**(Q)SAR tools for priority setting: a case study with printed paper and board food contact material substances**

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**Abstract**

Over the last years, more stringent safety requirements for an increasing number of chemicals across many regulatory fields (e.g. industrial chemicals, pharmaceuticals, food, cosmetics,...) have triggered the need for an efficient screening strategy to prioritize the substances of highest concern. In this context, alternative methods such as *in silico* (i.e. computational) techniques gain more and more importance. In the current study, a new prioritization strategy for identifying potentially mutagenic substances was developed based on the combination of multiple (quantitative) structure-activity relationship ((Q)SAR) tools. Non-evaluated substances used in printed paper and board food contact materials (FCM) were selected for a case study. By applying our strategy, 106 out of the 1723 substances were assigned ‘high priority’ as they were predicted mutagenic by 4 different (Q)SAR models. Information provided within the models allowed to identify 53 substances for which Ames mutagenicity prediction already has *in vitro* Ames test results. For further prioritization, additional support could be obtained by applying local i.e. specific models, as demonstrated here for aromatic azo compounds, typically found in printed paper and board FCM. The strategy developed here can easily be applied to other groups of chemicals facing the same need for priority ranking.

**Keywords**

(Q)SAR; mutagenicity; prioritization; food contact materials; alternative methods

**Abbreviations**

AD(I), applicability domain (index); ECHA, European Chemicals Agency; EFSA, European Food Safety Authority; FACET, Flavours, Additives and food Contact materials Exposure Task; FCM, food contact materials; FIG, FACET Industry Group; k-NN, k-Nearest Neighbors; IRFMN, Instituto di Ricerche Farmacologiche Mario Negri; (Q)SAR, (quantitative) structure-activity relationship; RASFF, Rapid Alert System for Food and Feed; REACH, Registration, Evaluation, Authorisation and restriction of Chemicals; SA, structural alert; SMILES, simplified molecular-input line-entry system

**1. Introduction**

Together with the high and continuously growing number of chemical substances subject to safety assessment, comes the need to establish adequate screening strategies to prioritize those of highest concern for human and/or environmental health. One notable example of a large group of substances urgently requiring a prioritization ranking for in-depth safety evaluation, are those used in food contact materials (FCM). Food contamination due to leakage of substances from FCM has become an increasing source of concern for human health (Liu et al., 2016, Muncke et al., 2014). Since 2011, an updated list of substances authorized as starting product or additive for the manufacture of plastic FCM is available (European Union, 2011). For non-plastic FCM, however, no harmonized European regulation has been established yet. Although national legislation exists in several Member States for different types of FCM, a broad range of substances currently used in FCM have not been evaluated for their safety (European Parliament, 2016).

Printing inks and paper(board) constitute large groups of non-plastic FCM substances. They are often used in combination and have been at the origin of multiple contamination issues, examples being the isopropylthioxanthone and the 4-methylbenzophenone crises (EFSA, 2005 and 2009). Most of the substances that can be present in printed paper and board FCM have not been officially evaluated for their potential toxicity. Consequently, these non-evaluated substances could give rise to future food crises (Van Bossuyt et al., 2016).

Regarding plastic FCM, the European Food Safety Authority (EFSA) requires a core set of test data in order to be able to evaluate consumer safety of these materials. Genotoxicity data are always requested, regardless of the (estimated) migration level (EFSA, 2012). Indeed, genotoxicity i.e. the ability to cause DNA damage, can induce adverse human health effects including cancer (Claxton et al., 2010). In line with new EFSA Scientific Committee’s recommendations on genotoxicity testing strategies, a battery of 2 *in vitro* genotoxicity tests is required, i.e. a gene mutation test in bacteria and an *in vitro* mammalian cell micronucleus test. If one of these tests yields a positive or equivocal result, further (*in vivo*) testing may be needed in order to investigate the genotoxic potential of the substance (EFSA, 2016).

The bacterial reverse mutation assay (Ames test) is the most commonly used *in vitro* test to detect gene mutations (OECD, 2008). Although it is a suitable test to identify gene mutation-inducing chemicals, its technical characteristics (in particular the test duration and the high quantity of test compound required) do not allow testing of 1000+ substances in a short period of time at reasonable cost. The same obstacles are also encountered with the other assay required in the genotoxicity testing battery. A promising approach to detect mutagens without animal nor *in vitro* testing lies in the application of *in silico* tools. These computer-assisted methodologies are based on available experimental data, and are increasingly adopted in regulatory toxicology because of their time-, cost- and animal-saving nature. In particular, (quantitative) structure activity relationship ((Q)SAR) systems represent promising predictive computational techniques to evaluate potential genotoxicity and carcinogenicity of chemical substances (Serafimova et al., 2010).

(Q)SARs comprise both statistical QSAR and rule-based SAR systems. Rule-based models perform predictions via detection of so-called ‘structural alerts’ (SA), i.e. chemical fragments responsible for the toxic effect as determined earlier based on human expert knowledge. Statistical models, on the other hand, predict toxicity using an algorithm obtained by investigating the mathematical correlation between chemical properties (translated into molecular descriptors) and toxic activity (Bakhtyari et al., 2013). In both systems, chemicals are typically processed by means of their simplified molecular-input line-entry system (SMILES) representation. Most commercial (e.g. Derek Nexus®) and free (e.g. Toxtree) *in silico* software programs include statistical QSAR and/or rule-based SAR models to predict the induction of gene mutations in the Ames test (‘Ames mutagenicity’). Furthermore, due to the abundance of consistent Ames test results and due to the binary result type: mutagenic/non-mutagenic, robust models for Ames mutagenicity are available and therefore the prediction performance for this endpoint is substantially better compared to other toxicological endpoints (Kamath et al., 2015). Indeed, *in silico* models for genotoxic endpoints other than Ames mutagenicity (e.g. chromosome damaging potential in the micronucleus test) exist, but until now their accuracy is limited and needs to be improved before these models can become a more reliable screening tool.

