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Comparison of Cramer classification between Toxtree, the OECD QSAR Toolbox and expert judgment

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ABSTRACT

The Threshold of Toxicological Concern (TTC) is a pragmatic approach in risk assessment. In the absence of data, it sets up levels of human exposure that are considered to have no appreciable risk to human health. The Cramer decision tree is used extensively to determine these exposure thresholds by categorizing non-carcinogenic chemicals into three different structural classes. Therefore, assigning an accurate Cramer class to a material is a crucial step to preserve the integrity of the risk assessment. In this study the Cramer class of over 1000 fragrance materials across diverse chemical classes were determined by using Toxtree (TT), the OECD QSAR Toolbox (TB), and expert judgment. Disconcordance was observed between TT and the TB. A total of 165 materials (16%) showed different results from the two programs. The overall concordance for Cramer classification between TT and expert judgment is 83%, while the concordance between the TB and expert judgment is 77%. Amines, lactones and heterocycles have the lowest percent agreement with expert judgment for TT and the TB. For amines, the expert judgment agreement is 45% for TT and 55% for the TB. For heterocycles, the expert judgment agreement is 55% for TT and the TB. For lactones, the expert judgment agreement is 56% for TT and 50% for the TB. Additional analyses were conducted to determine the concordance within various chemical classes. Critical checkpoints in the decision tree are identified. Strategies and guidance on determining the Cramer class for various chemical classes are discussed.

1. Introduction

Risk assessment is a scientific process that characterizes the magnitude of risk that chemicals or biologics pose to human and environmental health. Conventionally, risk assessment factors for most human health endpoints are derived from animal studies. The shift towards application of alternative methods has encouraged and enabled risk assessors to incorporate non-animal approaches such as *in vitro* studies and *in silico* methods. Another approach is the Threshold of Toxicological Concern (TTC) concept that may be applied to evaluate materials for their potential toxicity when exposure is very low.

The TTC, as an approach to risk assessment, includes the application of a judicious assurance of safety in the absence of chemical-specific toxicity data. Specifically, there may be no significant risk to human health when exposure is below a threshold level. This approach is based on the Threshold of Regulation (Hattan and Rulis, 1986), which was later expanded to consider the chemical structure in conjunction with toxicity data (Munro, 1990; Munro et al., 1996; Kroes et al., 2004). These analyses focused on systemic exposure following oral administration. The TTC approach was originally developed to address human carcinogenicity and systemic toxicity endpoints. It has a relatively long history of use in the evaluation of food contact chemicals and indirect additives, flavors and contaminants in foods, and impurities in pharmaceuticals (WHO, 2002). Recently, the TTC approach was extended to the safety evaluation of topically applied cosmetic ingredients, including fragrance materials (Blackburn et al., 2005; Kroes et al., 2007). The European Food Safety Authority (EFSA) uses the TTC approach for evaluation of flavors and evaluation of pesticide metabolites in groundwater (EFSA, 2012). Additionally, three independent non-food committees (SCCP, SCHER and SCHENIHR) evaluated

Abbreviations: RIFM, Research Institute for Fragrance Materials, Inc.; TTC, Threshold of Toxicological Concern; TT, Toxtree; TB, OECD QSAR Toolbox; SCCS, Scientific Committee on Consumer Safety; SCHER, Scientific Committee on Health and Environmental Risks; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks.

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0273-2300/

the potential applications of the TTC and concluded that the TTC approach is scientifically acceptable for human health risk assessment of systemic toxic effects caused by chemicals present at very low levels of exposure. Furthermore, for cosmetics the TTC approach can be used for those compounds which belong to a sufficiently represented structural class in the TTC database and where appropriate exposure data are available (SCHER/SCCS/SCENIHR, 2012). It is important to note that the TTC approach does not apply to proteins, metals, inorganic substances, high molecular weight substances (e.g., polymers), nanomaterials and radioactive substances. Furthermore, polyhalogenated dioxins/dibenzo furans and dioxin-like polyhalogenated biphenyls are excluded. Finally highly potent genotoxic carcinogens and materials that show evidence for high potency are excluded from the TTC approach (EFSA, 2012).

The Cramer decision tree (Cramer et al., 1978) is used for categorizing non carcinogenic chemicals in order to determine their TTC level (Munro et al., 1996; Kroes et al., 2004). The original Cramer decision tree consists of 33 'yes' (Y) or 'no' (N) questions or rules (Q) (see Appendix I). The answer to each question leads to another question until a final Cramer classification for the chemical of interest is established (Cramer et al., 1978). The Cramer decision tree classifies materials into one of three classes (I – low, II – intermediate and III – high). In 2009, a plug-in called "Cramer rules with extensions" was introduced. This plug-in included five extra questions (i.e., 40–44; please note there are no questions 34–39) and an expanded list of natural body constituents for answering Q1. Of all the Cramer rules, Q1 and Q22 are essentially look-up lists. All the other rules are structure-based except rules Q16 and 17 which are only partly structure-based, as they require reference to the literature or searching databases.

Once the Cramer class is determined, a corresponding TTC threshold for non-genotoxicity endpoint is chosen and compared with the exposure to determine whether the material is above or below the TTC threshold. Therefore, assigning the appropriate Cramer class to a chemical that lacks toxicity data is a crucial step to ensure the integrity of its risk assessment. Usually, freely available *in silico* programs, such as Toxtree and the QSAR Toolbox (TB) are used to determine Cramer class. Toxtree is an open source freely available software that was commissioned for development by the European Commission Joint Research Centre's European Chemicals Bureau (ECB) solely for the purpose of determining Cramer classification of chemicals (http://toxtree.sourceforge.net/). Later versions of Toxtree included additional schemes such as the BfR/SICRET skin irritation and corrosion rules and Verhaar scheme. OECD QSAR Toolbox (http://www.qsartoolbox.org/) was commissioned for development by the Organization for Economic Co-operation and Development (OECD). The Cramer classification scheme was included as a module. Although TT and the TB were developed based on the same Cramer decision tree (Cramer et al., 1978), the interpretation of each rule in the two *in silico* programs may vary. Hence, some discrepancies in Cramer classifications by *in silico* programs have been observed. These discrepancies could be due to technical problems such as bugs or interpretation of the rules (Lapenna and Worth, 2011; Patlewicz et al., 2008). Cramer classification discrepancies may lead to unnecessary testing or may cause incorrect waiving of testing. Therefore, it is important to obtain insight into the potential problems of any *in silico* programs that may lead to discrepancies in the Cramer classification.

