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An evaluation of the implementation of the Cramer classification scheme in the Toxtree software

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Risk assessment for most human health effects is based on the threshold of a toxicological effect, usually derived from animal experiments. The Threshold of Toxicological Concern (TTC) is a concept that refers to the establishment of a level of exposure for all chemicals below which there would be no appreciable risk to human health. When carefully applied, the TTC concept can provide a means of waiving testing based on knowledge of exposure limits. Two main approaches exist; the first of these is a General Threshold of Toxicological Concern; the second approach is a TTC in relation to structural information and/or toxicological data of chemicals. The structural scheme most routinely used is that of Cramer and co-workers from 1978. Recently this scheme was encoded into a software program called Toxtree, specifically commissioned by the European Chemicals Bureau (ECB). Here we evaluate two published datasets using Toxtree to demonstrate its concordance and highlight potential software modifications. The results were promising with an overall good concordance between the reported classifications and those generated by Toxtree. Further evaluation of these results highlighted a number of inconsistencies which were examined in turn and rationalised as far as possible. Improvements for Toxtree were proposed where appropriate. Notable of these is a necessity to update the lists of common food components and normal body constituents as these accounted for the majority of false classifications observed. Overall Toxtree was found to be a useful tool in facilitating the systematic evaluation of compounds through the Cramer scheme.

Keywords: Cramer classification; threshold of toxicological concern; Toxtree

1. Introduction

1.1 *Background to the TTC*

Risk assessment for most human health endpoints is based on the threshold of a critical toxicological effect, usually derived from animal experiments. The threshold of toxicological concern (TTC) is a concept that refers to the establishment of a human exposure threshold values for all chemicals below which there would be no appreciable risk to health. When carefully applied, the TTC concept can provide a means of waiving testing based on knowledge of exposure limits. A threshold is based from a statistical analysis of the toxicological data of a broad range of different and/or structurally related chemicals

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and extrapolation of the no effects doses obtained from the underlying animal experiments for these chemicals considered to be of negligible risk to human health. The TTC concept has been incorporated into some risk assessment processes in regulatory schemes in particular in the area of food additives and food contact agents [1–5]. More recently the approach has been applied to evaluate impurities in pharmaceuticals [6–8], cosmetic ingredients [9] as well as personal and household care products [10].

There are two main approaches from which TTCs can be derived. The first of these is a General Threshold of Toxicological Concern; an example of this approach is the Threshold of Regulation for food contact materials used by the US Food and Drug Administration (FDA). The second approach is a structured based TTC where generic thresholds are derived through an analysis of toxicological data of chemicals but according to their chemical structure. For a comprehensive account of the history and development of the TTC principle and in particular its application to chemicals in food, the reader is referred to the International Life Sciences Institute (ILSI) monograph [11]. Here we present a brief overview of the evolvement of TTC approaches as background context.

1.2 *General TTCs*

One of the first attempts to develop TTCs for food packaging materials was that published by Frawley in 1967 [12]. He evaluated a large dataset of 220 diverse chemicals for which the 2-year chronic toxicity study information was available. The chemicals were grouped into five categories on the basis of their NOELs (No observable effect levels). The categories were as follows: >1, >10, >100, >1000, $>10000\,mg\,kg^{-1}$ of diet. Most of the chemicals (180/220) had NOELs greater than $100\,mg\,kg^{-1}$ of diet, 19 had NOELs below $10\,mg\,kg^{-1}$ of diet but all of these were pesticides or heavy metals. There were five chemicals with NOELs below $1\,mg\,kg^{-1}$ of diet but again these were all pesticides with known toxicity. On the basis of his findings, Frawley suggested a level of $10\,mg\,kg^{-1}$ of diet for food packaging materials. Applying an additional safety factor of 100 gave a level of $0.1\,mg\,kg^{-1}$ in the human diet.

Rulis [13] conducted a similar analysis using the FDA's Priority Based Assessment of Food Additives (PAFA) database containing 159 compounds with subchronic or chronic data, LD50 values from 18,000 oral rodent studies from the Register of Toxicology Effects of Chemical Substances (RTECS) database (RTECS) [14] and TD50 values for 130 compounds taken from the Gold Carcinogenicity Potency database (CPD) [15]. He also determined that an intake of between 1–10 $\mu g\,kg^{-1}$/bw day of various chemicals might not pose a risk to humans.

The next major developments were in 1993 and 1995 when the FDA proposed and adopted the threshold of regulation for food contact substances [15,16]. These were substances that would result in minimal migration into food but which would be exempted from regulation as food additives. The threshold was set at 0.5 ppb or less for substances used in food contact articles i.e., an intake of $1.5\,\mu g\,person^{-1}\,day^{-1}$ ($0.025\,\mu g\,bw^{-1}\,day^{-1}$). Below this level FDA requires no specific toxicity testing and performs an abbreviated safety assessment mainly focussed on intake assessment.

Munro *et al.* [18] re-analysed the data assessed by Rulis [13] using the same methodology and also applied the approach to three alternative carcinogenicity datasets

including an updated CPD. Overall, the results of the analysis indicated that there was a low probability that a dietary level of 1 ppb of a substance of unknown toxicity would present a greater than one in a million risk of cancer.

1.3 *Structural TTCs*

Efforts to derive structural based TTCs have typically made use of the structural decision rules defined by Cramer *et al*. [19]. We will refer to these structural rules as Cramer decision tree rules or Cramer classes in the remainder of the document.

Munro *et al*. [20] explored the relationship between structure and toxicity by compiling a large database of over 600 substances that had been tested for a variety of endpoints. The resulting database contained 2941 NOELs for a total of 613 organic substances. The substances were then assigned to one of three structural classes as defined by Cramer *et al*. [19]. The distributions of NOELs were found to differ for the three classes of chemicals revealing how structural class has an important bearing on toxicity. Human exposure thresholds were derived for the three classes namely $1.8\,\mathrm{mg\,day^{-1}}$ for Class I, $0.54\,\mathrm{mg\,day^{-1}}$ for Class II and $0.09\,\mathrm{mg\,day^{-1}}$ for Class III substances.

In reference [21] a procedure for evaluating the safety of flavouring substances which integrated data on intake, structure-activity relationships, metabolism and toxicity was outlined. The procedure was developed for use by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) to evaluate flavouring substances. The approach of deriving human exposure limits using the Cramer structural classes was also described and the same approach was applied to examine limits for neurotoxicity and immunotoxicity endpoints. The thresholds derived from the CPD were found to be more conservative than those from other non-cancer endpoints.

Cheeseman *et al*. [22] examined whether structural parameters or the result of certain short term toxicity tests could be used to define a subset of less potent substances that supported higher threshold levels. A threshold of 4–5 ppb was proposed for substances without carcinogenicity structural alerts or with an Ames negative assay. Substances with a negative Ames test, no structural alerts and a LD50 greater than $1000\,\mathrm{mg\,kg^{-1}}$ had a proposed threshold of 10–15 ppb. In addition, results of reproductive tests for 3306 compounds and other multiple dose toxicity tests for 2542 compounds were compared with the database of carcinogenic potencies to establish whether carcinogenic endpoints were the most conservative toxicity endpoints for establishing thresholds of regulations. For each chemical, the lowest dose at which a toxic effect was seen was identified. This dose was divided by a safety/uncertainty factor of 1000 to derive a range of pseudo acceptable daily intakes, PADIs. The most likely medium value derived from the PADIs was found to be 8300 fold above the threshold derived from the CPD. This supported the premise that a virtually safe dose (VSD) based on carcinogenicity data really did protect against other toxicity effects.

The Cramer structural decision tree [19] has been applied in various studies including work by Renwick [23] who described the JECFA (Joint FAO/WHO Expert Committee on food additives) procedure for a safety evaluation of flavouring agents; Smith *et al*. [24,25] who discussed a 12-step procedure for the safety evaluation of Natural Flavour Complexes (NFCs), Dolan *et al*. [6] who outlined the use of the TTC for pharmaceutical manufacturing operations, Blackburn *et al*. [10] who examined the validity of developing

TTCs for personal and home care products and Kroes *et al*. [9] who applied the approach to cosmetic ingredients.

The Cramer *et al*. [19] decision tree relies primarily on chemical structures and estimates of total human intake to establish priorities for testing. The procedure uses recognised pathways for metabolic deactivation and activation, data on toxicity and the presence of a substance as a component of traditional foods and as an endogenous metabolite. It results in placing substances into one of three classes. The three classes are as follows.

Class I are substances of simple chemical structure with known metabolic pathways and innocuous end products which would suggest a low order of oral toxicity. Examples include simple alcohols, ketones, aldehydes, substances which occur naturally in food or are endogenous.

Class II contains substances that are intermediate. They possess structures that are less innocuous than in the Class I but they do not contain structural features that are suggestive of toxicity like those of Class III. Most substances that belong in Class II belong to either two categories, one includes substances within functional groups similar but more reactive than that in Class I, the other includes substances with more complex structures than substances in Class I but they are common components of food. This category includes heterocyclic substances and terpene ketones.

Class III are substances of a chemical structure that permit no strong initial impression of safety and may even suggest a significant toxicity. Examples include heterocyclic and heteroaromatic substances and cyclic ethers, many of these have side chains with reactive functional groups.

The Cramer scheme consists of 33 questions, each answered yes or no. Each answer leads to another question or to a final classification into one of the three classes. The tree is organised into branches dealing with major chemical classifications and is intended for use with all ingested, structurally defined organic and metallo-organic substances. Answering the questions does assume a reasonable competence in chemistry or biochemistry as the scheme relies on features of chemical structure as well as known data on metabolism or toxicity. The Cramer question set is provided as background information in Appendix I.