Numerous publications on (Q)SAR evaluation of chemicals/chemical groups are available, however mostly in the context of model validation. Besides one study in which 2 SAR models were used to rank heat-generated food contaminants (Cotterill et al., 2008), to our knowledge, no study reports are available on the application of (Q)SARs for prioritization of potential human genotoxicants. In the current study, a screening strategy based on (Q)SAR tools is applied to identify, within the large number of non-evaluated substances that can be used in printed paper and board FCM, those that represent the highest concern for human health. The non-evaluated substances were first selected from a recently compiled inventory containing all substances which may be used in this type of FCM (Van Bossuyt et al., 2016). Next, their potential to induce gene mutations was predicted using a battery of Ames mutagenicity (Q)SAR models. The models were selected by taking into account existing recommendations such as the use of complementary systems (in terms of prediction method). Moreover, the combination of a SAR and a QSAR is already mandatory in certain regulatory domains, for example in the case of impurity testing of pharmaceuticals as described in the ICH M7 guidelines (ICH, 2014). Using the combined (Q)SAR results, a priority list could be composed of non-evaluated printed paper and board FCM substances requiring an urgent in-depth safety evaluation.

**2. Materials and methods**

**2.1. Study substances**

Substances that have not been officially evaluated were selected from a recently compiled inventory including 6073 unique substances which may be used in printed paper and board FCM (Van Bossuyt et al., 2016). Out of the 4690 non-evaluated compounds, 1769 single substances were retained for the current analysis. The remaining 2921 non-evaluated substances are not eligible for straightforward *in silico* processing, due to their chemical structure (e.g. polymers, mixtures, complexes, inorganic substances). Subsequently, the ChemSpider (Royal Societies of Chemistry, 2016), ChemIDplus (National Institutes of Health, 2016a), PubChem (National Institutes of Health, 2016b) and European Chemicals Agency (ECHA, 2016) databases were consulted to collect missing CAS numbers and SMILES for the 1769 non-evaluated single substances. ChemSpider was used as the primary information source, whereas the ChemIDplus, PubChem and ECHA databases were consulted in case ChemSpider yielded no or ambiguous results. Afterwards, the compound selection was further refined by excluding substances for which no definite CAS number or SMILES could be identified, reducing the final number to 1723 (**Figure 1**).

Figure 1. Selection of study substances

**2.2. (Q)SAR models**

The selected (Q)SAR models, specified in **Table 1**, are diverse not only regarding their prediction method (SAR/QSAR), but also with respect to their availability (free/commercial). For each system, the prediction model(s) related to Ames mutagenicity was (were) applied.

Table 1. Model description

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Abbreviation | Software (version) | Model name | Method | AD | Availability |
| *Global (Q)SARs* | | | | | |
| Toxtree | Toxtree (2.6.0) | *In vitro* mutagenicity alerts (Ames test) by ISS | SAR | / | Freeware |
| VEGA | VEGA (1.1.1) | Mutagenicity (Ames test) model (CAESAR) v.2.1.13  Mutagenicity (Ames test) model (SarPy/IRFMN) v.1.0.7  Mutagenicity (Ames test) model (ISS) v.1.0.2 | QSAR  QSAR  SAR | VEGA ADI  VEGA ADI  VEGA ADI | Freeware |
| Derek | Derek Nexus™ (4.1.0) | Mutagenicity *in vitro* | SAR | / | Commercial |
| Sarah | Sarah Nexus™ (1.2.0) | Ames mutagenicity | QSAR | Sarah AD | Commercial |
| *Local QSARs for aromatic azo compounds* | | |  |  |  |
| CORAL | CORAL | Ames mutagenicity | QSAR | DefectSMILES | Freeware |
| istKNN | istKNN (0.9) | Ames mutagenicity | QSAR | / | Commercial |

AD(I): applicability domain (index); (Q)SAR: (quantitative) structure-activity relationship

* *Toxtree*

Toxtree (www.toxtree.sourceforge.net) is an open source software application of the Joint Research Centre of the European Union (European Commission, 2016b). Toxic hazard of test compounds is predicted based on a decision tree-approach, constructed through the definition of rules flagging alerts for the selected endpoint. For Ames mutagenicity, 44 SAs are incorporated. A QSAR module for aromatic amines and αβ-unsaturated aliphatic aldehydes is also available and allows to refine the prediction of these specific chemical classes (not considered in the current study). Toxtree does not feature an applicability domain functionality.

* *VEGA*

The VEGA platform (www.vega-qsar.eu) has been developed by the Istituto di Ricerche Farmacologiche Mario Negri (IRFMN) and can be downloaded for free. It comprises an array of toxicity estimation models, including 3 for the evaluation of Ames mutagenicity i.e. CAESAR, SarPy and ISS (IRFMN, 2016a). CAESAR and SarPy, both QSAR models, were developed using the same training set of 4337 compounds. However, their prediction technique differs in the sense that CAESAR combines a machine-learning algorithm with 2 sets of sequential SAs (Ferrari and Gini, 2010), whereas SarPy follows a purely quantitative approach to determine whether test compounds are mutagenic or non-mutagenic (Ferrari et al., 2013). The third Ames mutagenicity prediction model, ISS, contains a set of SAs extracted from Toxtree, more specifically the SAs related to mutagenicity as implemented in the Benigni-Bossa rulebase for mutagenicity and carcinogenicity. In theory, this should result in the same model as the Toxtree model described above. However, in practice the outcome sometimes differs in VEGA/ISS and Toxtree. A possible explanation for these differences may be found in the rebuilding process that was used to translate the Toxtree rulebase into VEGA/ISS.

In the current study, the separate results of the 3 Ames mutagenicity models were combined into 1 final ‘VEGA consensus’ result, since this approach increases the prediction performance compared to the use of the individual models (Cassano et al., 2014). The output of the single models is integrated through their corresponding applicability domain index (ADI) by means of the following equation:

CONSENSUS = (±1)∗ADICAESAR + (±1)∗ADISarPy + (±1)∗ADIISS

ADICAESAR + ADISarPy + ADIISS

Each ADI in the numerator is multiplied by +1 for a positive prediction and by -1 for a negative prediction. In case the final outcome is negative, only prediction results with an ADI of at least 0.75 in all 3 models were considered negative. A similar approach has been proposed by Cassano and colleagues (2014).