In this work, we evaluated 1016 fragrance materials by conducting Cramer classification using Toxtree, OECD QSAR Toolbox and expert judgment. We highlighted discrepancies in Cramer classification with certain chemical classes due to differences in the interpretation of the Cramer questions. We also outlined key strengths and weaknesses of each *in silico* program. We also present strategies to further refine the Cramer questions within the *in silico* programs, to reduce ambiguity and improve concordance with human expert assignments. (expert judgment). In this work, our experts determined Cramer classes by using the original Cramer rules (i.e., without extensions, as shown in Cramer et al., 1978). Hence, programs with the extensions are not discussed in our current work.

2. Methodology

2.1. Data set of fragrance materials

A total of 1016 fragrance ingredients from diverse generic chemical classes (i.e.: acetals, alcohols, aliphatic aldehydes, aromatic aldehydes, amines, carboxylic acids, esters, ethers, heterocyclics, hydrocarbons, ionones, ketones, lactones, phenols, and sulfur containing fragrance materials) were selected. These 1016 ingredients were low molecular weight organic chemicals and did not include any of the exclusions that are not covered by the TTC approach. Materials in each chemical class were categorized by expert assessment into several subclasses. The categorization of these materials followed the principles as outlined in the ECHA technical guidance and OECD guidance on grouping (OECD, 2014; ECHA, 2008). Briefly, materials were clustered together with similar chemical structure/functional groups, similar reactivity, similar metabolism and similar physicochemical properties (Belsito et al., 2011a,b,c, 2012, 2013a,b).

2.2. Determination of Cramer classification

The chemical structures of these 1016 fragrance materials were represented using the simplified molecular-input line-entry system (SMILES) (Weininger, 1988). The SMILES were used as input for Toxtree (TT), Version 2.6.0 and the OECD QSAR Toolbox (TB), Version 3.1. The Cramer class of each material was determined by the Cramer rule decision tree feature in TT and toxic hazard classification by Cramer (original) feature in TB. The path information of the Cramer class from these programs was also generated. In addition, these 1016 materials were manually classified by experts into different Cramer classes based on the original Cramer rules (Cramer et al., 1978). A quality control process was conducted to verify the manual classification by assigning some materials to two independent experts in a blind manner.

2.3. Concordance analysis of in silico programs with expert judgment

As mentioned above, all of the 1016 materials had a Cramer class assigned by TT, TB and expert judgment. The overall concordances of 1016 materials were calculated between TT vs the TB, TT vs expert judgment, and the TB vs expert judgment.

Further, the concordances of each chemical class between the two *in silico* programs and expert judgment were calculated to identify those chemical classes with potential discrepancy issues. For deeper insight, the subclass concordances of each subclass in those three chemical classes with the lowest concordance, between *in silico* programs and expert judgment, were also calculated.

3. Results

3.1. Overall concordance

The Cramer modules in TT and TB were developed based on the same Cramer decision tree (i.e., rules). The interpretation of each rule in the two *in silico* programs may vary. For example – ethyl 2-tert-butyl-cyclohexyl carbonate is assigned as Class III by the TB

and Class II by TT (Fig. 1). In the TB, Q17 is the key determining factor that shows discrepancy leading to the path: 19N, 23N, 24N, 25N, 26N, 22N and 33N. Interestingly, Q19 in TT is answered "yes" leading to a different path. In TT, because of Q17, the hydrolyzed residues are examined separately. The terpene moiety filters through Q18 and the nonterpenoid moiety through Q19, Q20, Q21, and Q18. Based on these two residues, TT assigns the most conservative class (Fig. 1).

Table 1 shows how many out of the 1016 materials were in concordance and discordance between TT and the TB. Six hundred and two (602) materials were assigned as Class I by both TT and the TB, while 94 were assigned as Class II, and 155 as Class III. A total of 165 materials out of 1016 (16%) showed different results from the two programs. The majority of the discordance (28 + 45, 44%) was between Class I and II assignments. Comparatively, 30% (13 + 36) of the 165 materials showed discordance for Class II

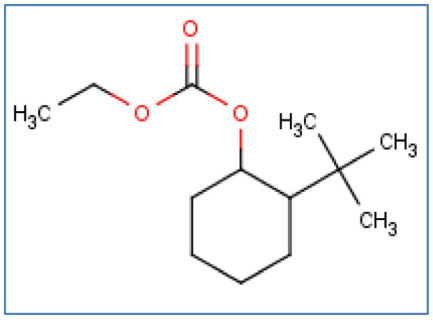
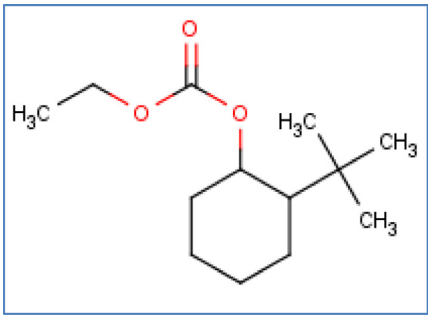
Ethyl-2-tert-butyl-cyclohexyl carbonate **CAS# 67801-64-3** **Expertbase→ I Toxtree→ II**	**Ethyl-2-tert-butyl-cyclohexyl carbonate** **CAS# 67801-64-3** **Expertbase→ I QSAR Toolbox→III**
	