The concept of establishing concept levels has also been investigated further by BIBRA International to evaluate food chemicals more generally [26]. They established concern levels for several food additives, plastic monomers and flavouring substances. In their opinion, the Cramer *et al*. [19] scheme misclassified several substances and required some modification. Yet despite this conclusion, the Cramer scheme in its original form still continues to be routinely applied.

Processing a large number of compounds through the scheme can be a time-intensive activity, in an effort to automate such evaluations, the European Commission Joint Research Centre's European Chemicals Bureau (ECB) commissioned the development of a software tool – Toxtree to implement the Cramer scheme. This is part of a wider ECB initiative to develop various *in silico* tools for the assessment of chemicals and to make them freely and publicly accessible [27]. The Toxtree software was developed by Ideaconsult Ltd (Sofia, Bulgaria). Since its original version which only encoded the Cramer scheme, additional rule-based classification schemes including the Verhaar scheme [28–30] as well as the BfR/SICRET skin irritation and corrosion rules [31,32] have been implemented. The Toxtree program is available as a free download from the ECB website (http://ecb.jrc.it/qsar/qsar-tools/index.php?c=TOXTREE).

1.4 *Objectives*

The objective of this work was to process two TTC datasets reported in the literature; the original Cramer *et al*. [19] dataset and the Munro *et al*. dataset [20] through Toxtree and to evaluate the extent to which the reported structural classes were correctly reproduced. In addition, where inconsistencies were identified, modifications were proposed.

2. Methods

The datasets were extracted from both papers. Structures were identified for as many of the compounds as possible. The structures were saved as sdf files and processed through Toxtree Version 1.2 (Ideaconsult Ltd, Sofia, Bulgaria). Results were saved as excel files together with the path information (i.e., the answers to the questions). This transparent audit trail allows the output to be examined further as necessary. Within Toxtree, this is termed the ''verbose explanation''. Figure 1 shows a screenshot of Toxtree once launched to show where the audit trail information is visible.

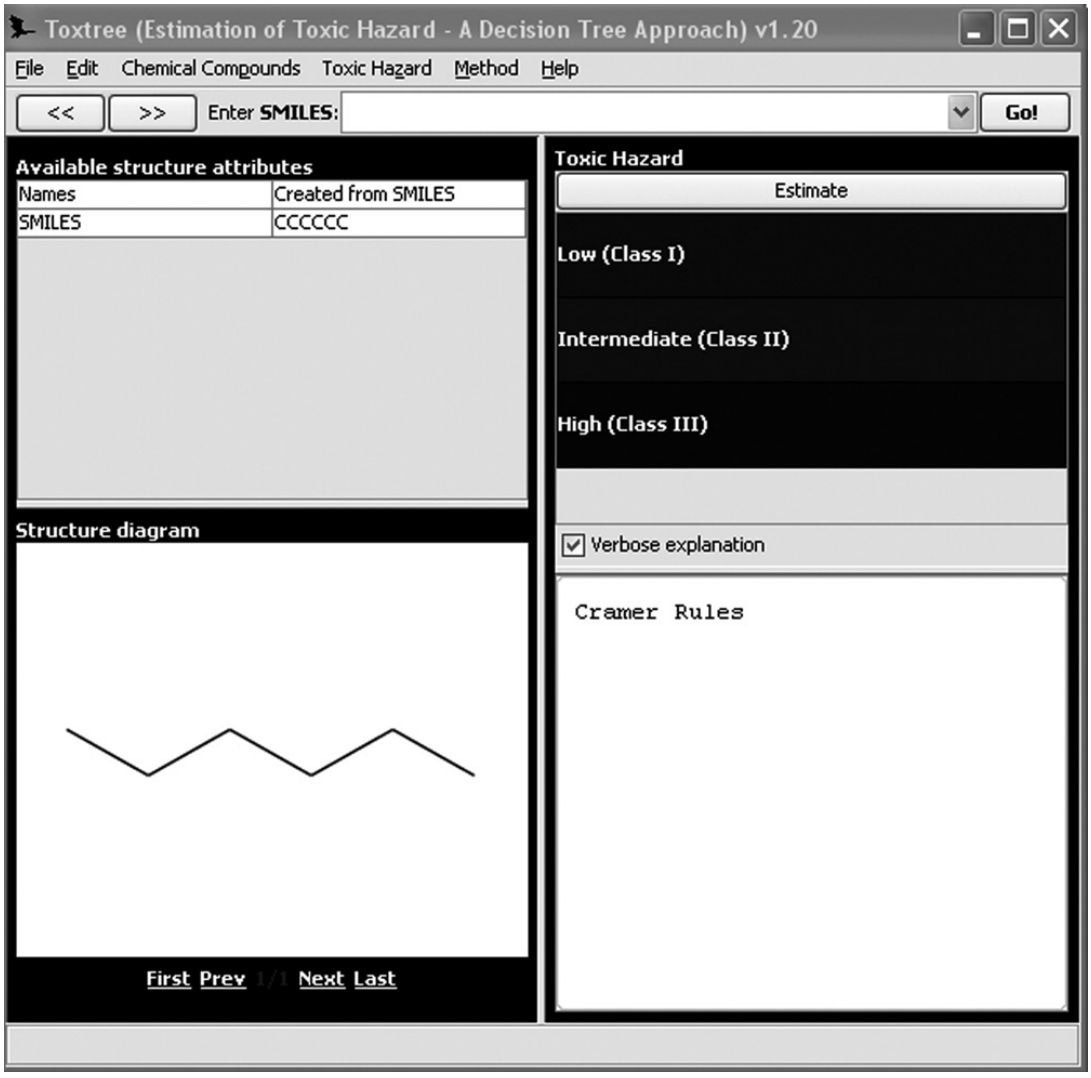


Figure 1. Screenshot of Toxtree.

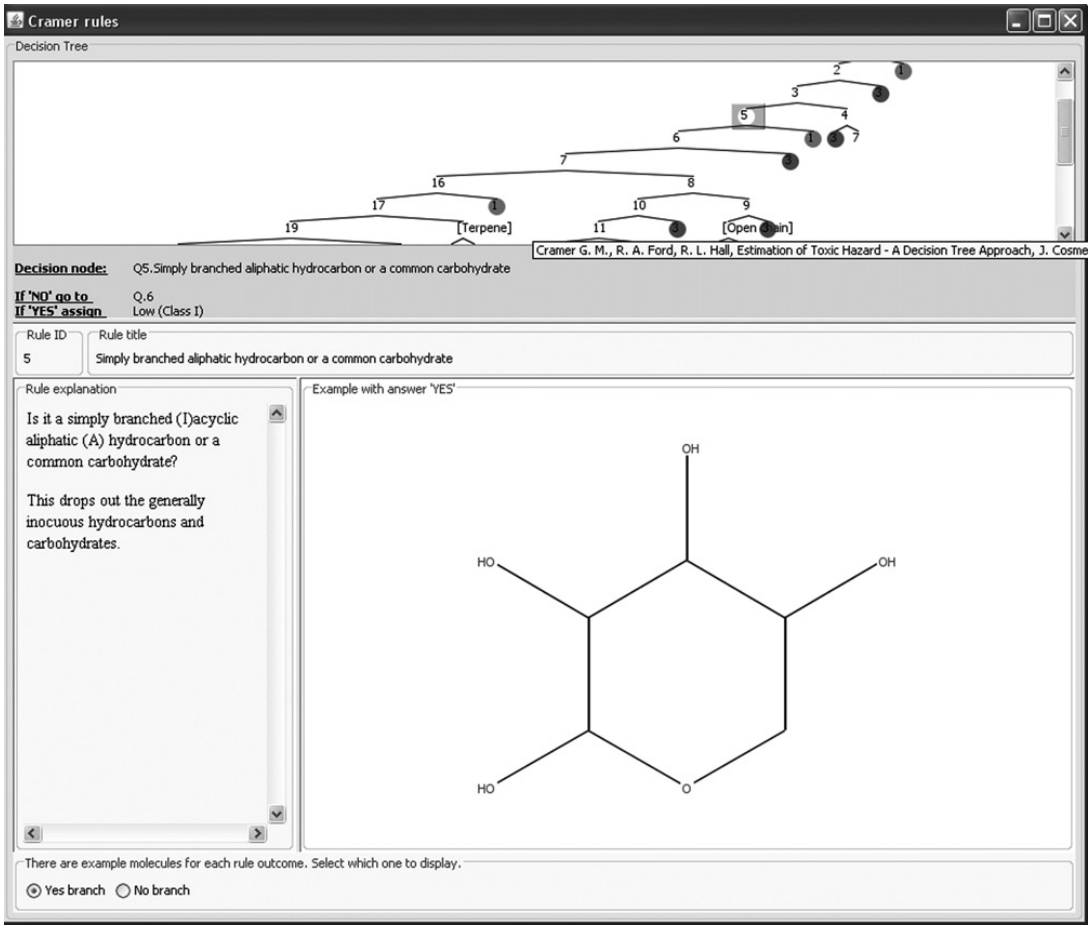


Figure 2. The decision tree as implemented in Toxtree together with one of the questions.

In the original Cramer *et al*. [19] publication, the path was provided as part of the appendix tabulating the substances, structures, NOELs and structural class information. This facilitated a direct comparison to be made with the Toxtree output and allowed for any inconsistencies to be rapidly identified and rationalised. In the Munro *et al*. publication [20], the final structural class information is noted but the path was not reported. In this case, the path was determined manually, in an effort to ensure that the structural class derived was consistent with that reported by Munro *et al*. [20] and then was compared with the output from Toxtree.

The decision tree implemented in Toxtree together with one of the questions is shown in Figure 2.