* *Derek Nexus™*

Derek is commercially available as part of the Lhasa Knowledge Suite® (Lhasa Limited, 2016a) and is a SAR tool that runs predictions for, among others, *in vitro* mutagenicity through expert-based rules. The latter were developed from a variety of open literature and confidential data. For this reason and because it is a rule-based system, no defined training set nor applicability domain are available. However, a recently implemented structure classification feature allows to substantiate negative predictions (Williams et al., 2016). In case no alert for Ames mutagenicity is found, the software labels the test compound as ‘inactive’ (i.e. negative). Additionally, the compound structure is screened for ‘misclassified’ and ‘unclassified’ features. If it contains a chemical fragment that is not retrieved in the set of compounds on which the expert rules are based, the graphic display will highlight this part of the molecule and indicate that the structure contains unclassified features. Misclassified features, on the other hand, refer to chemical substructures that are not SAs, but have been found in experimentally positive reference compounds that lack a SA in Derek. Since negative predictivity generally remains high for both (median=84%), misclassified and unclassified features are regarded as negative predictions that are flagged for expert review. However, for the current prioritization strategy that does not include elaborate expert reviewing, we followed a precautionary approach. Hence only negative predictions without warnings were considered negative.

* *Sarah Nexus™*

The Lhasa Knowledge Suite® also contains a QSAR-based Ames mutagenicity model named Sarah (Lhasa Limited, 2016b). In this statistical tool, the query compound is fragmented, after which the fragments are reviewed for activity *versus* inactivity. A network of hypotheses is then created by arranging meaningful fragments, followed by the application of relevant hypotheses to inform an overall mutagenicity prediction. A confidence score and applicability domain check complete the final conclusion.

* *CORAL*

CORAL (www.insilico.eu/coral) is a freely available standalone application software for building regression or classification QSAR models based on the Monte Carlo optimization method. It was developed as part of the EU-funded CHEMPREDICT project (IRFMN, 2016b). A complete description of the Ames mutagenicity model for aromatic azo compounds, built using the CORAL software, is provided by Manganelli et al. (2016). In brief, this model was generated using local and global SMILES-based descriptors on 3 random splits of data in training, calibration and validation sets. Test compounds are checked for falling into the applicability domain by calculating their DefectSMILES that should not exceed a predefined threshold value.

* *istKNN*

istKNN is a recently developed commercial software tool that can be used to build, evaluate and apply k-Nearest Neighbors (k-NN) models. The k-NN approach identifies a number (k) of neighboring compounds for the target compound to make a prediction. Each ‘neighbor’ is assigned a similarity index, allowing to extract the first k molecules with the closest similarity. In addition, specific similarity thresholds are defined, resulting in predictions solely based on molecules with a similarity index higher than the selected threshold. This method and the istKNN program are described by Manganaro et al. (2015). Details concerning the istKNN model for Ames mutagenicity prediction of aromatic azo compounds were recently published by Manganelli et al. (2016).

**2.3. Model output processing**

Each of the models described in section 2.2. introduces its own particular denomination method to label negative and positive compounds (or -in the case of Derek- the probability of toxicity). In the present study, the original model-specific classifications were converted into 3 categories (negative, positive or undefined) for reasons of uniformity (**Table 2**). The undefined category is composed of:

* substances outside domain (in the case of Sarah),
* substances lacking sufficient similar compounds to make a prediction (in the case of istKNN),
* substances for which negative predictions are less convincing due to a low ADI (in the case of VEGA),
* substances with high DefectSMILES (in the case of CORAL) and
* substances with mis-/unclassified features (in the case of Derek).

This conservative approach was adopted in order to minimize the number of false negatives. Indeed, labelling mutagens incorrectly as non-mutagenic should be avoided as much as possible.

Table 2. Harmonization of positive, negative and undefined predictions

|  |  |  |  |
| --- | --- | --- | --- |
| Model | Positive | Negative | Undefined |
| *Global QSARs* | | | |
| Toxtree | Structural alert for S. typhimurium mutagenicity | No structural alerts for S. typhimurium mutagenicity | N/A |
| VEGA | 1 | 0 with ADI ≥0.75 for all models | 0 with ADI <0.75 for at least 1 model |
| Derek | ‘Equivocal’ to ‘Certain’ | ‘Inactive without mis-/unclassified features’ to ‘Doubted’ | Inactive with mis-/unclassified features |
| Sarah | Positive | Negative | Outside domain |
| *Local QSARs for aromatic azo compounds* | | |  |
| CORAL | 1 | 0 with DefectSMILES <1.83485 | 0 with DefectSMILES ≥1.83485 |
| istKNN | 1 | 0 | No molecules were suitable for prediction |

N/A: not applicable; ADI: applicability domain index

**2.4. Determination and characterization of priority substances**

All compounds were processed in 4 global (Q)SARs (i.e. Toxtree, Vega, Derek Nexus™ and Sarah Nexus™), which are based on structurally diverse compounds, reflecting a range of different action mechanisms (Chaudry et al., 2010). Aromatic azo compounds were also examined in 2 local QSAR models (i.e. CORAL and istKNN), built from structurally similar compounds i.e. all containing an aromatic azo structure. Substances positive in the 4 global tools are considered of highest priority with respect to further safety testing. Among these, priority ranking was refined based on the amount and reliability of available experimental mutagenicity data. This is ideally investigated through database and literature searches. However, a fair amount of information can already be deduced by a more detailed investigation of the (Q)SAR results. For example, substances predicted positive with a confidence score of 100% in Sarah or an ADI of 1 in VEGA are chemicals for which positive experimental Ames test results are already available.

