] was prepared, presented and discussed at the Alarie Lecture and can be downloaded from the same site noted above. Table 1 contains a list of 102 chemicals for which both RD50 and TLV values are available. Since several chemicals were evaluated in different laboratories with different types of mice, a total of 184 entries are presented. This dataset was then used to update the correlation between RD50 and TLV values. The regression analysis results are presented and discussed in the Alarie Lecture. This was done using both the 1992 TLVs (used by Schaper) and the current 2015 TLVs. Again a good correlation was found, but not as high as found earlier when: 1) the number of chemicals was lower, 2) the TLV values were higher, and 3) not so many types of mice were used. The statistical analysis results (graphs of regression analysis and graphs of residuals) can also be downloaded at the Alarie Lecture site given above.

It should be noted that as given above, a total of 145 VOCs are available from the Alarie et al. (1998b) article and these were used by Luan et al. (2006) and Gupta et al. (2015). If QSAR approaches are contemplated, the same database should be used, not the updated Table 1 with less number of chemicals since Table 1 is really for RD50/TLV correlation and contains five inorganic gases and 97 VOCs.

An update of the Alarie et al. (1998b) database can also be completed. RD50 values, published after 1998 until 2006, are listed in Table 1 of Nielsen et al. (2007) and can be incorporated for QSAR modeling.

Available Data/Procedure to Test The Level of Uncertainty for a New Chemical From The Calculated Estimate From a QSAR

Obviously, decisions for new chemicals introduced in industry will need to be made on a case-by-case basis. It is difficult to get or project a measure of uncertainty (or unreliability). In this case, it can be done. In order to do this, Table 2 was prepared [*List of Chemicals with Threshold Limit Values (TLV) primarily based on sensory irritation. No RD50 values are available for these chemicals. This Table includes only volatile organic chemicals (VOCs)*]. It was presented and discussed at the Alarie Lecture and can be downloaded at the Lecture site given above.

Table 2 is a list of 114 VOCs for which a TLV has been established and as listed in Table 2, the basis for each TLV is to prevent sensory irritation. As shown in Table 2, we have a range of TLV values from 0.005 to 600 ppm. There are no RD50 values for these chemicals. Such chemicals were never evaluated with the RD50 bioassay.

- First, the RD50 value should be calculated for each chemical listed in Table 2 using a QSAR procedure, for example as published by Gupta et al. (2015).

- Second, the relationship 0.03 x RD50 should then be used to calculate the estimated TLV for each chemical.

- Third, the ratio of estimated/actual TLV should be calculated for each chemical.

- And fourth, the distribution of the ratio values should then be statistically examined. It is probable that some “outliers” will be found, being outside of the applicability domain of the QSAR or for other reasons. This can be evaluated and corrected. These are required steps before accepting a QSAR.

As a note, if we add the chemicals listed in Table 1 (102) with chemicals listed in Table 2 (114), we have 216 chemicals of the total of about 665 chemicals for which a TLV has been established on the basis of sensory irritation.

Trust but Verify

The importance of this section will depend on the findings in the above sections on verification of the QSAR estimates. There is also the possibility of using read-across to verify estimates of RD50s.

This issue was presented and discussed at the Alarie Lecture. At the beginning of accepting TLV values from a QSAR technique, we can experimentally verify that the calculated RD50 is indeed correct. This will require only four mice, exposed at the calculated RD50 concentration and for an exposure period of 30 minutes. If a maximum or plateau response has not been reached, as with toluene diisocyanate (Sangha and Alarie, 1979), the exposure can be extended to two hours.

The original method published in 1966 has been updated and computerized so toxic effects can be detected at all three levels of the respiratory tract: 1) upper respiratory tract (inhibition of respiration at the end of inspiration, sensory irritation due to trigeminal nerve stimulation), 2) conducting airways (airflow limitation during expiration due to smooth muscle constriction, tissue inflammatory reaction or mucus accumulation) and 3) alveolar level (pulmonary irritation due to inflammation, edema) (Vijayaraghavan et al., 1993, 1994; Alarie, 2000). In this manner, not only can the estimated RD50 value be experimentally verified if desired, but also other possible deleterious effects can be detected. Even pulmonary irritation as with ozone (Nielsen et al. 1999) can be detected within 30 minutes. Then the animals are evaluated again the next day to determine if the breathing pattern is normal or not. Reviews and articles are available using the updated computerized method, used to demonstrate and quantify the effects at all three levels of the respiratory tract for a wide variety of inhaled chemicals, and mixtures of chemicals, Alarie et al. (2000), Alarie, (2000), Castranova et al. (2002), Glaab, et al. (2002, 2005, 2007), Hoyman et al. (2012), nanoparticles, Leppanen et al. (2015) as well as induced pulmonary infections, Anh et al. (2006) and Wolbeling et al. (2010, 2011).