Q1. Normal constituent of the body? No,→ Q2 Q2. Any of these functional groups: aliphatic sec-amine, cyano, N-nitroso, diazo, triazeno, or quaternary N? No, → Q3 Q3. Any elements other than C, H, O, N, divalent S? No, → Q5 Q5. Is it a simply branched acyclic aliphatic hydrocarbon or a common carbohydrate? No,→ Q6 Q6. Is it a benzene derivative…? No,→ Q7 Q7. Is it heterocyclic? No, → Q16 Q16. Is it a common terpene? No, → Q17 Q17. Is it readily hydrolyzed to a common terpene, -alcohol, -aldehyde or -carboxylic acid? Yes Q19.Open chain Yes 'Residue 1' Q20.Aliphatic with some functional groups (see explanation) Yes 'Residue 1' Q21. 3 or more different functional groups No 'Residue 1' Q18.One of the list (see explanation) No Class Low (Class I) 'Residue 1' Q18.One of the list (see explanation) **Yes** Class Intermediate (Class II) 'Residue 2'	Q1. Normal constituent of the body? **No,**→ Q2 Q2. Any of these functional groups: aliphatic sec-amine, cyano, N-nitroso, diazo, triazeno, or quaternary N? **No,** → Q3 Q3. Any elements other than C, H, O, N, divalent S? **No,** → Q5 Q5. Is it a simply branched acyclic aliphatic hydrocarbon or a common carbohydrate? **No,**→ Q6 Q6. Is it a benzene derivative…? **No,**→ Q7 Q7. Is it heterocyclic? **No,** → Q16 Q16. Is it a common terpene? **No,** → Q17 Q17. Is it readily hydrolyzed to a common terpene, -alcohol, -aldehyde or -carboxylic acid? **No** Q19. Open chain →**No** Q23 Aromatic →**No** Q24 Monocarbocyclic with simple substituent→**No** Q25 Cyclopropane →**No** Q26 Monocycloalkanone or bicyclo compound →**No** Q22 Common component of food→**No** Q33 Has sufficient number of sulfonate or sulfamate groups →**No** Class III (High)

Fig. 1. Example comparing classification pathway in TT and TB (answers in large font are wrong). *Note:* Rule numbers (Q) are listed in the order based on the Cramer decision tree (Appendix I).

Table 1
The Matrix of Cramer classification concordance/discordance between the *in silico* tools (TT and TB).

		TT version 2.6		
		Class I	Class II	Class III
TB version 3.1	Class I	602	45	7
	Class II	28	94	36
	Class III	36	13	155

and III, while 26% (36 + 7) of the 165 materials showed discordance for Class I and III. Although the number of disagreements was relatively low between Class I and III, this amounts to a significant impact in their TTC value. This means that depending on the tool that is used by the risk assessor, a different Cramer class may be assigned that would impact the TTC level for these materials.

Of the 1016 assessed materials, 171 materials (17%) have Cramer classes assigned by TT that differ from expert judgment. Of the 171, 68 of them have different class assignment by TT and the TB. Of the 1016 assessed materials, 227 materials (22%) have Cramer classes assigned by the TB that differ from expert judgment. Of the 227, 124 materials have different assignment by TT and the TB. These results suggested that only part of the Cramer class discrepancies can be identified through the discordance of TT and the TB. Moreover, there are materials that are in Cramer classification agreement between TT and the TB but disagree with expert judgment. One also needs to keep in mind that both *in silico* tools may assign the same incorrect classification. In total, about 20% of the 1016 materials have discrepancies in Cramer class assignment when using *in silico* programs.

3.2. Concordance within chemical class between in silico programs and expert judgment

As shown in Table 1, *in silico* programs may have discrepancies in determining Cramer classifications. As such, it is important to identify possible misinterpreted rules in these programs. Additional analyses were conducted to determine the concordance within various chemical classes.

As illustrated in Fig. 2, amines, lactones and heterocycles have the lowest percentage agreement with expert judgment for TT and the TB. For amines, the expert judgment agreement is 45% for TT and 55% for the TB. For heterocycles, the expert judgment agreement is 55% for TT and the TB. For lactones, the expert judgment agreement is 56% for TT and 50% for the TB. The materials that belonged to the chemical classes of aliphatic aldehydes, and esters showed ⩾80% agreement for both *in silico* programs with expert judgment. Some chemical classes such as phenols, ionones and carboxylic acids, have a higher agreement (>90%) between expert judgment and both *in silico* programs. Clearly there is a high likelihood that TT and the TB, would assign the accurate Cramer classes for materials that belong to these chemical classes.

Materials that belonged to the generic class of aromatic aldehydes showed 94% concordance of TT with expert judgment and 78% concordance for the TB with expert judgment. Materials that belonged to the generic class of hydrocarbons showed 100% concordance of TT with expert judgment and 70% concordance of TB with expert judgment. These results further show that the performance of the two programs varies for different chemical classes. In order to explore the potential reasons that may cause discrepancies in Cramer classifications, the three chemical classes that showed the lowest percent agreement were evaluated in greater detail. The materials in these chemical classes (i.e., amines, heterocyclics and lactones) were divided into subclasses. The concordance of each subclass is presented in Figs. 3–5.

For the general chemical class amines, there are five subclasses (Fig. 3). Among these subclasses, amines/amides and amines/esters/anthranilates/N-acetyl have 100% agreement with expert judgment for TT and the TB. However, amines/esters/anthranilates/amino has 0% agreement with expert judgment for TT and only 40% agreement with expert judgment for the TB. The subclass, amines/esters/anthranilates has 50% agreement with expert judgment for both TT and the TB. While, amines/esters/anthranilates/N-methyl has 100% agreement with expert judgment for TT and 50% agreement with expert judgment for TB.

For the general chemical class lactone, there are six subclasses (Fig. 4). Among these subclasses, lactones/gamma-lactones (5-membered ring/saturated) and lactones/delta-lactones (6-membered-ring/unsaturated) have 50% agreement with expert judgment for TT and the TB, while lactones/delta-lactones (6-membered-ring/saturated) and lactones/epsilon-lactones (7-membered-ring) show 100% agreement with expert judgment in the TB and 0% agreement with expert judgment for TT. Macrocyclic lactones and lactides show 100% agreement with expert judgment in TT and 0% agreement with expert judgment for the TB and lactones/bicyclic show 100% agreement with expert judgment for TT and 25% agreement with expert judgment for the TB.