Furthermore, a recently compiled inventory of substances which may be used in printed paper and board FCM was consulted to roughly estimate the likelihood of the high priority substances to migrate into the food and become bioavailable after oral intake. In addition, the Flavours, Additives and food Contact materials Exposure Task (FACET) tool was used to obtain a first indication of their actual use. The inventory and FACET tool have been described earlier (Van Bossuyt et al., 2016).

Also, the application of 2 local QSARs was investigated for substances containing an aromatic azo bond, with the goal of priority ranking refinement. More specifically, aromatic azo substances positive in one or two of the additional QSAR tools were considered of higher priority than those predicted negative by both.

**3. Results and discussion**

**3.1. Individual models**

An overview of the prediction outcome of the 1723 study substances as a function of (Q)SAR system used is presented in **Figure 2**. At least 229 up to 366 of the substances are predicted mutagenic *in silico*. It must be noted that, in the case of VEGA, most of the substances (758) are outside domain when applying the ADI requirements set out in 2.2. This is due to the differences between the prediction methods and applicability domains of the 3 individual VEGA tools constituting the consensus model. Apparently, several substances do not reach an ADI ≥0.75 in all 3 models in order to consider them as negative in the current approach (**Table 2**). For these substances, together with the undefined substances in Derek and Sarah, a positive outcome cannot be ruled out.

Figure 2. Number of substances predicted negative (green), positive (red) and undefined (grey) for Ames

mutagenicity in a series of (Q)SAR tools.

The positive rate is most divergent between Toxtree and Sarah. Interestingly, these tools are at the same time most dissimilar with regards to their prediction mechanism: Toxtree is a generic rule-based SAR model without the possibility of AD determination, whereas Sarah is a statistically-based QSAR model in which each compound is checked for being in- or outside a predefined Ames mutagenicity AD. **Figure 2** also shows that the numbers of positives found in the individual models are very similar in Toxtree (366) and VEGA (350) on the one hand, and in Derek (255) and Sarah (229) on the other hand. This suggests that there might be a substantial overlap in the compounds predicted positive by Toxtree and VEGA, and by Derek and Sarah, respectively. As such, we found that for Toxtree and VEGA, 269 of the substances were overlapping. The fact that VEGA has implemented the Toxtree model contributes to this overlap. For Derek and Sarah, the overlap was limited to 119 compounds (**Table 3**). This observation demonstrates that the different methods lead to a different outcome for several compounds. Detailed examination of the non-overlapping compounds with contradictory prediction results can therefore reveal chemical classes for which Ames mutagenicity prediction needs improvement. Evidently, if a substance is positive in multiple tools - based on various data sets and subsequent prediction rules - this could be expected to imply solid reasoning, in turn associated with increased prediction confidence for experimental mutagenicity. Therefore, substances positive in a variety of (Q)SAR models are of higher concern.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Battery of 2 tools** | | | | | **Battery of 3 tools** | |  |
| *(Q)SAR* | VEGA | | Derek | | Sarah | VEGA | Toxtree |
| Toxtree | 269 | | 205 | | 154 | 183 |  |
| VEGA |  | | 195 | | 172 |  | 147 |  |
| Derek |  | |  | | 119 |  | 110 |
| Sarah |  | |  | |  | 112 |  |  |
|  | |  | | Derek | Sarah | *(Q)SAR* |

Table 3. Number of substances predicted positive for Ames mutagenicity in a battery of 2 (left panel) and 3 (right panel) (Q)SAR tools.

**3.2. Combination of models**

The combined prediction outcome using the 4 (Q)SARsystems is depicted in **Figure 3**. Out of the 1723 non-evaluated substances, 106 are predicted mutagens by all tools, whereas 572 are predicted to be non-mutagenic. A substantial part of 1045 study substances was not clearly identified as mutagenic or non-mutagenic, but either positive in at least 1 but not in all tools, or negative in all tools but with an outside domain notification in at least 1 tool. These substances were considered as ‘undefined’. In **Figure 4**, a more detailed overview of the prediction results is provided. The majority of substances (1191 compounds) do not trigger a positive prediction in any of the four tools. However, many of these compounds (619 compounds) were outside the AD of at least one tool. Consequently, in the context of this study, ‘negative’ should be interpreted as ‘no positive response reported’. On the other hand, only 8 out of the 128 substances predicted positive in 3 tools are outside the domain of the 4th tool of the *in silico* battery (4 in Derek and 4 in VEGA). It can be debated what significance should be given to results based on outside domain warnings. From a precautionary point of view, it is more appropriate to consider compounds outside the AD as potential mutagens, since this indicates a general lack of knowledge on the (toxicological) properties of the specific chemical class. Hence, after the 106 substances found to be positive in all tools, the 8 compounds found as positive in 3 tools and outside domain in the 4th tool are of second highest concern. **Figure 4** represents the detailed priority ranking following this strategy.

Undefined (#1045)

Figure 3. Distribution of the substances according to overall negative (green), positive (red) or undefined

(orange) prediction for Ames mutagenicity when combining 4 (Q)SAR tools (large pie). The undefined

results are subdivided in substances generating a positive outcome in 1 up to 3 tools or substances

negative in all 4 but outside domain (small pie).

**PRIORITY**

Figure 4. Priority ranking and distribution of non-evaluated printed paper and board substances according to

Ames mutagenicity prediction outcome using 4 (Q)SAR tools; the fraction of substances outside domain in at least 1 remaining tool is mentioned between brackets. V: VEGA, T: Toxtree, D: Derek, S: Sarah.