3. Results and discussion

3.1 *Cramer dataset*

The dataset as reported in Cramer *et al*. [19] comprises 82 substances in total. This was processed through Toxtree in order to classify the compounds into one of the three structural classes. Only one compound was misclassified (i.e., 99% of the Toxtree classifications were correct). Isobornylacetate (IBA) (see Figure 3) was

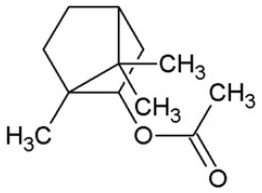


Figure 3. Structure of isobornylacetate (IBA).

Table 1. Paths for IBA.

Name	*Cramer reported class*	*Cramer reported pathway*	*Toxtree estimated pathway*	*Toxtree generated class*
IBA	II	1N,2N,3N,5N,6N,7N,16N,17N, 19N,23N,24N,25N,26N,22Y II	1N,2N,3N,5N,6N,7N,16N,17Y (19Y,20Y,21N,18N)(18N) I	I

incorrectly assigned to Class I by Toxtree whereas Cramer *et al*. [19] had classified it as Class II.

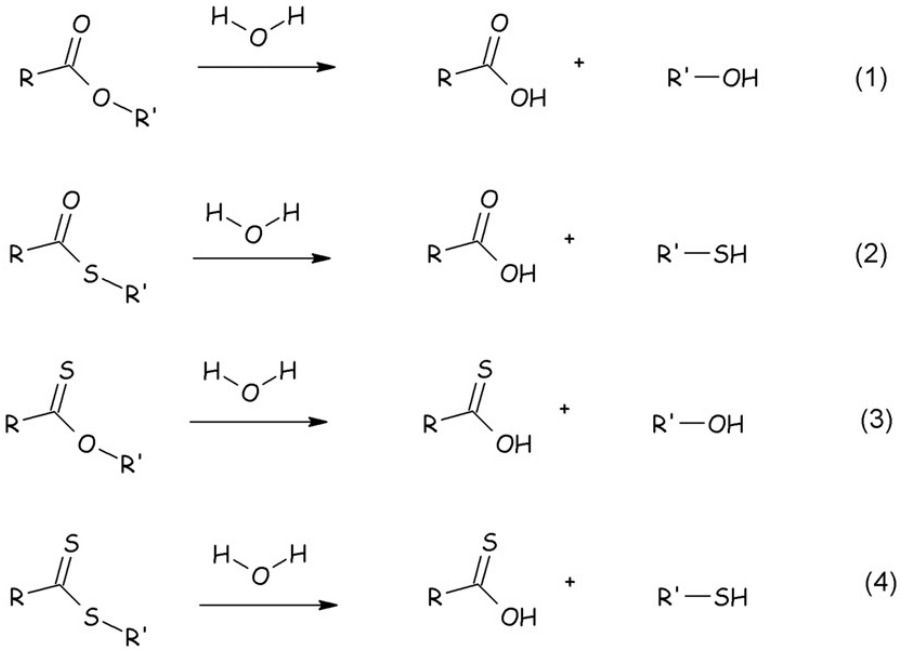
Examining the paths (Table 1) reveals a discrepancy in the answer to Question 17 – ''is the substance readily hydrolysed to a common terpene?'' The Cramer decision tree implies that IBA will not to be readily hydrolysed to a common terpene whereas Toxtree estimated the contrary.

According to the fixed set of four hydrolysis reactions implemented in Toxtree, (see Scheme 1), IBA is able to undergo one of hydrolysis reactions shown. The hydrolysis product is recognised by Toxtree as common terpene, although this is not correct, according to its stereo configuration. A refinement to the way in which Toxtree recognises common terpenes would resolve this type of discrepancy.

On the other hand the Cramer *et al*. [19] decision tree rule assumes easy hydrolysis by physiological processes after consumption. This hydrolysis will not be possible for IBA, since it is not the natural stereoisomer and will not be hydrolysed by enzymes existing in human body. This is not captured by Toxtree. If the hydrolysis was not constrained to physiological processes, a possible explanation for the discrepancy might be that the rules for hydrolysis encoded into Toxtree ignore the potential that steric and inductive effects can play in modifying behaviour. The constrained nature of the alkyl R' group in IBA might possibly hinder attack at the carbonyl group, resulting in IBA being less susceptible to hydrolysis. The set of rules for hydrolysis in Toxtree could be refined to reflect such subtleties.

3.2 *Munro dataset*

The Munro *et al*. dataset [20] comprised over 600 compounds which had been assigned into the three Classes for the purposes of deriving human exposure levels. The full list of compounds is available as an appendix in the primary reference. Here, we will only make reference to those compounds that were misclassified by Toxtree for each of the three classes in turn.



Scheme 1. General hydrolysis rules as implemented within Toxtree.

3.2.1 *Class I*

There were 136 compounds assigned as Class I by Munro *et al*. [20]. Toxtree correctly assigned 122 of them i.e., 89.7% (122/136) were correctly classified. Of the 14 compounds that were misclassified; four were assigned as Class II (Table 2) and 10 as Class III (Table 3).

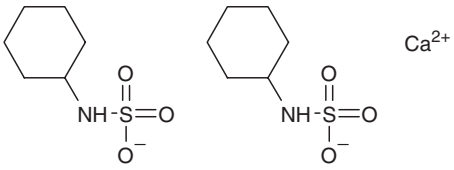
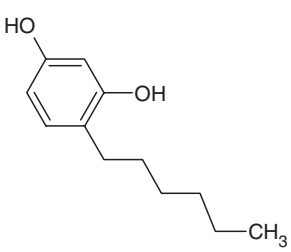
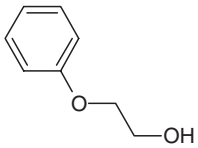
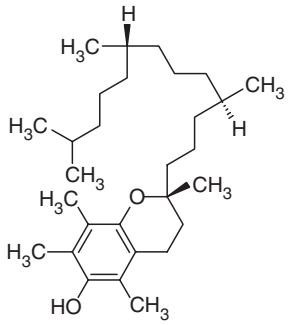
3.2.1.1 *Class I substances misclassified by Toxtree as Class II*. Table 2 lists the misassigned compounds together with their CAS numbers, structures and NOEL values as determined by Munro *et al*. [20]. The compounds are discussed in turn below.

A related compound to calcium cyclamate [139-06-0], sodium cyclamate was identified in the Cramer *et al*. [19] dataset. Inspection of its reported path (1N,2N,3Y,4Y,7N, 16N,17N,19N,23N,24Y,18N – I) and comparing it with that produced by Toxtree (1N,2N,3Y,4Y,7N,16N,17N,19N,23N,24N,25N,26Y – II) for calcium cyclamate [139-06-0] provided some insights to help rationalise the discrepancy observed.

As can be seen from the paths, the discrepancy lies in Question 24 (''Is the substance monocarbocylic with ring or aliphatic side chains, unsubstituted or containing only alcohol, aldehyde, side chain ketone, acid, ester or sodium, potassium or calcium sulphonate or sulphamate or acyclic acetal). In this case Toxtree counted two rings instead of one due to the structural representation of calcium cyclamate. A refinement within Toxtree to prevent rings on disconnected structures from being counted would resolve this type of discrepancy.

4-Hexylresorcinol [136-77-6] was manually re-evaluated and the following path was proposed (1N,2N,3N,5N,6N,7N,16N,17N,19N,23Y,27Y,28N,30N,18N – I). Toxtree on the other hand produced 1N,2N,3N,5N,6N,7N,16N,17N,19N,23Y,27Y,28N,30Y,31N,32Y – II as its path. A conflict with the response to Question 30 might account for the

Table 2. Munro Class I compounds that were misclassified by Toxtree as Class II.

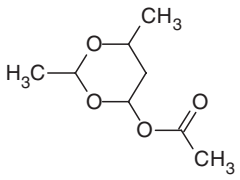
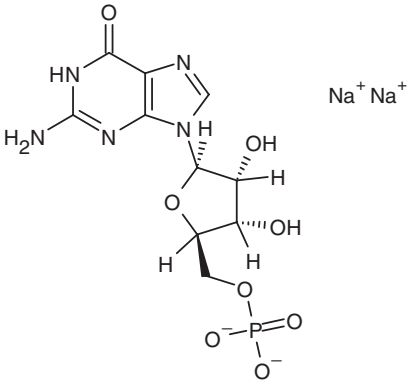
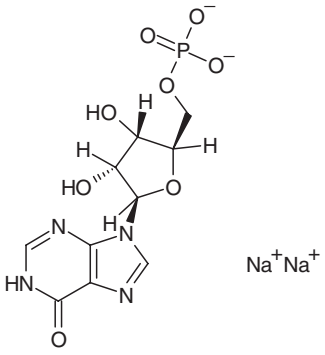
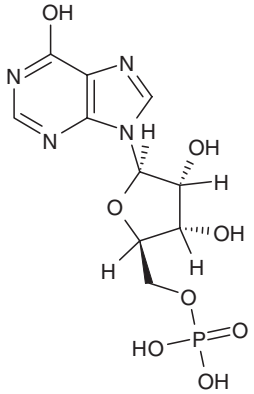
Structure	*Name*	*CASRN*	*NOEL (mg kg^{-1} bw day^{-1})*
	Calcium cyclamate	139-06-0	7203
	Hexylresorcinol, 4-	136-77-6	62.5
	Phenoxyethanol	122-99-6	80
	Tocopherol, alpha-	59-02-9	310

misclassification in this case. Question 30 seeks to determine the presence of substituents that may be hydrolysed to ring substituents of five or less carbons. However, the cut-off of alkyl chain in Question 30 is set at five carbons not six as is the case in the structure here. A negative response to Question 30 for 4-hexylresorcinol would be followed with a negative response to Question 18 and will ultimately give rise to a Class I assignment. More work is required using further examples to elucidate the modifications that may be necessary for Toxtree.

The same reasoning (i.e., a conflict with Question 30) was proposed for phenoxyethanol [122-99-6]. The Toxtree path and that derived manually were identical to that for 4-hexylresorcinol.