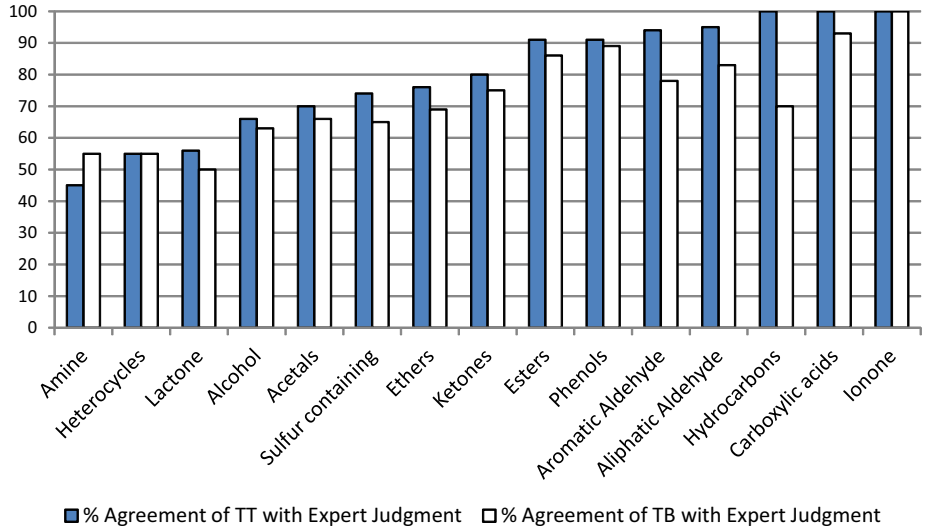


Fig. 2. Concordance evaluation of TT and TB with expert judgment.

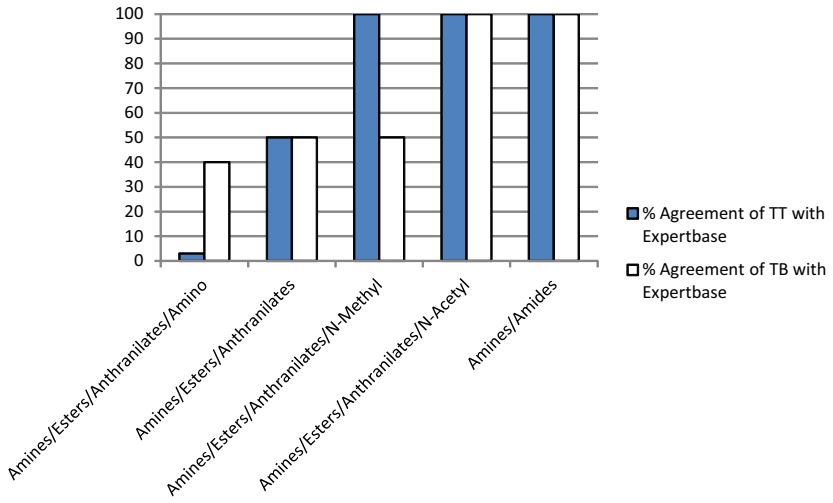


Fig. 3. Concordance evaluation on TT and TB with expert judgment for the amines chemical class.

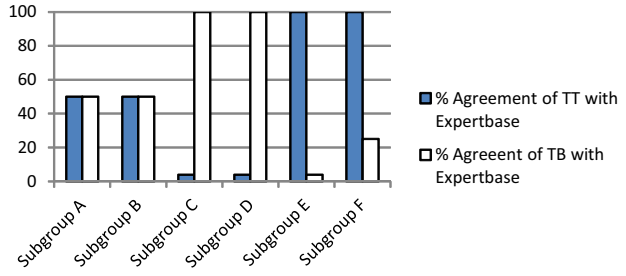


Fig. 4. Concordance evaluation of TT and the TB with expert judgment for the lactones chemical class. Subgroup A: lactones/gamma (γ)-lactones (5 membered ring)/saturated; Subgroup B: lactones/delta (δ)-lactones (6 membered ring)/unsaturated; Subgroup C: lactones/delta (δ)-lactones (6 membered ring)/saturated; Subgroup D: lactones/epsilon (ε)-lactones (7 membered ring); Subgroup E: macrocyclic lactones and lactides (7 membered ring); Subgroup F: lactones/bicyclic.

For the general chemical class of heterocyclic materials there are sixteen subclasses (Fig. 5). Among these subclasses, heterocyclic/oxygen containing/furan/tetrahydro bicyclic/bridged, heterocyclic/oxygen containing/pyrans/dihydropyrans/monocyclic and heterocyclic/oxygen containing/pyrans/tetrahydro bicyclic/bridged show 0% agreement for TT and the TB with expert judgment while heterocyclic/oxygen containing/furan/monocyclic/saturated subclasses show only 25% agreements for TT and the TB with expert judgment.

3.3. The possible reasons and critical questions that caused discrepancies in classification

The above demonstrate that the disconcordance of each chemical class may be caused by only specific subclasses. Therefore, further analysis was also conducted on other classes. Table 2 summarizes selected examples from different generic classes with discrepancies of Cramer classes assigned by the two *in silico* programs. The rules that may be misinterpreted by either program are listed (Table 2).

The alcohol class was further subdivided into subclasses based on the structural fragments (e.g., branched chain, aryl alkyl, straight chain, and cyclic) and the position of the functional group (e.g., primary, secondary, tertiary or if there was unsaturation). The majority of the disparities in the classifications were among the terpenes, secondary, tertiary and aryl alcohol materials. Essentially, most of the tertiary alcohols that were assigned as Class III