**3.3. Priority substances**

The 106 substances predicted positive in all 4 (Q)SAR tools are considered of highest priority for further investigation of potential mutagenicity. Fifty-three of these are found in the model training sets (51 have a confidence score of 100% in Sarah and 2 have an ADI of 1 in VEGA), hence they are presumed experimental Ames mutagens (**Table 4a**). For these 53 compounds, if possible the primary literature should be consulted to verify the positive outcome, and if confirmed, *in vivo* data are required to either endorse or overrule the *in vitro* positive results. In case mutagenicity is confirmed *in vivo* or no reliable negative *in vivo* data are available, they are of highest priority for migration testing. Indeed, the mutagenic potential of a FCM compound is only of concern in case it migrates into the food. Furthermore, migrants need to become bioavailable to be able to cause (mutagenic) effects. Consultation of the combined inventory described in 2.4. shows that migration into food followed by oral bioavailability is very likely for all these 53 compounds (**Table 5**). The combination of the specific physicochemical parameters considered in the current study has not yet been described elsewhere, nevertheless all are historically known as being indicative for migration and/or oral bioavailability (Van Bossuyt et al., 2016). In line with the precautionary principle, a combination of these parameters is thus highly relevant. Ideally, an elaborate migration and bioavailability model could contribute to a more complete picture, however so far no generally accepted model is available.

Besides the 53 experimental Ames positives, for another 53 substances no experimental data are available in the (Q)SAR systems (**Table 4b**). Subsequently, their experimental mutagenicity potential should be investigated urgently. All of them are likely to migrate into food due to a molecular weight below 1000 g/mol, and at least 42 out of the 53 meet typical criteria for bioavailability (**Table 5**).

Table 5. Distribution of non-evaluated printed paper and board FCM priority substances according to

physicochemical parameters related to migration and bioavailability. Substances with results below the cut-off are likely to migrate (in the case of low molecular weight) or become bioavailable (other cases).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameter | Cut-off | ≤ cut-off | | > cut-off | | ≤ cut-off | | > cut-off | |
|  |  | ***53 experimental Ames positives*** | | | | ***53 experimental unknown*** | | | |
|  |  | # | % | # | % | # | % | # | % |
| Molecular weight | 1000 g/mol | 53 | 100 | 0 | 0 | 53 | 100 | 0 | 0 |
| Lipinski rule of 5 violations | 1 | 52 | 98 | 1 | 2 | 42 | 79 | 11 | 21 |
| Polar surface area | 140 Angström2 | 52 | 98 | 1 | 2 | 45 | 85 | 8 | 15 |
| Rotating bonds | 10 | 53 | 100 | 0 | 0 | 46 | 87 | 7 | 13 |

The majority (99) of the 106 priority compounds are printing ink substances, in many cases (29) pigments or dyes (**Table 4a and b**). It can be noted that in the context of food contamination with non-plastic FCM, in particular constituents of printing inks are found as a major contamination source. This is among others reflected in a high number of notifications through the Rapid Alert System for Food and Feed (RASFF) (European Commission, 2016a; Lago et al., 2015). Up to now, the latter notifications mainly concern photo-initiators originating from the UV-curing treatment of printing inks.

Table 4a. Overview of substances, listed for use in printed paper and board FCM, predicted positive for Ames mutagenicity in 4 (Q)SAR tools and confirmed Ames mutagens according to (Q)SAR model training data. \* aromatic azo compound predicted positive in 1 local QSAR, \*\* aromatic azo compound predicted positive in 2 local QSARs.

|  |  |  |  |
| --- | --- | --- | --- |
| CAS number | Chemical name | FACET number | Use in FCM |
| 57-14-7 | N,N-Dimethylhydrazine | 5914 | Monomer in printing ink |
| 74-87-3 | Chloromethane | 6081 | Monomer in printing ink and additive in paper and board |
| 75-00-3 | Chloroethane | 5491 | Monomer in printing ink |
| 75-55-8 | 2-Methylaziridine | 4921 | Monomer in printing ink |
| 77-78-1 | Dimethyl sulphate | 7268 | Monomer in printing ink, paper and board and additive in paper and board |
| 78-87-5 | 1,2-Dichloropropane | / | Additive in paper and board |
| 78-94-4 | Butenone | 6090 | Monomer in printing ink |
| 80-40-0 | Ethyl toluene-4-sulphonate | 6903 | Additive in printing ink |
| 80-48-8 | Methyl toluene-4-sulphonate | / | Monomer in paper and board |
| 85-83-6 | 1-(2-Methyl-4-(2-methylphenylazo)phenylazo)-2-naphthol\*\* | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 85-86-9 | 1-(4-(Phenylazo)phenylazo)-2-naphthol\*\* | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 96-23-1 | 1,3-Dichloropropan-2-ol | / | Monomer in paper and board |
| 98-88-4 | Benzoyl chloride | 5050 | Additive in printing ink |
| 100-44-7 | α-Chlorotoluene | 7367 | Monomer in printing ink, paper and board |
| 101-77-9 | 4,4'-Methylenedianiline | 1079 | Monomer in printing ink |
| 101-80-4 | 4,4'-Oxydianiline | 4902 | Monomer in printing ink |
| 106-50-3 | p-Phenylenediamine | 6832 | Monomer in printing ink |
| 106-87-6 | 7-Oxa-3-oxiranylbicyclo[4.1.0]heptane | 4456 | Solvent in printing ink |
| 106-88-7 | 1,2-Epoxybutane | 5094 | Monomer in printing ink, paper and board and additive in paper and board |
| 106-90-1 | 2,3-Epoxypropyl acrylate | 2094 | Monomer in printing ink, paper and board |
| 106-92-3 | Allyl 2,3-epoxypropyl ether | 4807 | Monomer in printing ink |
| 107-02-8 | Acrylaldehyde | 4586 | Monomer in printing ink, paper and board |
| 107-05-1 | 3-Chloropropene | 6867 | Monomer in printing ink, paper and board |
| 107-07-3 | 2-Chloroethanol | 5471 | Monomer in printing ink |
| 111-44-4 | Bis(2-chloroethyl) ether | 5480 | Monomer in printing ink and additive in paper and board |
| 111-64-8 | Octanoyl chloride | 6256 | Monomer in printing ink |
| 122-60-1 | 2,3-Epoxypropyl phenyl ether | 4128 | Monomer in printing ink |
| 123-73-9 | (E)-crotonaldehyde | / | Monomer in paper and board |
| 128-95-0 | 1,4-Diaminoanthraquinone | / | Dye and pigment in printing ink |
| 130-15-4 | 1,4-Naphthoquinone | 3961 | Monomer in printing ink |
| 140-95-4 | 1,3-Bis(hydroxymethyl)urea | 2563 | Additive in printing ink, paper and board and monomer in paper and board |
| 286-20-4 | 1,2-Epoxycyclohexane | 4457 | Additive in printing ink |
| 302-01-2 | Hydrazine | 2647 | Monomer in printing ink |
| 556-52-5 | 2,3-Epoxypropan-1-ol | 4127 | Monomer in printing ink |
| 558-30-5 | 2,2-Dimethyloxirane | 6838 | Monomer in printing ink |
| 1854-26-8 | 4,5-Dihydroxy-1,3-bis(hydroxymethyl)imidazolidin-2-one | / | Additive in paper and board |
| 2210-79-9 | 2,3-Epoxypropyl o-tolyl ether | 4129 | Monomer in printing ink |
| 2224-15-9 | 2,2'-[Ethylenebis(oxymethylene)]bisoxirane | 5412 | Additive in printing ink |
| 2386-87-0 | 7-Oxabicyclo[4.1.0]hept-3-ylmethyl 7-oxabicyclo[4.1.0]heptane-3-carboxylate | 3447 | Solvent in printing ink |
| 2426-08-6 | Butyl 2,3-epoxypropyl ether | 5067 | Monomer in printing ink |
| 2451-62-9 | 1,3,5-Tris(oxiranylmethyl)-1,3,5-triazine-2,4,6(1H,3H,5H)-trione | 7407 | Monomer in printing ink |
| 2461-15-6 | [[(2-Ethylhexyl)oxy]methyl]oxirane | 4197 | Monomer in printing ink |
| 3101-60-8 | p-Tert-butylphenyl 1-(2,3-epoxy)propyl ether | 6834 | Monomer in printing ink |
| 3252-43-5 | Dibromoacetonitrile | / | Additive in paper and board |
| 3266-23-7 | 2,3-Epoxybutane | 5097 | Monomer in printing ink |
| 4016-14-2 | 2,3-Epoxypropyl isopropyl ether | 6839 | Additive in printing ink |
| 4170-30-3 | Crotonaldehyde | 4171 | Monomer in printing ink |
| 6471-49-4 | 3-Hydroxy-4-[(2-methoxy-5-nitrophenyl)azo]-N-(3-nitrophenyl)naphthalene-2-carboxamide\*\* | 2822 | Dye and pigment (with aromatic azo structure) in printing ink |
| 7665-72-7 | (Tert-butoxymethyl)oxirane | 6293 | Monomer in printing ink |
| 17557-23-2 | 1,3-Bis(2,3-epoxypropoxy)-2,2-dimethylpropane | 6840 | Monomer in printing ink |
| 21490-63-1 | Trans-2,3-dimethyloxirane | 6297 | Monomer in printing ink |
| 26249-20-7 | Epoxybutane | 5098 | Monomer in printing ink |
| 857892-58-1 | Oxirane | 6299 | Additive in printing ink |