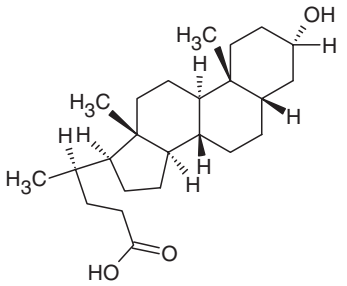
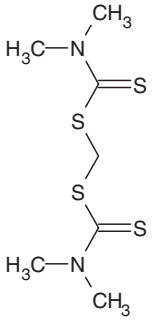
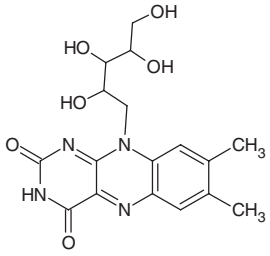
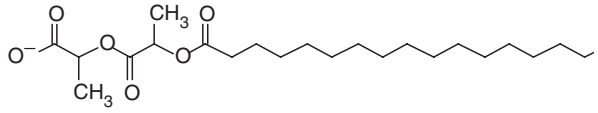
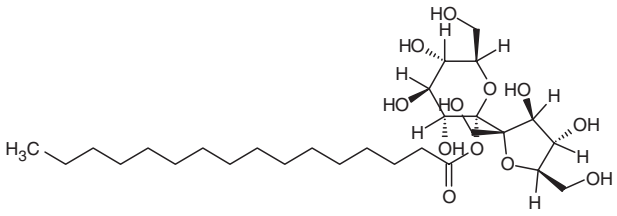
Another compound misclassified by Toxtree was alpha-tocopherol [59-02-9]. Also known as Vitamin E, this compound is naturally present in the body and therefore proposed to be Class I by virtue of a positive response to Question 1 (Is the substance a normal constituent of the body or an optical isomer of such?). The list of normal components in the body could be extended within Toxtree to include this compound as

Table 3. Munro Class I compounds that were misclassified by Toxtree as Class III.

Structure	*Name*	*CASRN*	*NOEL (mg kg^{-1} bw day^{-1})*
	Dimethoxane	828-00-2	62.5
	Disodium 5′-guanylate	5550-12-9	100
	Disodium 5-inosinate	4691-65-0	1441
	Inosine monophosphate	131-99-7	1774

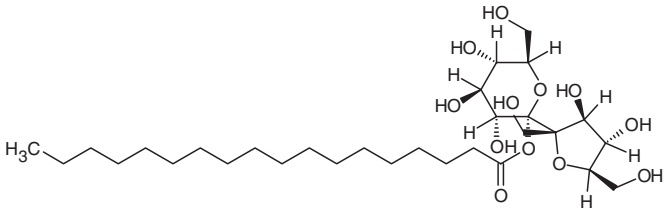
(*Continued*)

Table 3. Continued.

Structure	Name	CASRN	NOEL ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Lithocholic acid	434-13-9	250
	Methylenebis, 2,2′-	22656-77-5	187
	Riboflavin	83-88-5	4
	Sodium stearoyl lactylate	25383-99-7	4435
	Sucrose monopalmitate	26446-38-8	720

(*Continued*)

Table 3. Continued.

Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}$ $bw\,day^{-1}$)
	Sucrose monostearate	25168-73-4	2000

another example. The list of normal body components currently implemented within Toxtree is limited to a set of 67 examples taken from several internet sources crosschecked by an expert.

3.2.1.2 *Class I substances misclassified by Toxtree as Class III*. Table 3 lists those substances reported as Class I by Munro *et al*. [20] but classified as Class III by Toxtree.

Dimethoxane [828-00-2] the path by Toxtree is 1N,2N,3N,5N,6N,7Y,8N,10N, 11N,12N,22N,33N – III, which appears plausible given what toxicity data has been reported for Dimethoxane [33–36]. We therefore question the classification reported by Munro *et al*. [20].

Disodium 5′-guanylate [5550-12-9] is a food additive used to enhance flavour. It is listed on EAFUS which contains ingredients added directly to food that US FDA has either approved as food additives or are listed or affirmed as GRAS (generally recognised as safe). EAFUS is an inventory often referred to as *''Everything'' Added to Food in the United States* comprising of more than 3000 substances. The information was generated from a database maintained by the US Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition (CFSAN) under an ongoing program known as the Priority-based Assessment of Food Additives (PAFA) [37]. The following path was proposed following manual inspection 1N,2N,3Y,4Y,7Y,8N,10N,11Y,33Y – I but only if phosphate salts were considered to be part of the scope of Question 4 (Do all elements not listed in Question 3 occur only as (a) a sodium, potassium, calcium, magnesium or ammonium salt of a carboxylic acid or (b) a sulphate or hydrochloride of an amine or (c) a sodium, potassium or calcium sulphonate, sulphamate or sulphate?). The answer generated by Toxtree was as follows: 1N,2N,3Y,4N – III. It is feasible that the evaluation by Munro *et al*. [20] reached a similar conclusion to ensure that a known food additive was not misclassified to be of higher concern.

Disodium 5-inosinate [4691-65-0] is also a food additive listed on EAFUS [37]. It often added to foods in conjunction with disodium 5′-guanylate. By analogy to disodium 5′-guanylate, the scope of Question 4 might have been the reason for the misclassification generated by Toxtree.

Inosine monophosphate (IMP) [131-99-7] is a nucleoside, formed from ribose 5-phosphate in the initial step of purine metabolism. As such, it is plausible that this

compound would evoke a positive response to Question 1 (Is the substance a normal constituent of the body?). The path generated by Toxtree was 1N,2N,3Y,4N. An extension to the list of 67 body constituents currently implemented within Toxtree would avoid such misclassifications.

Lithocholic acid [434-13-9] is a naturally occurring bile acid [38] which would suggest it should fall into Class I by virtue of a positive response to Question 1.

Examining the path for 2,2′-methylenebis, (methylenebis(N,N-dimethyldithiocarbamate)) [22656-77-5] manually gives rise to the same classification as that generated by Toxtree (1N,2N,3N,5N,6N,7N,16N,17N,19Y,20N,22N,33N – III). No reasoning could be determined for the misclassification of this compound by Toxtree.

Riboflavin [83-88-5], also known as vitamin B_2, is an easily absorbed micronutrient with a key role in maintaining health. It is probably synthesised by intestinal bacteria like other B vitamins. It is continuously excreted in the urine of healthy individuals [39]. An addition to the list of normal body constituents (as per Question 1) should prevent this compound being misclassified by Toxtree.

Sodium stearoyl lactylate [25383-99-7] is assigned to Class III by Toxtree in accordance with the following path; (1N,2N,3Y,4N – III). Re-examination of the path manually (1N,2N,3Y,4Y,7N,16N,17N,19Y,20Y,21N,18N – I) suggests a potential conflict with the answer to Question 4. A modification to the implementation of Question 4 in Toxtree would be worthwhile.

Manual re-evaluation of sucrose monopalmitate [26446-38-8] and sucrose monostearate [25168-73-4] (1N,2N,3N,5Y – I) suggests that the scope of the implementation to Question 5 might account for the misclassification made by Toxtree of these two compounds (1N,2N,3N,5N,6N,7Y,8N,10N,11Y,33N –III).

3.2.2 *Class II*

There were 28 compounds assigned to Class II (Intermediate) by Munro *et al*. [20]. Of these, 16 were correctly assigned as Class II (57.14%), 2 (7%) were incorrectly assigned as Class I and 10 (35.71%) as Class III.

3.2.2.1 *Class II substances misclassified by Toxtree as Class I*.

Table 4 lists the compounds which were misclassified as Class I.

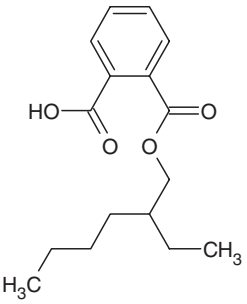
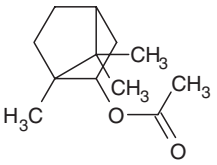
Isobornyl acetate [125-12-2] appeared in the Cramer *et al*. [19] dataset and as such has already been discussed.

Ethylhexyl phthalate mono-2-[4376-20-9] was re-examined manually resulting in the same pathway as that generated by Toxtree. A related compound ethylhexyl phthalate di-2-, was found in the Cramer *et al*. [19] dataset and used to explore whether there was an alternative explanation for the misclassification between the Munro *et al*. [20] publication and Toxtree. In fact, the related compound was assigned as Class I by virtue of the same path as that derived by Toxtree. We are therefore in agreement with the assignment in Cramer *et al*. [19] and believe the classification in Munro *et al*. [20] to be incorrect.

3.2.2.2 *Class II substances misclassified by Toxtree as Class III*.

Table 5 lists the compounds which were misclassified as Class III.

Table 4. Munro Class II compounds misclassified by Toxtree as Class I.

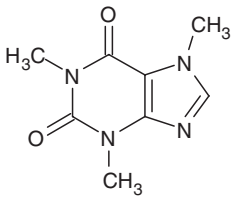
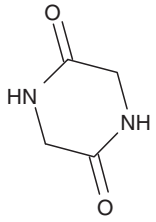
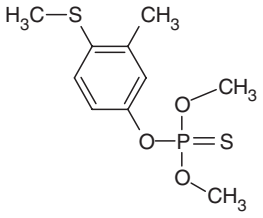
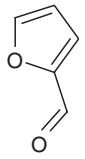
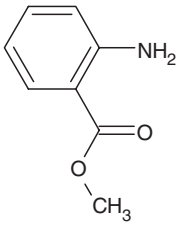
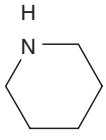
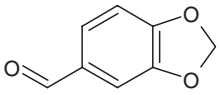
Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Ethylhexyl phthalate, mono-2-	4376-20-9	50
	Isobornyl acetate	125-12-2	15

Caffeine [58-08-2] was assigned to Class III by Toxtree (1N,2N,3N,5N,6N,7Y,8N, 10N,11Y,33N – III). Comparison with the path generated manually (1N,2N,3N,5N,6N, 7Y,8N,10N,11N,12Y,13Y,14N,22Y – II) reveals a discrepancy in the answer to Question 11 (Does the hetereocyclic ring contain or bear substituents other than simply branched hydrocarbons, alkyl alcohols, aldehydes, acetals, ketones, ketals, acids, esters, mercaptans, sulphides, methyl ethers, hydroxy or single rings with no substituents other than those listed?). Close examination of the scope of Question 11 would be worthwhile to determine whether a modification in Toxtree is required.