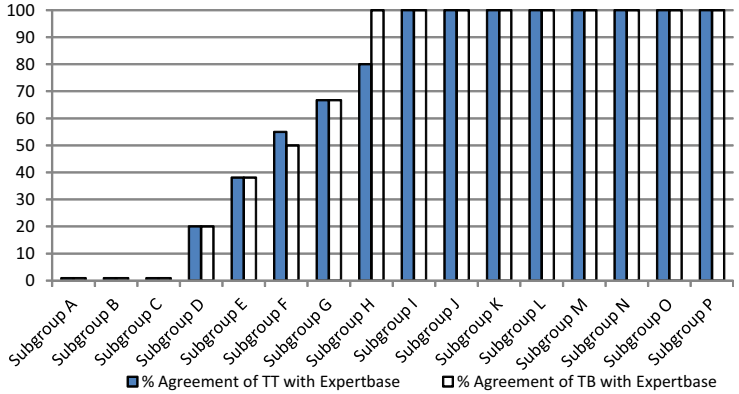


Fig. 5. Concordance evaluation on TT and TB with expert judgment for the heterocyclic chemical class. Subgroup A: heterocyclic/oxygen containing/furan/tetrahydro bicyclic/bridged; Subgroup B: heterocyclic/oxygen containing/pyrans/dihydropyrans/monocyclic; Subgroup D: heterocyclic/oxygen containing/furan/monocyclic/saturated; Subgroup E: heterocyclic/nitrogen containing/pyrazines; Subgroup F: heterocyclic/nitrogen containing; Subgroup G: heterocyclic/oxygen containing/furan/tetrahydro monocyclic; Subgroup H: heterocyclic/oxygen containing/pyrans/keto-pyrans; Subgroup I: heterocyclic/nitrogen containing/quinolines; Subgroup J: heterocyclic/oxygen containing/furan/benzofuran/orphan; Subgroup K: heterocyclic/oxygen containing/furan/bicyclic fused; Subgroup L: heterocyclic/oxygen containing/furan/monocyclic/α,β-unsaturated; Subgroup M: heterocyclic/oxygen containing/furan/monocyclic/sulfur alkyl; Subgroup N: heterocyclic/oxygen containing/furan/monocyclic/sulfur alkyl/orphan; Subgroup O: heterocyclic/oxygen containing/furan/tetrahydro tricyclic/fused; Subgroup P: heterocyclic/oxygen containing/pyrans/tetrahydro monocyclic.

Table 2
Examples of materials from selected generic classes with discrepancies in Cramer class assignment and specific rules that were potentially misinterpreted.

CAS No.	Material name	Generic class	Chemical sub-class	Expert judgment	TT (v 2.6)	TB (v 3.1)	Hypothesis for classification discrepancy in TT	Hypothesis for classification discrepancy in TB
141-92-4	Hydroxycitronellal dimethyl acetal	Acetals	Acetals	I	III	II	Misinterpretation of Q20	Misinterpretation of Q18
515-00-4	Myrtenol	Alcohol	Alcohol/alkyl cyclic/unsaturated/primary α,β-unsaturated	I	I	II	Not applicable	Misinterpretation of Q16
639-99-6	Elemol	Alcohol	Alcohol/alkyl cyclic/unsaturated/tertiary	I	III	II	Misinterpretation of Q24	Misinterpretation of Q18
100-86-7	α,α-Dimethylphenethyl alcohol	Alcohol	Alcohol/aryl alkyl	I	III	II	Misinterpretation of Q30	Misinterpretation of Q18
13254-34-7	2,6-Dimethyl-2-heptanol	Alcohol	Alcohol/branched chain/saturated/tertiary	I	III	II	Misinterpretation of Q20	Misinterpretation of Q18
1113-21-9	Geranyl linalool	Alcohol	Alcohol/branched chain/unsaturated/tertiary α,β	I	III	II	Misinterpretation of Q20	Misinterpretation of Q18
619-01-2	Dihydrocarveol (isomer unspecified)	Alcohol	Alcohol/cyclic/monocyclic/secondary alcohols/unsaturated	I	I	II	Not applicable	Misinterpretation of Q18
138-87-4	*p*-Menth-8-en-1-ol	Alcohol	Alcohol/cyclic/monocyclic/tertiary alcohols/unsaturated	I	III	II	Misinterpretation of Q24	Misinterpretation of Q18
107-74-4	Hydroxycitronellol	Alcohol	Alcohol/diols	I	III	II	Misinterpretation of Q20	Misinterpretation of Q18
21834-92-4	5-Methyl-2-phenyl-2-hexenal	Aromatic Aldehyde	Aldehydes/aryl alkyl/branched/α,β-unsaturated	II	II	I	Not applicable	Misinterpretation of Q30
107-75-5	Hydroxycitronellal	Aliphatic Aldehyde	Aldehydes/branched chain/saturated/medium chain	I	III	II	Misinterpretation of Q20	Misinterpretation of Q18
116-26-7	2,6,6-Trimethyl cyclohexa-1,3-dienyl methanal	Aliphatic Aldehyde	Aldehydes/cyclic/monocyclic/α,β-unsaturated	I	I	II	Not applicable	Misinterpretation of Q24
122-59-8	Phenoxy acetic acid	Carboxylic acids	Carboxylic acids/aryl alkyl	III	III	I	Not applicable	Incomplete path in Q25
4728-82-9	Allyl cyclohexaneacetate	Esters	Esters/allyl/alkyl/branched saturated/even carbon chain length/orphan	II	II	III	Not applicable	Misinterpretation of Q24
105-95-3	Ethylene brassylate	Esters	Macrocyclic lactones and lactides	I	I	III	Not applicable	Misinterpretation of Q8
65505-24-0	Isobutyl N-methyl anthranilate	Amines	Esters/anthranilates/N-methyl	III	III	II	Not applicable	Misinterpretation of Q32
6066-49-5	3-*n*-Butyl phthalide	Lactone	Heterocycles/lactones/delta (δ)-lactones (6 membered ring)/bicyclic/aryl saturated	III	III	I	Not applicable	Misinterpretation of Q9
25524-95-2	5-Hydroxy-7-decenoic acid delta;-lactone	Lactone	Heterocycles/lactones/delta (δ)-lactones (6 membered ring)/unsaturated	I	II	I	Misinterpretation of Q18	Not applicable
611-13-2	Methyl 2-furoate	Heterocycles	Heterocycles/oxygen containing/furan/monocyclic/saturated	II	III	III	Misinterpretation of Q22	Misinterpretation of Q11
141-97-9	Ethyl acetoacetate	Ketones	Ketone/aliphatic/keto esters/straight chain/saturated	I	I	III	Not applicable	Misinterpretation of Q21
4695-62-9	*d*-Fenchone	Ketones	Ketone/cyclic/bicyclic/bridged/saturated/fenchones	II	III	III	Misinterpretation of Q26	Misinterpretation of Q26
4674-50-4	Nootkatone	Ketones	Ketone/cyclic/bicyclic/α,β-unsaturated/cyclohexyl	II	III	III	Misinterpretation of Q26	Misinterpretation of Q26
88-15-3	2-Acetyl thiophene	Sulfur-containing	Sulfur containing	II	III	III	Misinterpretation of Q22	Misinterpretation of Q22