Table 4b. Overview of substances, listed for use in printed paper and board FCM, predicted positive for Ames mutagenicity in 4 (Q)SAR tools and requiring

experimental testing. \* aromatic azo compound predicted positive in 1 local QSAR, \*\* aromatic azo compound predicted positive in 2 local QSARs.

|  |  |  |  |
| --- | --- | --- | --- |
| CAS number | Chemical name | FACET number | Use in FCM |
| 82-38-2 | 1-(Methylamino)anthraquinone | / | Dye and pigment in printing ink |
| 136-84-5 | 1,3-Bis(hydroxymethyl)imidazolidin-2-one | 4216 | Additive in printing ink |
| 624-65-7 | 3-Chloropropyne | 6897 | Monomer in printing ink |
| 938-18-1 | 2,4,6-Trimethylbenzoyl chloride | 5051 | Monomer in printing ink |
| 1208-52-2 | 2,4'-Methylenedianiline | 4134 | Monomer in printing ink |
| 1606-83-3 | 1,1'-[But-2-yne-1,4-diylbis(oxy)]bis[3-chloropropan-2-ol] | 4252 | Additive in printing ink |
| 1719-57-9 | Chloro(chloromethyl)dimethylsilane | 7055 | Monomer in printing ink |
| 1742-95-6 | 4-Aminonaphthalene-1,8-dicarboximide | 6178 | Additive in printing ink |
| 2095-03-6 | 2,2'-[Methylenebis(p-phenyleneoxymethylene)]bisoxirane | 6296 | Additive in printing ink |
| 2238-07-5 | 2,2'-[Oxybis(methylene)]bisoxirane | 5479 | Additive in printing ink |
| 2478-20-8 | 6-Amino-2-(2,4-dimethylphenyl)-1H-benz[de]isoquinoline-1,3(2H)-dione | / | Dye and pigment in printing ink |
| 2530-83-8 | [3-(2,3-Epoxypropoxy)propyl]trimethoxysilane | 2638 | Monomer in printing ink and additive in paper and board |
| 2602-34-8 | [3-(2,3-Epoxypropoxy)propyl]triethoxysilane | 2893 | Monomer and additive in printing ink |
| 2897-60-1 | [3-(2,3-Epoxypropoxy)propyl]diethoxymethylsilane | 7052 | Additive in printing ink |
| 3049-71-6 | 2,9-Bis[4-(phenylazo)phenyl]anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone\* | 2999 | Dye and pigment (with aromatic azo structure) in printing ink |
| 3126-95-2 | (Propoxymethyl)oxirane | 6292 | Monomer in printing ink |
| 3176-79-2 | 1-[[3-Methyl-4-[(3-methylphenyl)azo]phenyl]azo]-2-naphthol\*\* | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 3271-22-5 | 2,4-Dimethoxy-6-pyren-1-yl-1,3,5-triazine | 4135 | Additive in printing ink |
| 3454-29-3 | 1-(2,3-Epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy)methyl]butane | 7403 | Additive in printing ink |
| 4378-61-4 | 4,10-Dibromodibenzo[def,mno]chrysene-6,12-dione | 5304 | Dye and pigment in printing ink |
| 4482-25-1 | 5,5'-[(4-Methyl-1,3-phenylene)bis(azo)]bis[toluene-2,4-diamine]\*\* | 3907 | Additive (with aromatic azo structure) in printing ink |
| 5026-74-4 | p-(2,3-Epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline | 6358 | Monomer in printing ink |
| 6410-38-4 | 4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-N-(2-methoxyphenyl)naphthalene-2-carboxamide\* | 2847 | Dye and pigment (with aromatic azo structure) in printing ink |
| 6448-95-9 | 3-Hydroxy-4-[(2-methyl-5-nitrophenyl)azo]-N-phenylnaphthalene-2-carboxamide\* | 2829 | Dye and pigment (with aromatic azo structure) in printing ink |
| 6471-50-7 | 4-[(4-Chloro-2-nitrophenyl)azo]-3-hydroxy-N-(2-methylphenyl)naphthalene-2-carboxamide\* | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 6539-67-9 | 3-[[2-(Acetylamino)-4-[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino]phenyl]azo]naphthalene-1,5-disulphonic acid | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 6655-84-1 | 3-Hydroxy-4-[(2-methyl-5-nitrophenyl)azo]-N-(o-tolyl)naphthalene-2-carboxamide\* | 2995 | Dye and pigment (with aromatic azo structure) in printing ink |
| 7328-97-4 | 2,2',2'',2'''-[Ethane-1,2-diylidenetetrakis(p-phenyleneoxymethylene)]tetraoxirane | 5411 | Additive in printing ink |