Other Evaluations of the RD50 Method for Regulations

The Office of Environmental Health Hazard Assessment, California Environmental Protection Agency published a further evaluation of RD50s vs TLVs as well as comparisons with LOAELs (lowest observed adverse effects levels reported for

human subjects) and RELs (concentration level at or which no adverse health effect anticipated for a specified exposure duration in humans) Kuwabara et al. (2007). They concluded "RD50 has benefits for use in setting protective levels for the health of workers and the general population". Nielsen et al. (2007) updated the database of Schaper (1993) and presented the various considerations for using RD50s for risk assessment. Gaffney and Paustenbach (2007) also prepared a discussion on using sensory irritation for setting occupational exposure limits. Woudenberg and van der Torn (1992) published a review of available toxicological data to establish EELs (Emergency Exposure Limits). They concluded "An animal experimental EEL of high toxicological adequacy available for many irritant chemicals is the concentration causing a 50% decrease in respiratory rate (RD50)". The method was approved by the American Society of Testing and Materials in 1984 (now ASTM International), and re-approved every 4 years "Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals, Designation E981-04 (Reapproved 2012)" and now includes the computerized RD50 method. The method was also recently reviewed by ECHA (European Chemical Agency) (2014).

Recent Research on Stimulation of Trigeminal Nerve Endings and Stimulation of Other Sensory Nerve Endings In the Conducting Airways and Alveolar Level of the Respiratory Tract

The nasal mucosa and cornea are innervated by the trigeminal nerve/trigeminal nerve endings. Other sensory nerve endings are present in the larynx, conducting airways and alveolar level. The respiratory tract is very well-innervated, from the tip of the nose to the alveolar level. Stimulation of such nerve endings at any of these locations will also induce specific reflex reactions (Alarie, 1973). Research during the past 15 years has revealed the specific and the essential role of receptors, TRP (Transient Receptor Potential) channels, at all these levels for chemoreception as well for inflammation. Two very comprehensive reviews were published on this topic by Bessac and Jordt (2008, 2010) regarding stimulation by known sensory irritants including nrVOCs, rVOCs and inorganic sensory irritants such as hydrogen peroxide and chlorine, listed in Table 1 cited above, and evaluated with the RD50 bioassay. Both reviews emphasized the essential role of TRPA1 and TRPV1.

These reviews also serve as an introduction to a recent article by Lehmann et al. (2016) proposing the involvement of TRP channels in sensory irritation and the use of an *in-vitro* assay to evaluate sensory irritants and possibly replace the RD50

method. To evaluate if such an *in-vitro* assay, using trigeminal sensory neurons, would present the same results as the *in-vivo* RD50 assay (i.e., potency results of the RD50 assay), seven VOCs were selected. Five of the seven were listed in the Schaper database (RD50s from 5 to 730 ppm). Qualitatively, five VOCs were found positive in the bioassay for stimulating TRPA1 and/or TRPV1 and two were negative. Quantitatively, the same potency order as RD50s was not observed. So, why did it fail? The *in-vitro* bioassay tested chemicals dissolved in different solvents: dimethyl sulfoxide, distilled water or polyethylene glycol. When using an *in-vitro* assay, if a liquid phase exposure is used (chemicals dissolved in solvents, even if the same solvent is used), there is no possibility to match the concentration realized in an *in-vivo* bioassay using a vapor phase exposure. Therefore, we cannot expect a potency correlation between the two. The reasons have been presented and are applicable for both nrVOCs and rVOCs (Ferguson, 1939) and (Tichy, 1983). The first factor is the partition coefficient of the vapor/receptor liquid phase being tested and then chemical reactivity of the rVOCs. With a vapor phase exposure, there will be a distribution of the vapor between the air phase and the liquid phase of the receptors which will result in an exposure concentration completely different than using a solvent exposure.

Nevertheless, it should be possible to use the *in-vitro* assay, as presented by Lehman et al. (2016) by changing the solvents exposure system to a vapor phase-liquid receptor interface. This would also permit evaluation of potent and reactive sensory irritants with a very high vapor pressure such as acrolein, methyl isocyanate, etc. that are impossible to test using solvents. Then, such *in-vitro* receptors assays may be able to replace the RD50 bioassay.

Conclusion

The concept of nonreactive/reactive mechanisms in inhalation toxicology is certainly not new. The use of physicochemical descriptors, electronic constants, steric constants and other miscellaneous constants for QSAR in inhalation toxicology has been well-described by Tichy (1983), and used by Nielsen et al. (1990) and Alarie et al. (1995, 1998a) for sensory irritating airborne chemicals.
With the recent advances in both computational chemistry and computational statistical procedures, we can now realistically handle much more complex databases than congeners or homologous series to be evaluated. This is the case here. We have reached the point of being able to propose that QSAR techniques

are able to estimate the potency of both nrVOCs and rVOCs for widely used, stored and transported industrial chemicals having a specific toxicological and biological endpoint: ***SENSORY IRRITATION***.

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