by TT and Class II by the TB were determined to be Class I by expert judgment. For example, 2,6-dimethyl-2-heptanol was assigned as Class III by TT and Class II by the TB. In TT the path of concern was 16N, 17N, 19Y, 20N, 22N, 33N, while in the TB the path of concern was 16N, 17N, 19Y, 20Y, 21N, 18Y. However, expert judgment determined this as Class I based on the path of 20Y, 21N, 18N. In the TB, tertiary alcohols are consistently treated in the same way as secondary alcohols, leading to a misinterpretation of Q18b. In most cases, Class III classification is due to the answer No to Q20 (when part of an open chain compound) or No answer to Q24 (when part of an alicyclic compound) or a Yes answer to Q30 (when part of an aromatic compound). However, when tertiary alcohols are identified as common terpenes or readily hydrolyze to common terpenes, the tertiary alcohol does not go through Q20, Q24 or Q30. We also determined that, in most cases, the basic structural group did not have a significant impact on classification – the exceptions were for bicyclic alcohols and aryl alcohols. In TT, for aryl alcohols, the classification problem was due to Q30-Aromatic ring with substituents other than those listed as qualifying for I classification. Specifically, the problem was in determining whether an aromatic substituent is complex or not. For example, α,α-dimethylphenethyl alcohol was assigned as Class III by TT and Class II by the TB. In TT the path of concern was 16N, 17N, 19N, 23Y, 27Y, 28N, 30Y, 31N, 32N, 22N, 33N while in the TB the path of concern was 16N, 17N, 19Y, 20Y, 21N, 18Y. However, expert judgment notes α, α-dimethylphenethyl alcohol should be a Class I substance by the path 27Y, 28N, 30N, 18N. In this case, Q30 was misinterpreted in TT and Q19 and 18 were misinterpreted in the TB.

The aldehyde class was sub-divided into two subclasses – aromatic aldehydes and aliphatic aldehydes. Each sub-class was further sub-divided by basic structure and reactivity of the CHO group. For aliphatic aldehydes, expert judgment had 95% agreement with TT and 83% agreement with the TB. For aromatic aldehydes, expert judgment had 94% agreement with TT and 78% with the TB. The disparities in classifications for aliphatic aldehydes were largely due to the misinterpretation of Q18, Q20 and Q24. For example, 5-methyl-2-phenyl-2-hexenal was predicted as Class II by expert judgment and TT. However, in the TB, it was predicted as Class I. This was due to the misinterpretation of Q30. In the TB, Q30 was answered as "no" and thus followed the path – 30N, [aromatic]Y, 18N. However, the likely path appears to be 30Y, 31N and 32Y. Essentially this material was Class II by answering "yes" to Q32, as the aliphatic substituent chain was longer than 5 C-atoms.

The heterocyclic class of materials includes materials in heterocycles/nitrogen containing and heterocyclic/oxygen containing classes. Most of the disparities in classification were noted in the heterocyclic/oxygen containing materials. As an example for methyl 2-furoate (see Table 3), both *in silico* tools predicted Class III. In contrast, expert judgment determined this to be Class II by "yes" to Q22, based on the presence of this material in almonds, cocoa, coffee, honey and various fruits.

In the ketone chemical class, most of the classification problems were due to bicyclic or cyclic substructure. For example, *d*-Fenchone was determined as Class II by expert judgment but both TT and the TB predict this as Class III. This was due to an interpretation problem in Q26. Our expert judgment determined the answer to Q26 is "Yes"

Table 3
Overview of the critical Cramer questions that lead to discrepancy in classification.

Chemical subclass or substructure	Cramer rules that may lead to discrepancy	Comments
Beta-lactones	Q9	The original Q9 states "Is it a lactone fused to another ring or a 5- or 6-membered β-unsaturated lactone" can be clarified further by including β-lactone in this question
Heterocycles/furans	Q11	The intention of Q11 is to essentially ask, if the ring contains any other atoms apart from O, S, or N. If there are multiple rings, then each ring is treated differently
Terpene	Q16	Q16 and 17 are only partly structure-based. The *in silico* tools usually assigns No to these questions. Users need to reference to the literature or search databases. Since a Yes response to any of these questions will either reduce the classification or leave it the same, it is best to assume the default response No unless the user searches databases or literature
Terpene	Q17	
Secondary alcohol & esters of secondary alcohol	Q18b	Rule 18 (b) "a secondary alcohol or ester of a secondary alcohol attached to a terminal vinyl group" should be replaced by "a secondary alcohol attached to a terminal vinyl group or an ester of a secondary alcohol attached to a terminal vinyl group"
Open chain ketones without any substituents	Q18h	Usually open chain ketones without substituents reach question 18, where 18h decides their classification: "Is the substance…an acyclic aliphatic ketone, ketal or ketoalcohol with no other functional groups and with four or more carbon atoms on either side of the keto group." The problem of interpretation of this question arises from the context-dependent ways in which the word "either" is used in English. The question could be interpreted: (a) Y if there are four or more carbon atoms on one side or on both sides; (b) Y only if both sides have 4 or more carbon atoms; (c) Y only if one side has 4 or more carbons and the other side has less than 4. The *in silico* tools are inconsistent with the interpretation applied in answering this question
Tertiary alcohol (open chain)	Q20	In most cases, Class III classification is due to the answer No to Q20 (when part of an open chain compound
Tertiary alcohol (part of alicyclic compound)	Q24	Q24 asks if the substance is monocyclic (*excluding cyclopropanes and cyclobutanes) and contains only substituent groups from a short list. Yes usually gives I, via 18N, and No forwards to Q25. This asks if the compound is a cyclopropane or a cyclobutane with only the functional groups listed in Q24
Ketones alicyclic without any substituents		
Ketones cyclic/bicyclic	Q26	There are many cases where a bicyclic compound should trigger Yes to Q26 where the rings are not fused but are connected by single or double bonds or in a *spiro*- configuration. In most cases *in silico* tools are unable to count the rings unless they are separate (i.e. Having no more than one carbon atom shared)
Aryl alcohol Aryl aldehyde Carboxylic acids with an aryl component Essentially any substance with an Aryl component	Q30	Often times *in silico* tools are unable to assess the number and types of substituents. Therefore the issue arises in determining whether an aromatic substituent is complex or not. That is Q30Y, instead of Q30N
Amines	Q32	The intention of this question is to assign simple derivatives of tetralin as Class II while assigning polycyclic substances (excluding normal food components) such as steroid as Class III