| 12225-06-8 | N-(2,3-dihydro-2-oxo-1H-benzimidazol-5-yl)-3-hydroxy-4-[[2-methoxy-5-[(phenylamino)carbonyl]phenyl]azo]naphthalene-2-carboxamide | 2997 | Dye and pigment (with aromatic azo structure) in printing ink |
| 12236-64-5 | N-[4-(acetylamino)phenyl]-4-[[5-(aminocarbonyl)-2-chlorophenyl]azo]-3-hydroxynaphthalene-2-carboxamide | 2979 | Dye and pigment (with aromatic azo structure) in printing ink |
| 13236-02-7 | 1,2,3-Tris(2,3-epoxypropoxy)propane | 6836 | Additive in printing ink |
| 14228-73-0 | 1,4-Bis[(2,3-epoxypropoxy)methyl]cyclohexane | 5250 | Additive in printing ink |
| 16096-30-3 | 2,2'-[(1-Methylethylene)bis(oxymethylene)]bisoxirane | 6294 | Additive in printing ink |
| 16096-31-4 | 1,6-Bis(2,3-epoxypropoxy)hexane | 3967 | Additive and solvent in printing ink |
| 16403-84-2 | 4-[(5-Carbamoyl-o-tolyl)azo]-3-hydroxynaphth-2-anilide | 2828 | Dye and pigment (with aromatic azo structure) in printing ink |
| 25188-42-5 | 7-Benzamido-4-hydroxy-3-[[4-[(4-sulphophenyl)azo]phenyl]azo]naphthalene-2-sulphonic acid | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 28804-47-9 | Methyl toluenesulphonate | / | Additive in paper and board |
| 31482-56-1 | 3-[Ethyl[4-[(4-nitrophenyl)azo]phenyl]amino]propiononitrile\*\* | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 36215-07-3 | 1-Chloro-3-methoxypropane | 6841 | Monomer in printing ink |
| 36968-27-1 | 4-[[4-(Aminocarbonyl)phenyl]azo]-3-hydroxy-N-(2-methoxyphenyl)naphthalene-2-carboxamide | 2827 | Dye and pigment (with aromatic azo structure) in printing ink |
| 39817-09-9 | 2,2'-[Methylenebis(phenyleneoxymethylene)]bisoxirane | 2347 | Monomer in printing ink |
| 50593-68-5 | 3-Chloro-6-nitro-1H-indazole | 4033 | Additive in printing ink |
| 52373-93-0 | 1-Amino-4-(ethylamino)-9,10-dihydro-9,10-dioxoanthracene-2,3-dicarbonitrile | 4125 | Additive in printing ink |
| 56396-10-2 | 4-[[5-(Anilino)carbonyl-2-methoxyphenyl]azo]-3-hydroxynaphthalene-2-carboxamide | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 59487-23-9 | 4-[[5-[[[4-(aminocarbonyl)phenyl]amino]carbonyl]-2-methoxyphenyl]azo]-N-(5-chloro-2,4-dimethoxyphenyl)-3-hydroxynaphthalene-2-carboxamide | 2814 | Dye and pigment (with aromatic azo structure) in printing ink |
| 61847-48-1 | Methyl 4-[[(2,5-dichlorophenyl)amino]carbonyl]-2-[[2-hydroxy-3-[[(2-methoxyphenyl)amino]carbonyl]-1-naphthyl]azo]benzoate | 2815 | Dye and pigment (with aromatic azo structure) in printing ink |
| 61951-98-2 | N-(2,3-dihydro-2-oxo-1H-benzimidazol-5-yl)-3-hydroxy-4-[[5-methoxy-2-methyl-4-[(methylamino)sulphonyl]phenyl]azo]naphthalene-2-carboxamide | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 62570-50-7 | 1-Amino-4-(ethylamino)-9,10-dihydro-9,10-dioxoanthracene-2-carbonitrile | 3142 | Dye and pigment in printing ink |
| 67990-05-0 | N-(5-chloro-2-methoxyphenyl)-3-hydroxy-4-[[2-methoxy-5-[(phenylamino)carbonyl]phenyl]azo]naphthalene-2-carboxamide | 2829 | Dye and pigment (with aromatic azo structure) in printing ink |
| 68227-78-1 | N-(5-chloro-2-methylphenyl)-3-hydroxy-4-[[2-methoxy-5-[(phenylamino)carbonyl]phenyl]azo]naphthalene-2-carboxamide | 2992 | Dye and pigment (with aromatic azo structure) in printing ink |
| 68516-75-6 | N,N'-naphthalene-1,5-diylbis[4-[(2,3-dichlorophenyl)azo]-3-hydroxynaphthalene-2-carboxamide] | / | Dye and pigment (with aromatic azo structure) in printing ink |
| 68818-86-0 | 9,10-Diethoxyanthracene | 4905 | Additive in printing ink |
| 74336-59-7 | 3-[(4-Chloro-2-nitrophenyl)azo]-2-methylpyrazolo[5,1-b]quinazolin-9(1H)-one\*\* | 2984 | Dye and pigment (with aromatic azo structure) in printing ink |