Diketopiperazine [29990-68-9] is a breakdown product of the sweetener aspartame. This might explain the observed discrepancy between the Munro classification and that arising from Toxtree. A positive answer to Question 22 (which is concerned with identification of common components of food) would lower the concern level from Class III (as determined by Toxtree – 1N,2N,3N,5N,6N,7Y,8N,10N,11N,12N,22N, 33N – III) to Class II (as determined manually).

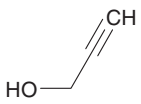
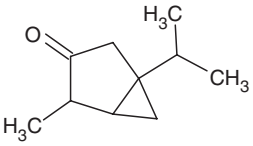
Manual inspection of the Cramer decision tree for fenthion [55-38-9] could give rise to the same pathway (i.e., 1N,2N,3Y,4N) as that proposed by Toxtree but which is in conflict with the class reported in Munro *et al*. [20]. If Munro *et al*. [20] considered the phosphate group as part of the scope of Question 4 (as discussed previously for sodium stearoyl lactylate [25383-99-7]), then the following paths could be envisaged; 1N,2N,3Y,4Y, 7N,16N,17N,19N,23Y,27Y,28N,30Y,31N,32N,22Y – II or 22N,33Y – III. In the former case, a positive answer to Question 22 would imply that fenthion was a common component of food or structurally related to a common component of food. In the case of a negative answer to Question 22, the subsequent answer to Question 33 is likely to be negative too and thus would assign fenthion as Class III. Since fenthion is classified by the U.S. Environmental Protection Agency (EPA) as a Restricted Use Pesticide (RUP) due to

Table 5. Munro Class II compounds misclassified by Toxtree as Class III.

Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Caffeine	58-08-2	10.1
	Diketopiperazine	29990-68-9	500
	Fenthion	55-38-9	16
	Furfural	98-01-1	30
	Methyl anthranilate	134-20-3	150
	Piperidine	110-89-4	69
	Piperonal	120-57-0	360

(*Continued*)

Table 5. Continued.

Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Propargyl alcohol	107-19-7	5
	Pyridine	110-86-1	1
	Thujone	546-80-5	5

the special handling warranted by its toxicity, we propose that a Class III is the correct one in this instance and hence disagree with the classification proposed in Munro *et al*. [20].

Furfural [98-01-1] is a chemical derived from a variety of agricultural byproducts, including corncobs, oat and wheat bran [40]. However, Toxtree generated the following path (1N,2N,3N,5N,6N,7Y,8N,10N,11N,12Y,13Y,14N,22N,33N – III). In light of its food origin, and listing on the EAFUS inventory [37], a positive response to Question 22 would account for the Class II classification proposed in Munro *et al*. [20]. The list of food components implemented within Toxtree could be usefully extended with this example.

Methyl anthranilate [134-20-3], also known as MA, methyl 2-aminobenzoate or carbomethoxyaniline, is an ester of anthranilic acid. It is used as a bird repellent to protect corn, sunflowers, rice and fruit etc. It also occurs naturally in the Concord grapes, and in bergamot, champaca, gardenia, jasmine, lemon, mandarin, neroli, oranges, rue oil, strawberry, tuberose, and ylang ylang. It is used for flavouring of sweet, soft drinks, gums, and drugs. The US FDA considers it as GRAS and it is listed on the EAFUS inventory [37]. This would explain the classification proposed by Munro *et al*. [20]. Re-examination of methyl anthranilate suggests the following plausible path 1N,2N,3N,5N,6N, 7N,16N,17N,19N,23Y,27Y,28N,30Y,31N,32N,22Y – II. This differs significantly from the path generated by Toxtree from. 1N,2N,3N,5N,6N,7N,16N,17N,19N,23Y,27Y, 28N,30Y(31N,32Y – II)(31N,32N,22N,33N – III) in the answer to Question 30. Question 30 raises the possibility of hydrolysis of simple esters, such that the resulting hydrolysis products are treated separately in subsequent questions. Toxtree determines methyl anthranilate as having the potential to be hydrolysed, by virtue of its rulebase for reactivity. However inspection of the structure suggests that whilst hydrolysis is theoretically possible through attack at the carbonyl group, the methoxy group is such a poor leaving group, that the reaction is not favoured. Thus the Cramer decision path of the reaction product resulting in a Class III assignment is not feasible. A modification to the hydrolysis rule within Toxtree would be a useful improvement. In addition, the

extension of the food component list to include methyl anthranilate as an example would also be worthwhile.

The path generated by Toxtree for piperidine [110-89-4] was 1N,2N,3N,5N,6N, 7Y,8N,10N,11N,12N,22N,33N – III. However since piperidine is the main active chemical agent in black pepper a positive response to Question 22 would result in a Class II classification which corresponds to that reported in Munro *et al*. [20].

Piperonal [120-57-0] (heliotropine, protocatechuic aldehyde methylene ether) is used as a flavouring and in perfume. It is also a minor natural component of the extract of vanilla [41]. This most likely explains the classification proposed in Munro *et al*. [20] and accounts for the discrepancy observed in the Toxtree path. A refinement to Question 22 within Toxtree would address this misclassification.

Re-examining propargyl alcohol [107-19-7] reveals a conflict in Question 20 between the two paths that derived manually and that generated by Toxtree. (1N,2N,3N,5N,6N, 7N,16N,17N,19Y,20N,22N,33N – III for Toxtree *vs*. 1N,2N,3N,5N,6N,7N,16N,17N, 19Y,20Y,21N,18Y – II manually). Question 20 explores whether the compound is linear or simply branched and contains a combination of specific functional groups such as alcohols, aldehydes, carboxylic acids etc. It is feasible that propargyl alcohol (1-hydroxy-2-propine) was treated as two separate functional groups – the triple bond (C#C) and alcohol. Since the former was not listed as an example, a negative response was generated to Question 20. A refinement in the implementation of Question 20 might be required within Toxtree. In this case, the impact of a modification should be carefully reviewed to ensure that other rules are not adversely affected.

Comparison between the classification by Toxtree and that derived manually for pyridine [110-86-1] reveals no conflict 1N,2N,3N,5N,6N,7Y,8N,10N,11N,12Y,13N – III. Pyridine is assigned as Class III which is inconsistent with the classification made in Munro *et al*. [20]. The latter may in fact be incorrect but without the path information, the apparent discrepancy can not be accounted for.

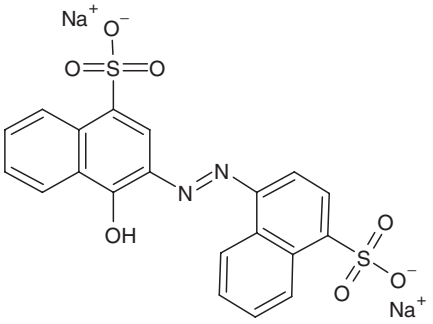
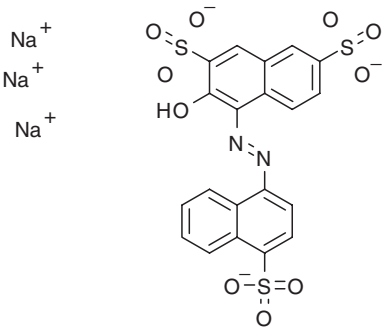
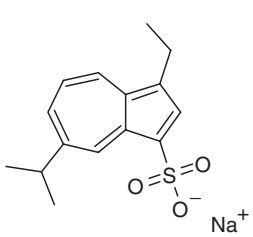
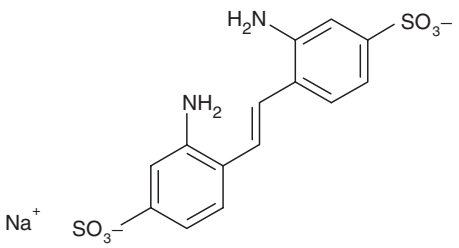
Thujone [546-80-5] is found in a number of plants, such as arborvitae, as well as some junipers, sage, and wormwood. It is most known for being a chemical in the drink absinthe, a distilled, highly alcoholic, anise-flavoured spirit derived from herbs. Certain levels of thujone are permitted in foodstuffs in the EU. This potentially explains the classification made in Munro *et al*. [20] since the discrepancy arose in the response to Question 22.

3.2.3 *Class III*

There were 446 chemicals assigned to Class III. A structure could not be determined for trenbolone hydroxide, 17-alpha-. Azorubine and carmoisine were listed with invalid CAS numbers (3567-69-0 and 3567-64-9, respectively), though subsequent searching identified that they both shared the same CAS number as C.I. Acid Red 14 (3567-69-9). Since the NOELs reported for these three compounds were different, it was considered best to exclude azorubine and carmoisine from the analysis. For the remaining 443 chemicals, 429 (96.8%) were correctly assigned as Class III, 12 (2.7%) were incorrectly as Class I (Table 6) and 3 (0.68%) as Class II (Table 7).