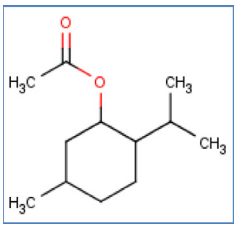
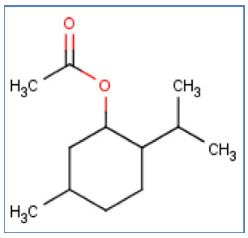
Menthyl acetate (CAS # 89-48-5)	**Menthyl acetate (CAS # 89-48-5)**
	
Toxtree → Class II; Expert judgment→Class I	**Toolbox → Class I; Expertbase→Class I**
Q1. Normal constituent of the body? **No**,→ Q2	Q1. Normal constituent of the body? **No**,→ Q2
Q2. Any of these functional groups: aliphatic sec-amine, cyano, N-nitroso, diazo, triazeno, or quaternary N? **No**, →Q3	Q2. Any of these functional groups: aliphatic sec-amine, cyano, N-nitroso, diazo, triazeno, or quaternary N? **No**, → Q3
Q3. Any elements other than C, H, O, N, divalent S? **No**, →Q5	Q3. Any elements other than C, H, O, N, divalent S? **No**, → Q5
Q5. Is it a simply branched acyclic aliphatic hydrocarbon or a common carbohydrate? **No**,→ Q6	Q5. Is it a simply branched acyclic aliphatic hydrocarbon or a common carbohydrate? **No**,→ Q6
Q6. Is it a benzene derivative…? **No**, Q7	Q6. Is it a benzene derivative? **No**→ Q7
Q7. Is it heterocyclic? **No**, → Q16	Q7. Is it heterocyclic? **No**, → Q16
Q16. Is it a common terpene? **No**,→ Q17	Q16. Is it a common terpene? **No**,→ Q17
Q17. Is it readily hydrolyzed to a common terpene, -alcohol, -aldehyde or -carboxylic acid? **Yes**, giving **acetic acid** (→ Q19) and **menthol** (→ Q 18)	Q17. Is it readily hydrolyzed to a common terpene, -alcohol, -aldehyde or -carboxylic acid? **No**
Q19.Open chain **Yes** 'Residue 1'	Q19. Open chain →**No**
Q20.Aliphatic with some functional groups (see explanation) **Yes** 'Residue 1'	Q23 Aromatic →**No**
Q21.3 or more different functional groups **No** 'Residue 1'	Q24 Monocarbocyclic with simple substituents→ **Yes**
Q18. One of the list (see explanation) → **No** Class Low (Class I) 'Residue 1'	Q18. One of the list (see explanation) →**No** (Class I) Low
Q18.One of the list (see explanation) **Yes** Class Intermediate (Class II) 'Residue 2'	**2**

Fig. 6. Example outlining discrepancies due to Q18 in TT and Q17 in TB (answers in large font are wrong). *Note:* Rule numbers (Q) are listed in the order based on the Cramer decision tree (Appendix I).

leading to Class II. For open chain ketones without any substituents, most of the classification problems were due to discrepancy in the interpretation of Q18h, as discussed by Cramer et al. (1978). Q18h states, "Is the substance. . .an acyclic aliphatic ketone, ketal or keto alcohol with no other functional groups and with four or more carbon atoms on either side of the keto group." This question could be interpreted as: (a) Yes, if there are four or more carbon atoms on one side or on both sides; (b) Yes, only if both sides have 4 or more carbon atoms; (c) Yes, only if one side has 4 or more carbons and the other side has less than 4. TT appears to be inconsistent in how this question is interpreted. One may gain additional insight into the original by re-examining the full dataset that Cramer et al. used to test

their decision tree. In any event, *in silico* tools should be made consistent as to which interpretation is applied.

The acetals chemical class are often evaluated as the parent aldehyde, which may be a terpene. For example, hydroxycitronellal dimethyl acetal is predicted as Class III in TT by Q20N. However, Q20 refers to "linear or simply branched aliphatic containing certain functional groups". In this case, this material contains the combination of alcohol and acetal groups and should be Y to this question and finally to be Class I through 21N, 18N.

For the lactones chemical class, the following types of lactones are assigned as Class III: (1) lactone that is fused to another ring; (2) any gamma- or delta-lactones with α,β-unsaturation (e.g., α, β relative to the carbonyl group). However, a non-fused, non-α-β-unsaturated lactone is determined as Q9N: at this point the compound is treated as its hydrolysis product (e.g., a hydroxy-acid) and if it is a simple lactone (no complicating substituents) it should follow the path to Q18 and then to I classification. However, TT and the TB may misclassify as II or III if the hydroxyl group of the corresponding hydroxyacid is secondary or tertiary. There is a further anomaly, resulting from an ambiguity in the original wording of the Cramer rules. The text does not specifically address β-lactones, so on the basis of the wording, they would trigger N to Q9 and then be considered as β-hydroxy acids, leading to I classification. For example, based on the wording in the original paper, β-propiolactone (CAS # 57-57-8; not a fragrance ingredient) may be considered as Class I. However, Cramer et al. (1978) show in an illustrated example that the β-lactone structure should be Class III. The intention of Cramer decision tree Q9 is to classify lactones suspected of unusual toxicity in Class III, and this certainly applies to β-lactones. The original question "Is it a lactone fused to another ring or a five or six-membered β-unsaturated lactone" can be clarified further by including β-lactone.