The 106 compounds positive in the 4 (Q)SAR tools represent a relatively large number of substances requiring experimental (toxicological and/or migration) data. One option to establish a refined priority ranking lies in the investigation of the actual use of these substances. Although FCM manufacturing companies in general do not wish to disseminate detailed information on this matter, a first indication can already be found through consultation of the Flavours, Additives and food Contact materials Exposure Task (FACET) tool (Hearty et al., 2011). In the EU-funded FACET project, a probabilistic modelling tool was developed to estimate consumer exposure to food contact substances (Oldring et al., 2013). Information on substance application and relative use was obtained from a FACET Industry Group (FIG) consisting of 13 European FCM trade associations representing among others the printing ink and paper(board) industry (University College Dublin, 2012). As a result, substances with a FACET number indicate substances for which the FIG has confirmed current usage. Forty-four training set Ames positives and 42 positives without experimental data, have a FACET number, suggesting their priority is higher compared to the 20 substances without a FACET number. One weakness of the FACET tool is its limited coverage, which is restricted to FCM substances applied in primary packaging, whereas for a complete assessment secondary packaging and articles should also be considered. The application of substances without a FACET number cannot be ruled out either, as this information is currently lacking. Despite this limitation and even though this approach does not drastically minimize the number of priority substances to be evaluated in-depth, it is reasonable to consider the substances associated with a FACET number prior to the ones without such number.

Another interesting refinement method is the provisional exclusion of compounds predicted negative by local QSAR models, i.e. specific for a particular group of compounds. Indeed, the prediction capacity of a (Q)SAR model can be increased when the chemical domain is well-defined. For example, it was found that 25 of the 106 substances contain an aromatic azo bond, a chemical structure frequently found in pigments and dyes. Recently, the IRFMN developed 2 QSAR models to estimate Ames mutagenicity of aromatic azo substances, one based on CORAL software and a second one based on a k-nearest neighbors algorithm (Manganelli et al., 2016). Application of the local QSARs resulted in 13 compounds predicted negative in both models (low priority), 5 contradictory results (medium priority) and 7 positive in both (high priority). Upon combining this extended QSAR evaluation with the abovementioned consideration of the existence of a FACET number, 3 substances of highest concern (CAS# 6471-49-4 in **Table 4a**, CAS# 4482-25-1 and 74336-59-7 in **Table 4b**) could be identified. They are positive in the 6 (Q)SAR tools and have in addition been assigned a FACET number, confirming their current usage.

**3.4. General remarks**

Ideally, a (Q)SAR should meet the OECD principles for the validation of (Q)SAR tools in order to facilitate its consideration for regulatory purposes (OECD, 2014). The principles state that it should be associated with 1) a defined endpoint; 2) an unambiguous algorithm; 3) a defined domain of applicability; 4) appropriate measures of goodness-of-fit, robustness and predictivity and 5) a mechanistic interpretation, if possible. Moreover, a checklist with questions is available to facilitate the evaluation of a (Q)SAR for the abovementioned criteria. The guidance document itself points out that these criteria are very difficult to fulfil in practice, however they should be strived for as much as possible. All tools applied in the current work are linked to a well-defined toxicological endpoint, i.e. Ames mutagenicity. Most of the tools feature a clearly established algorithm. Some of the tools dispose of applicability domain indications and provide a mechanistic interpretation for the prediction results. None of the tools is completely transparent when it comes to providing full details of external validation performance. Although all tools have several shortcomings, their type and degree varies. Combining different tools can therefore prove beneficial, especially for priority setting among large groups of chemical substances, as demonstrated in the current study. Evidently, validation of a (combination of) (Q)SAR model(s) for a group of compounds with a specific application is difficult. Indeed, validation requires a substantial number of evaluated compounds with reliable experimental Ames test data. In the case of FCM substances, validation is not only complicated by the limited number of evaluated compounds, but also by the variety of chemical classes to which they belong. Due to the current lack of knowledge as to which model is most capable of generating trustworthy predictions for printed paper and board FCM substances, it is thus deemed appropriate to use a screening battery of complementary systems (i.e. SARs and QSARs).

**4. Conclusion**

In this study, the beneficial role of *in silico* tools in prioritization strategies was demonstrated using non-evaluated printed paper and board FCM substances as an example. However, a much wider range of application domains can be anticipated. For instance, the strategy could be useful in prominent issues among which the prioritization of long-standing industrial chemicals lacking a (recent) safety evaluation, and of secondary substances – found in most chemical formulations – such as impurities or degradation products. In the current work, the selection of model(s) had an impact on the number of positives, as this was substantially lower when using Derek Nexus™ or Sarah Nexus™ compared to using Toxtree or VEGA. One hundred and six substances were consistently predicted positive in a battery of 4 (Q)SAR Ames mutagenicity tools. Subsequent priority ranking to determine the urgency for an in-depth safety evaluation was established by investigating the availability (and quality) of experimental toxicological data within the (Q)SAR tools. Furthermore, local QSAR systems also proved useful for refining the prioritization of well-defined structurally similar molecules. To conclude, the prioritized printed paper and board FCM substances will be subjected to a more extensive investigation of their potential genotoxicity consisting of literature study and, if necessary, *in vitro* testing.

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