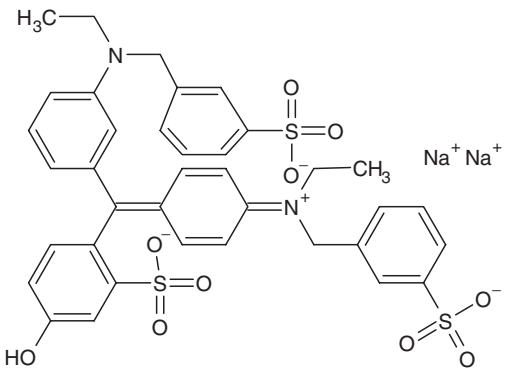
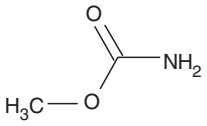
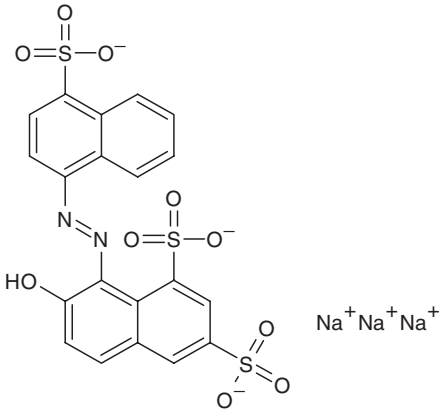
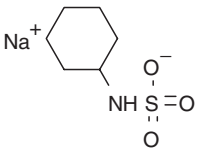
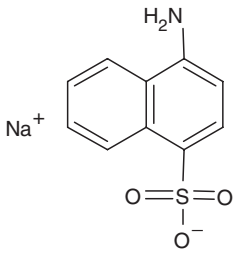
3.2.3.1 *Class III substances misclassified by Toxtree as Class I*. Table 6 listed the substances together with their structures and reported NOEL values. Many of the

Table 6. Munro Class III compounds misclassified by Toxtree as Class I.

Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	C.I. Acid Red 14	3567-69-9	1171
	Amaranth	915-67-3	7.5
	Azuletil sodium (KTI-32)	99287-30-6	10
	Diamino-2,2′-stilbenedisulfonic acid, 4,4′-, disodium salt	7336-20-1	1207

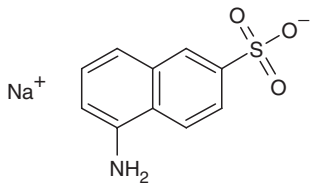
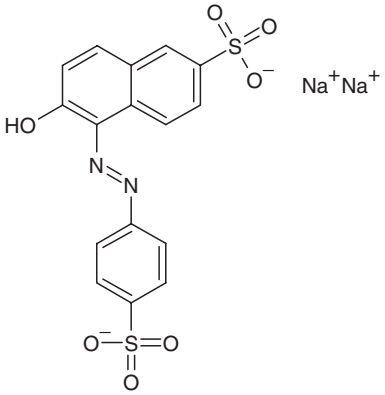
(*Continued*)

Table 6. Continued.

Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Fast Green FCF	2353-45-9	1716
	Methyl carbamate	598-55-0	100
	Ponceau 4R	2611-82-7	86
	Sodium cyclamate	139-05-9	720
	Sodium naphthionate (Sodium 4-aminonaphthalene-1-sulphonate)	130-13-2	30

(*Continued*)

Table 6. Continued.

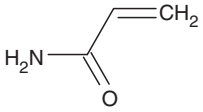
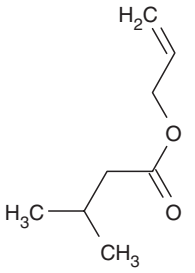
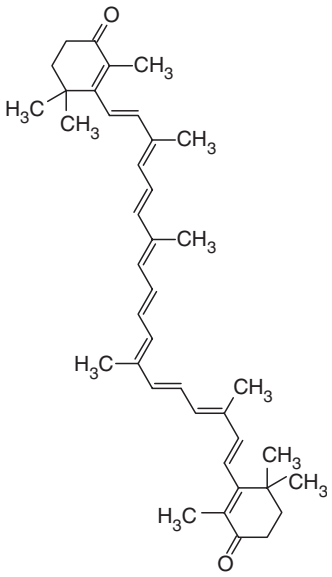
Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Sodium naphthionate (Sodium 5-aminonaphthalene-2-sulphonate)	28907-84-8	30
	Sunset Yellow FCF	2783-94-0	678

chemicals listed here are dyes. They appear to be misclassified on account of Question 33 in the Cramer decision tree. This question aims to discriminate between Class I and Class III compounds by considering the ratio of sulphonate/sulphamate groups to free primary amines and their position. Sodium, potassium and calcium sulphonate and sulphamate salts have a strong tendency to decrease the toxicity by promoting solubility and rapid excretion. This is particularly noticeable for certain food colourings. It is important that the compound bears sufficient sulphonate groups including one on each of the major structural fragments into which the original compound might be metabolised. This question serves to steer sulphonated compounds except those with amines non-adjacent to the sulphonate into a presumptively less toxic classification than those compounds would occupy if they were unsulphonated.

The current implementation within Toxtree uses a small set of metabolic reactions (Scheme 2) to find the products into which the original compound might be metabolised. If a sulphonate group is present in every metabolite, then the answer to Question 33 is considered positive and the compound is assigned as Class I. However Toxtree fails to assign compounds where the sulphonate group is adjacent to a primary amine as Class I. This is a necessary modification within Toxtree.

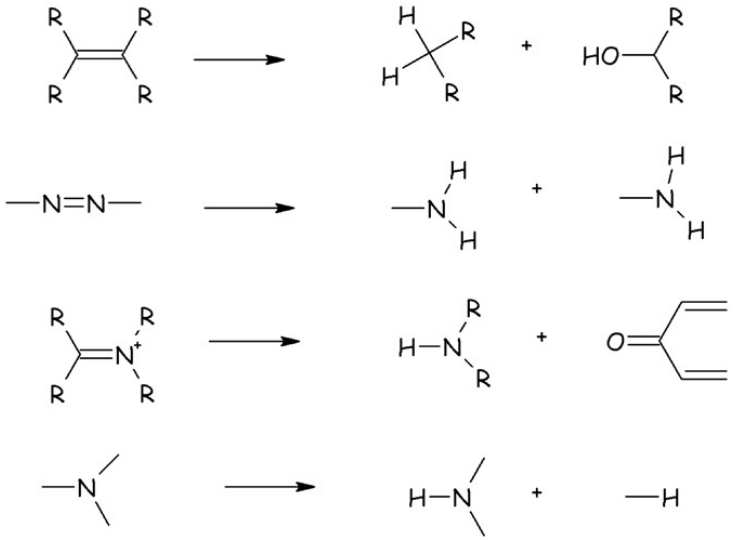
C.I. acid red 14 [3567-69-9], amaranth [915-67-3], sunset yellow FCF [2783-94-0], ponceau 4R [2611-82-7] and fast green FCF [2353-45-9] satisfy the conditions of Question 33 of sufficient sulphonate groups and without primary NH2 groups to be assigned as Class I. Two closely related structures (F&DC Yellow No. 6 [2753-94-0] and F&DC Red No. 4 [4548-53-2] were found in the Cramer *et al*. [19] dataset which were also

Table 7. Munro Class III compounds misclassified by Toxtree as Class II.

Structure	*Name*	*CASRN*	*NOEL* ($mg\,kg^{-1}\,bw\,day^{-1}$)
	Acrylamide	79-06-1	0.2
	Allyl isovalerate	2835-39-4	62
	Canthaxanthin	514-78-3	500

assigned as Class I. Their structures are shown in Figure 4. We believe the Munro *et al*. [20] assignment of Class III may be incorrect in these five cases. Diamino-2,2′-stilbenedisulfonic acid, 4,4′-, disodium salt [7336-20-1], sodium 4-aminonaphthalene-1-sulphonate [130-13-2] and sodium 5-aminonaphthalene-2-sulphonate [28907-84-8] are assigned by Toxtree as Class I. These 3 compounds should have been classified as Class III, since there are primary amino groups but they are not adjacent to the sulphonate group. This is not currently flagged by Toxtree and a modification is required to address this shortcoming.

Toxtree generated the following path for azuletil sodium (KTI-32) [99287-30-6], 1N,2N,3Y,4Y,7N,16N,17N,19N,23Y,27Y,28N,30N,18N – I. Re-examination of the path manually identifies the potential of an alternative path (1N,2N,3Y,4Y,7N,16N,17N,19N, 23Y,27Y,28Y,29N,33) if the scope of Question 28 was interpreted differently. Question 28



Scheme 2. General metabolic reactions within Toxtree (used in Question 33).

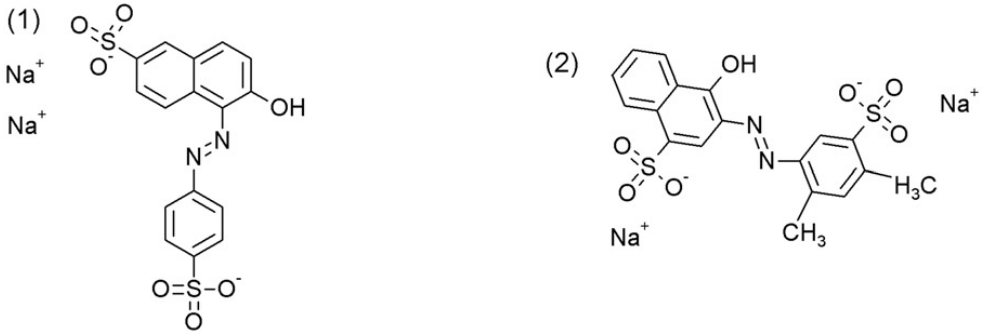


Figure 4. Related dye structures from Cramer *et al*. [19] with Class I assignment.
Note: (1) is FD & C Yellow No. 6 and (2) is FD & C Red No. 4.

asks whether the compound contains more than one aromatic ring. Azuletil sodium contains an aromatic azulene background which could have been interpreted in Munro *et al*. [20] to be comprised of aromatic cycloheptatriene and aromatic cyclopentadiene structures. This would mean a positive response to Question 28 which in turn would lead to Question 33 from which a Class III assignment is feasible. This may explain the classification made in Munro *et al*. [20]. Azuletil sodium is actually an anti-ulcer drug [42] suggesting that a Class I assignment might be entirely plausible. The Munro *et al*. [20] is questionable but without the path information, the apparent discrepancy can not be accounted for.