It was also found that a significant number of materials had discrepancy in classification due to questions 18 and 17. For example, the expert judgment determined menthyl acetate to be Class I, but TT predicted the material was Class II (Fig. 6). The Cramer decision tree sequence for these two materials reveals a different interpretation of Q18 by the TT software. The misinterpretation of Q18 may be due to the phrasing of Q18b in the original Cramer paper: Is it "a secondary alcohol or ester of a secondary alcohol attached to a terminal vinyl group?" In TT, a secondary alcohol of any sort (saturated, unsaturated, etc.), would lead to a "YES" – that answer would result in Class II. However, as per the original Cramer paper, it is clear that only those secondary alcohols that are attached to a terminal vinylic group (e.g. R—CHOH—CH=CH_2) should trigger the alert. In this case, a terpene residue is a secondary alcohol, but the hydroxyl group is not attached to a terminal vinyl group. Therefore, the answer should be "NO" which would lead to Class I (Fig. 6). To reduce ambiguity, Q18b should be understood as "a secondary alcohol attached to a terminal vinyl group or an ester of a secondary alcohol attached to a terminal vinyl group."

4. Discussion

Based on our experience, we have summarized the most critical Cramer rules for different chemical subclasses along with possible strategies to elucidate the discrepancies (see Table 3). This is based on our chemical inventory. There may be other discrepancies that may come to light when additional chemical inventories are used.

Cramer classification is crucial for determining the TTC levels of a material. Correct determination of TTC may in turn impact its risk assessment. If there is an incorrect Cramer classification, then this may lead to the use of an inappropriate TTC level, which subsequently may lead to unnecessary testing, or may cause incorrect waiving of testing. As such, based on our findings, we have outlined some check points that risk assessors can follow to reduce the potential for disparities in Cramer classifications:

- Use multiple methods to determine the Cramer class, including different *in silico* programs and expert judgment, if possible.
- If your material of interest falls in a low concordance group, such as heterocyclics, amines, lactones, acetals and alcohols (Figs. 3–5 and Table 2) or contains any of the substructure(s) as listed in (Table 3), exercise expert judgment.
- Rules numbers 1 and 22 depend on user-defined lists of compounds, which are normal constituents of the body or common components of food. The *in silico* tools contain a limited number of these components. Users should expand these files with appropriate molecules.
- Verify the answers for rule numbers 9, 11, 16, 17, 18, 20, 24, 26, 30 and 32.
- When in doubt, or if the material is of critical importance, use expert judgment.

We acknowledge that manually determining Cramer classification for a large inventory of materials is impractical. However, when TTC plays a key role in decision making (e.g., conducting or waiving animal testing) then expert judgment should be exercised. If the expert judgment gives a lower classification than the *in silico* programs, then a proper justification or explanation should be provided. In addition, when there is any doubt with interpretation of any Cramer rules, then for conservative purposes one may use the higher Cramer classification.

This work was undertaken to support the use of TTC in evaluating fragrance ingredients. As such, determining the most likely Cramer class is critical. Ongoing work by our group includes expansion of our expert judgment by evaluating other fragrance materials and evaluation of *in silico* tools with extensions.

5. Conclusions

The authors recognize that both TT and the TB are useful tools that, enable effective implementation of the Cramer decision tree. It is also important to recognize that the Cramer rules as written in 1978 were intended to guide chemists and risk assessor in exercising judgment.

The current evaluation shows that overall TT ver. 2.6 appeared to have a slightly better performance than the TB ver. 3.1 for the chemicals that were evaluated in our work. However, it is clear that the *in silico* performances are chemical class-dependent. In any case, upgrades to performances of both *in silico* tools based on refinements of the Cramer questions and possible coding changes will reduce disparities in classifications. In the meantime, following the "check points" we have recommended will reduce the potential for disparities in Cramer classifications and assure that the most likely class is assigned so that in turn the appropriate TTC value is employed.

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Appendix I. Summary of the original Cramer decision scheme

Rule No.	Rule title	If NO: assign class OR go to rule	If YES: assign class OR go to rule
1	Normal constituent of the body	2	Low (Class I)
2	Contains functional groups associated with enhanced toxicity	3	High (Class III)
3	Contains elements other than C, H, O, N, divalent S	5	4
4	Elements not listed in Q3 occurs only as a Na, K, Ca, Mg, N salt, sulfamate, sulfonate, sulfate, hydrochloride	High (Class III)	7
5	Simply branched aliphatic hydrocarbon or a common carbohydrate	6	Low (Class I)
6	Benzene derivative with certain substituents	7	III
7	Heterocyclic	16	8
8	Lactone or cyclic diester	10	9
9	Lactone, fused to another ring, or 5- or 6-membered á,â-unsaturated lactone?	23	High (Class III)
10	3-membered heterocycles	11	High (Class III)
11	Has a heterocyclic ring with complex substituents.	12	33
12	Heteroaromatic	22	13
13	Does the ring bear any substituents?	High (Class III)	14
14	More than one aromatic ring	22	15
15	Readily hydrolyzed	33	22
16	Common terpene (see explanation in Cramer et al., 1978)	17	Low (Class I)
17	Readily hydrolyzed to a common terpene	19	18
18	One of the list (see Cramer et al., 1978 for detailed explanation)	Low (Class I)	Intermediate (Class II)
19	Open chain	23	20
20	Aliphatic with some functional groups (see Cramer et al., 1978 for detailed explanation)	22	21
21	3 or more different functional groups	18	High (Class III)
22	Common component of food	33	Intermediate (Class II)
23	Aromatic	24	27
24	Monocarbocyclic with simple substituents	25	18
25	Cyclopropane (see explanation in Cramer et al., 1978)	26	Intermediate (Class II)
26	Monocycloalkanone or a bicyclo compound	22	Intermediate (Class II)
27	Rings with substituents	High (class III)	28
28	More than one aromatic ring	30	29
29	Readily hydrolyzed	33	30
30	Aromatic ring with complex substituents	18	31
31	Is the substance an acyclic acetal or ester of substances defined in Q30?	32	18
32	Contains only the functional groups listed in Q30 or Q31 and those listed below	22	Intermediate (Class II)
33	Has sufficient number of sulfonate or sulfamate groups	High (Class III)	Low (Class I)

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