Methyl carbamate [598-55-0] shows a conflict in the response to Question 20. Toxtree output 1N,2N,3N,5N,6N,7N,16N,17N,19Y,20Y,21N,18N – I which is in contrast to the path derived manually 1N,2N,3N,5N,6N,7N,16N,17N,19Y,20N,22N,33N – III). This explains the likely misclassification in this case. Question 20 outlines how the compound must be a linear or simply branched aliphatic compound containing any one or combination of specific functional groups such as carboxylic acids, esters, primary amines etc. In this case, the carbamate group (H_2N–COO) is not represented in the list of

functional groups given in Question 20 and for this reason a negative response was proposed. Functional groups should be treated in their entirety rather than as individual fragments, it is feasible that Toxtree considered this compound as possessing a primary amine group and ester group and hence assigned a positive response to Question 20. Whilst reviewing the implementation of Question 20 within Toxtree would be worthwhile, the practical solution is less clear as an explicit definition of all functional groups would need to be implemented.

Sodium cyclamate [139-05-9] an artificial sweetener [43], has been previously evaluated by Cramer *et al*. [19] and assigned Class I. A related compound, Calcium cyclamate was discussed earlier and also assigned Class I. We disagree with the classification proposed in Munro *et al*. [20] in this case.

3.2.3.2 *Class III substances misclassified by Toxtree as Class II*. Table 7 lists the three substances that were misclassified by Toxtree together with their structures and reported NOEL values. The possible reasons for the misclassifications observed are discussed for each compound in turn.

Acrylamide [79-06-1] was re-evaluated manually and a Class III assignment was proposed owing to a discrepancy in the response to Question 20. The implementation of this rule may need to be re-considered in Toxtree as discussed earlier for methyl carbamate in particular. The path proposed by manual inspection was 1N,2N,3N,5N,6N,7N, 16N,17N,19Y,20N,22N,33N – III as opposed to that generated by Toxtree (1N,2N,3N, 5N,6N,7N,16N,17N,19Y,20Y,21N,18Y – II).

Manually re-examining the path for allyl isovalerate [2835-39-4] gives rise to the same path as that generated by Toxtree (1N,2N,3N,5N,6N,7N,16N,17N,19Y,20Y,21N,18Y – II). It is plausible that the Munro evaluation considered the allyl functionality to be unaccounted explicitly in the list of functional groups under Question 20. A negative response to Question 20 would ultimately result in an assignment of Class III through the following path 1N,2N,3N,5N,6N,7N,16N,17N,19Y,20N,22N,33N – III. More plausible is the path proposed by Toxtree particularly since Question 18 lists a set of compounds including allylic compounds to help discriminate between Class I and Class II compounds. In addition, a closely related compound was identified in the Cramer *et al*. [19] dataset, allyl heptanoate. Inspection of the path reported there, revealed allyl heptanoate to be assigned to Class II by virtue of a positive response to Question 20 i.e., the same pathway as was generated for allyl isovalerate). No modification is proposed for Toxtree; the Class III assignment reported in Munro *et al*. [20] is believed to be incorrect.

Canthaxanthin [514-78-3] was classified by Toxtree as a Class II compound. Re-examination of the path manually gives rise to the same path (1N,2N,3N,5N, 6N,7N,16N,17N,19N,23N,24N,25N,26Y – II The discrepancy with the Munro *et al*. [20] assignment might lie with Question 26 and how it was interpreted. Question 26 raises whether the compound contains no other functional group other than aliphatic side chains, alcohol, aldehyde, ketone, acid, ester, acyclic acetal or ketal etc. If Question 26 was interpreted to exclude unsaturated aliphatic side chains from this list, then the next question in the path would query whether canthaxanthin was a common food component (i.e., Question 22) or not. Since canthaxanthin is in fact a food additive and listed on EAFUS [37], a positive response in Question 22 would result in a Class II assignment. The path being 1N,2N,3N,5N,6N,7N,16N,17N,19N,23N,24N,25N, 26N, 22Y – II. Whilst Question 26 might be open to interpretation, it is clear from exploring the other possible

pathway, that Canthaxanthin would still be assigned Class II. The listing on EAFUS supports a lower assignment i.e., Class II, thus the assignment reported by Munro *et al.* [20] is believed to be incorrect.

4. Conclusions

In this study, we have investigated the usefulness of the Toxtree software (version 1.2) as a tool for by applying the Cramer decision tree. On the basis of the results obtained, we highlight where refinements are needed to improve the way in which the Cramer decision tree is implemented in Toxtree.

We have evaluated the datasets that were reported in Cramer *et al.* [19] and Munro *et al.* [20] using Toxtree to demonstrate its concordance with the Class assignments reported. Toxtree was found to perform well, demonstrating that the rules had been implemented satisfactorily into the software. This is evidenced by the high levels of correct predictions for the two datasets (99% for the Cramer *et al.* [19] dataset and for the Munro *et al.* [20] dataset: 89.7% (Class I), 57.1% (Class II) and 96.8% Class III)). The poorest agreement was for the Class II subset in the Munro *et al.* [20] dataset though on further examination, 3 compounds were felt to be incorrectly assigned by Munro *et al.* [20] and the majority of the remaining incorrect assignments were due incorrect identification of food components (Question 22). The list of food components within Toxtree is limited at present to 110 examples. Toxtree was also found to misclassify in a number of cases on Question 1 which identifies natural constituents of the body. Toxtree has a limited list of 67 examples. Updating these lists with a larger more comprehensive set of examples will significantly improve the performance of Toxtree. Inventories such as EAFUS [37] would be one useful resource of food components that could be used to update the present list within Toxtree. Other modifications could include refinements to the implementation of Question 17 regarding the recognition of common terpenes (c.f., Isobornyl acetate), Question 5 which concerns the identification of carbohydrates, and Question 33, which discriminates between Class I and III on the basis of the number of sulphonate/sulphamate groups and location of primary amino groups. This could be the subject of further work but would require evaluation of more example sets with reported Cramer decision tree classifications in order to precisely define the requirements necessary.

It is perhaps worth highlighting that the transparent means of reporting the results within Toxtree does in fact enable expert judgement to override the inconsistencies observed. The coding of the Cramer decision tree in the form of structure-based rules did necessitate some subjective interpretations, hence some rules as has been observed in this study can sometimes prove to be too wide or narrow in their scope. Overall, Toxtree is found to be a useful tool to enable the systematic evaluation of Cramer structural classes.

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Appendix I. The Cramer decision tree

Q1: Is the substance a *normal constituent of the body*, or an optical isomer of such?

This question throws into Class I all normal constituents of body tissues and fluids, including normal metabolites. Hormones are excluded, as are, by implication, the metabolites of environmental and food contaminants or those resulting from disease state.
If YES, Class I; If NO, proceed to Q2

Q2: Does the substance contain any of the following functional groups: an aliphatic secondary amine or a salt thereof, cyano, N-nitroso, diazo, triazeno or quaternary nitrogen, except in any of the following forms: $>C=N+R_2$, $>C=N+H_2$ or the hydrochloride or sulphate salt of a primary or tertiary amine?

Questions 2, 3 and 4 are a means of placing in Class III those structures that contain elements or valency states often associated with enhanced toxicity. Halo-, nitro-, N-nitroso- and diazo-compounds, organophosphates, quaternary nitrogen compounds and similar xenobiotic structures should cause 'yes' answers to Question 2 and 3 and a 'no' answer to Question 4.
If YES, Class III; If NO, proceed to Q3

Q3: Does the structure contain elements other than C, H, O, N or divalent S?

If YES, proceed to Q4; If NO, proceed to Q5

Q4: Do all elements not listed in Q3 occur only as (a) a Na, K, Ca, Mg or ammonium salt of a carboxylic acid, or (b) a sulphate or hydrochloride of an amine, or (c) a Na, K, or Ca sulphonate, sulphamate or sulphate? (If the answer is yes, treat as free acid, amine, unsulphonated or unsulphated compound, except for the purposes of Q24 and Q33, and proceed).

This is intended to let through, for further consideration, certain acid, amine, sulphonate and sulphate salts. Sulphamate salts are treated as such because they are not readily hydrolysed.

If YES, proceed to Q7; If NO, Class III

Q5: Is it a *simply branched* acyclic aliphatic hydrocarbon or a common carbohydrate?

This drops out the generally innocuous hydrocarbons and carbohydrates.

If YES, Class I; If NO, proceed to Q6

Q6: Is the substance a benzene derivative bearing substituents consisting *only* of (a) hydrocarbon chains or 1′-hydroxy or hydroxy ester-substituted hydrocarbon chains and (b) one or more alkoxy groups, one of which must be para to the hydrocarbon chain in (a)?

This places in Class III safrole, myristicin and related substances.

If YES, Class III; If NO, proceed to Q7

Q7: Is the substance heterocyclic

If YES, proceed to Q8; If NO, proceed to Q16

Q8: Is it a lactone or cyclic diester?

This question separates the lactones and cyclic diesters from other heterocyclic compounds.

If YES, proceed to Q9; If NO, proceed to Q10

Q9: Is it a Lactone, fused to another ring, or 5- or 6-membered a,b-unsaturated lactone?

This places certain lactones known or suspected to be of unusual toxicity in Class III.

*If it is a lactone, from this point on, treat the structure as if it were the hydroxy acid in the form of its more stable tautomer and proceed to Q20 if it is open chain, to 10 if it is heterocyclic and to Q23 if it is carbocylic; if it is a cyclic diester, treat as the separate components.

If YES, Class III; If NO, *

Q10: Is it a 3-membered heterocycle?

This places such substances as epoxides and ethylenimine in Class III.

If YES, Class III; If NO, proceed to Q11

Q11: Disregarding only the heteroatoms on any one ring, does that heterocyclic ring contain or bear substituents other than *simply branched* hydrocarbons (including bridged chains and monocyclic aryl or alkyl structures),alkyl alcohols, aldehydes, acetals, ketones, ketals, acids, esters (including cyclic esters other than lactones), mercaptans, sulphides, methyl ethers, hydroxy or single rings (hetero or aryl) with no substituents other than those just listed?

Questions 11 15 separate out various categories of heteroaromatic substances. Under 11, set aside and do not consider the atom(s), usually O, N and S, making the ring heterocyclic. If there is more than one hetero ring, regard each ring separately, with the remainder of the structure as substituents of that hetero ring. Other than the heterocyclic atom(s), does the ring carry anything besides the simple groups listed?

If so, the answer is YES, and the next Question 33. If not, then classify further by Q12 *et seq*. Bridged-chain derivatives may be represented by structures like the bicyclic ether 1,4-cineole while monocyclic aryl derivatives may be represented by compounds like benzaldehyde propylene glycol acetal or 3-phenyl-2-furancarboxaldehyde.

If YES, proceed to Q33; If NO, proceed to Q12

Q12: Is it heteroaromatic?

This question separates the aromatic heterocyclics for the purpose of considering whether they are polynuclear (Q14) or unsubstituted (Q13).

If YES, proceed to Q13; If NO, proceed to Q22

Q13: Does the ring bear any substituents?

If YES, proceed to Q14; If NO, Class III

Q14: Does the structure contain more than one *aromatic* ring?

If YES, proceed to Q15; If NO, proceed to Q22

Q15: Is it *readily hydrolysed* to mononuclear residues? (If YES, treat the mononuclear heterocyclic residues by Q22 and any carbocyclic residue by Q16).

If YES, proceed to Q22; If NO, proceed to Q33

Q16: Is it a *common terpene* -hydrocarbon, -alcohol, -aldehyde or -carboxylic acid (not a ketone)?

Q16 and Q17 deal with terpenes. A hydrocarbon terpene that is a *common terpene* and has not already been put in Class I by Q5, would go into Class I by Q16.

If YES, Class I; If NO, proceed to Q17

Q17: Is the substance *readily hydrolysed* to a *common terpene*, -alcohol, -aldehyde or -carboxylic acid? (If the answer is YES, treat the hydrolysed residues separately and proceed to Q18 for the terpene moiety and to Q19 for any non-terpenoid moiety).

Since there may be substances that are hydrolysed to two or more residues, one of which is terpene, treat the residues separately from Q18 onward to conclusion.

If YES, proceed to Q18; If NO, proceed to Q19

Q18: Is the substance one of the following:

- a vicinal diketone; or a ketone or ketal of a ketone attached to a terminal vinyl group
- a secondary alcohol or ester of a secondary alcohol attached to a terminal vinyl group
- allyl alcohol or its acetal, ketal or ester derivative
- allyl mercaptan, an allyl sulphide, an allyl thioester or allyl amine
- acrolein, a methacrolein or their acetals
- acrylic or methacrylic acid
- an acetylenic compound
- an acyclic *aliphatic* ketone, ketal or ketoalcohol with no other functional groups and with four or more carbons on either side of the keto group
- a substance in which the *functional groups* (E) are all *sterically hindered*

Q18 examines the terpenes (and later the open-chain and mononuclear substances by reference) to determine whether they contain certain structural features generally thought to be associated with some enhanced toxicity.

If YES, Class II; If NO, Class I

Q19: Is the substance *open chain*?

Q19-21 deal with open-chain substances.

If YES, proceed to Q20; If NO, proceed to Q23

Q20: Is the structure a linear or *simply branched aliphatic* compound, containing any one or combination of only the following *functional groups*: (a) four or less, each, of alcohol, aldehyde, carboxylic acid or esters and/or (b) one each of one or more of the following: acetal, either ketone or ketal but not both, mercaptan, sulphide (mono- or poly-), thioester, polyoxyethylene [(OCH_2CH_2)x with x no greater than 4], or primary or tertiary amine

This question should be answered YES if the structure contains one or any possible combination of alcoholic, aldehydic or carboxylic acid or ester groups, provided there are no more than four of any one kind. It should be answered YES if the structure contains in addition to, or instead of, those just listed, any assortment of no more than one each of the following: acetal, either ketone or ketal but not both, mercaptan, mono- or polysulphide, thioester, polyoxyethylene, primary or tertiary amine. Answer the question NO if the structure contains more than four of any of the first set of groups, more than one of the second set, or any substituent not listed.

If YES, proceed to Q21; If NO, proceed to Q22

Q21: Does the structure contain $>= 3$ different types of functional groups (exclude methoxy and consider acids and esters as one functional type)?

Aliphatic compounds containing three or more different functional groups (excluding methoxy) are too complex to permit satisfactory prediction of toxicity. They should go therefore, into Class III. However, we do not wish to put into Class III polyesters and similar substances, so these and the methoxy compounds get passed along to Q18.

If YES, Class III; If NO, proceed to Q18

Q22: Is the substance *a common component of food or structurally closed related to a common component of food*?

This question places in Class II the natural, nature-identical and nearly nature-identical substances not already put into Class I by physiological occurrence or structural criteria. An artificial substance or one not closely related, goes to Q33.

If YES, Class II; If NO, proceed to Q33

Q23: Is the substance *aromatic*?

Questions 23-26 deal with alicyclic substances

If YES, proceed to Q27; If NO, proceed to Q24

Q24: Is the substance monocarbocyclic (excluding cyclopropane or cyclobutane and their derivatives) with ring or *aliphatic* side chains, unsubstituted or containing only alcohol, aldehyde, side-chain ketone, acid, ester, or Na, K or Ca sulphonate or sulphamate, or acyclic acetal or ketal?

If YES, proceed to Q18; If NO, proceed to Q25

Q25: Is the substance (a) a cyclopropane or cyclobutane with only the substituents mentioned in Q24 or (b) a mono- or bicyclic sulphide or mercaptan?

If YES, Class II; If NO, proceed to Q26

Q26: Does the structure contain no functional groups other than those listed in Q24 and is either a monocycloalkanone or a bicyclic compound with or without a ring ketone?

If YES, Class II; If NO, proceed to Q22

Q27: Do(es) the ring(s) have any substituents?

Questions 27 31 deal with aromatic compounds.

If YES, Class III; If NO, proceed to Q28

Q28: Does the structure contain more than one *aromatic* ring?

If YES, proceed to Q29; If NO, proceed to Q30

Q29: Is it *readily hydrolysed* to mononuclear residues? (If YES, treat the individual aromatic mononuclear residues by Q30 and any other residue by Q19).

If YES, proceed to Q30; If NO, proceed to Q33

Q30: Disregarding ring hydroxy or methoxy does the ring bear substituents *other* than 1-5-carbon *aliphatic* groups, either hydrocarbon or containing alcohol, ketone, aldehyde, carboxyl or simple esters that may be hydrolysed to ring substituents of 5 or less carbons? (If a simple ester that may be hydrolysed, treat the aromatic portion by Q18 and the residue by Q19).

This should be answered NO if the ring bears only aliphatic groups of 5 carbons or less, which are either hydrocarbon in nature or contain the groups listed. If the ring bears any other substituents than those listed, the question should be answered YES and one should proceed to Q31.

If YES, proceed to Q31; If NO, proceed to Q18

Q31: Is the substance an acyclic acetal, -ketal or -ester of any of the above substances (see Q30)?.

(If YES, assume hydrolysis and treat the non-aromatic residues by Q19 and the aromatic residue by Q18.)

This question is simply designed to see whether the substance would fit within the definition of Q30 if it were not an acetal, a ketal or an ester. In other words, would the substance carry only the groups listed in Q30.

If YES, proceed to Q18; If NO, proceed to Q32

Q32: Does the substance contain only the *functional groups* listed in Q30, or their derivatives listed in Q31, but with any or all of the following: (a) a single fused non-aromatic carbocyclic ring, (b) *aliphatic* substituent chains longer than 5 carbon atoms, or (c) a polyoxyethylene [(OCH_2CH_2)x, with x no greater than 4] chain either on the aromatic ring or on an *aliphatic* side chain?

Part (a) is intended to allow simple derivatives of tetralin into Class II while putting polycyclic compounds such as steroids ultimately into Class III except those that may be normal food components. Part (b) allows compounds with permitted functional groups but longer side chains into Class II instead of sending them eventually into Class III. Part (c) puts short-chain polyoxyethylene derivatives of aryl compounds into Class II rather than Class III.

If YES, Class II; If NO, proceed to Q22

Q33: Does the substance bear on every major structural component at least one Na, K or Ca sulphonate or sulphamate for every 20 or fewer carbon atoms without any free primary amines except those adjacent to the sulphonate or sulphamate.

Na, K, Ca sulphonate and sulphamate salts have a strong tendency to decrease toxicity by promoting solubility and rapid excretion. This is particularly noticeable, for example, with some of the food colourings. It is important that the substance bears sufficient sulphonate groups, including one on each major structural fragments into which the original compound might be metabolised. This question serves to steer sulphonated compounds except those with amines non-adjacent to the sulphonate into a presumptively less toxic classification than the compounds would occupy if unsulphonated.

If YES, Class III; If NO, Class I