LOA White Paper

Utility of Metabolomics to Support Read-Across for UVCB substances under REACH

1 Introduction

The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)[1] legislation in Europe was adopted in 2007 to improve the protection of human health and the environment from the risks posed by chemicals. REACH legislation applies to all chemical substances[2] and these substances or chemicals must be registered in Europe under REACH if companies want to manufacture, import or market chemicals in Europe. REACH places the burden of demonstrating chemical safety on companies and REACH is enforced by the European Chemicals Agency (ECHA) who maintain and evaluate substance (chemical) registrations. If the safe use of a substance cannot be demonstrated or the risks cannot be managed adequately, ECHA, via member state competent authorities, can restrict the use of hazardous substances in Europe. The Lower Olefins and Aromatics REACH Consortium (LOA) registers many substances in Europe, among them, over 100 substances that are of unknown or variable composition, complex reaction products or biological materials, collectively called UVCBs. The regulatory assessment of UVCB substances under REACH is challenging, as the information requirements and guidelines are mainly targeted at relatively simple, well-defined mono-constituent chemicals. By definition, UVCB substances are variable in composition with some unknowns. The numbers of known substance constituents >0.1% by weight can be many ten's (for example see Appendix 1), therefore, strictly meeting the REACH information requirements around substance identity and analytical composition is challenging, if not sometimes impossible for UVCB substances. To overcome this, UVCBs which have a similar chemical composition have been registered in categories under REACH.

UVCB substance identities or names are based on manufacturing processes and physical-chemical properties, and where applicable, their analytical chemical description covers groups of the main molecular constituents and specific marker[3] substances present in the UVCB. The LOA member companies manufacture the UVCB substances in their portfolio by a process called steam cracking. Steam cracking is a petrochemical industrial process used to refine a variety of feedstock to produce important chemicals for use in other industrial process. Examples of feedstock used in steam cracking

[1] REGULATION (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

[2] Note: in other documents accompanying this paper and in the recording of the webinar, the use of substances and streams may be, and has been, used interchangeably to describe the individual substances present in the Resin Oils and Cyclic Dienes category. Constituents refers to the individual molecules that collectively make a substance/stream.

[3] Marker substances can be mono-constituent substances or UVCB streams where the toxicology and chemical composition is well defined.

processes are ethane, propane or butane from natural gas, naphtha - a mixture of hydrocarbons from the distillation of crude oil, gas oil and residues also from the distillation of crude oil. One example of a UVCB product of the steam cracking process is Distillates (petroleum), steam-cracked, C5-12 fraction. Another example is Distillates (petroleum), steam-cracked, C8-12 fraction. These UVCBs differ by the carbon number of the main constituents C5 to C12 or C8 to C12, and illustrate how many of the substances produced are similar, although not the same (see Section 1.3). Therefore, to address the practicalities around meeting the regulatory information requirements under REACH and to reduce unnecessary use of animals in chemical safety testing, those UVCB substances that are similar, based on the relevant parameters as described earlier, have been grouped into categories for registration purposes under REACH. The example UVCBs mentioned are two substances registered in a category described technically as Resin Oils and Cyclic Dienes (also referred to as Category L), which contains twelve substances in total. See Appendix 1 for an illustration of the category information profile (CIP) for Resin Oils and Cyclic Dienes with a fuller explanation of the CIP in Section 1.2.

Apart from substance identity and registration purposes, one of the main reasons to group similar substances into categories is to help address the information needs for human health and environmental hazard endpoints. To avoid unnecessary, i.e., duplicate, animal testing on similar substances, the main alternative provided in the REACH regulation is a process called “read-across”, either between two substances or between a larger group of substances (see Section 1.1 for a more detailed explanation). However, to demonstrate that hazard data from one substance is applicable to (an)other(s), the relatively broad chemical analytical descriptors used for substance identity purposes are not always sufficient. Evidence from *in vitro* and *in vivo* data that the responses observed following exposure to these substances in a biological system are similar may help to address this issue. To generate these data, LOA have used metabolomics (discussed in more detail in Section 1.4) to analyse biological responses in rats after exposure to individual substances that make up the Resin Oils and Cyclic Dienes category, as well as to specific marker substances which are the main constituents of these UVCBs (Appendix 1). The aim of these data are (1) to determine if the biological responses observed are broadly similar between the UVCB substances in the category, and (2) to determine if the biological responses are consistent with the responses observed for the main constituents (maker substances) of the UVCB. Together these data help to underpin grouping and read-across hypotheses. This hypothesis is further discussed in Section 1.3.

Overall, the aim of the work summarised in this paper was to explore the utility of metabolomics to demonstrate that substances within the category of Resin Oils and Cyclic Dienes (in this case) all produce similar biological responses which are as expected, given what is known of their chemical composition. Doing so reinforces the LOA Category L (Resin Oils and Cyclic Dienes) human health testing strategy (outlined in Figure 1 and Figure 2), which proposes that only selected substances in the Resin Oils and Cyclic Dienes category would be required to be tested. The results obtained from the substances (referred to as ‘source’ records in ECHA’s read-across terminology, see Section 1.2) are used to assess the hazard profiles of the remainder of substances in the category that have received limited testing (referred to as ‘target’). This approach is expected to reduce the numbers of animals required to fulfil the information requirements of REACH, and avoids individual testing on substances that are similar, while demonstrating the safety of category substances in REACH. For example, the application of the proposed testing strategy would reduce the total number of animals needed to perform all the

mandated studies for the Resin Oils and Cyclic Dienes category from 63,208 to 26,082 (see Figure 2), without compromising the confidence or quality of the chemical safety information required under REACH. The approach integrates the analytical data, *in vitro* and *in vivo* testing on all substances, in combination with a selection of higher tier tests and Figure 2) to further strengthen the read-across used to bridge from the source to the target substances in the category.

1.1 What is Read-Across?

ECHA have published a framework which is used for evaluation of read-across and further information can be found there[4]. One of the aims of REACH is to promote alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals. For REACH, the most commonly used alternative is grouping of substances and read-across. As outlined in REACH Annex XI section 1.5, substances whose physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern because of structural similarity may be considered as a group or category of substances. Within such a group, the information requirements for REACH can be interpolated from substances which have the required data set (source substance(s)) to those which do not have the full data set (target substance(s))[5]. The LOA Resin Oils and Cyclic Dienes are an example of such a category, for which the testing strategy is a combination of the proposed safety assessment with the read-across and is outlined in Figure 1 and Figure 2. For this category, source substances are those coded with L-09, L-07, L-02 and L-11 and have a green box showing that all the required higher-tier human health information requirements will be provided for these substances (e.g. animal data from OECD 408, OECD 414, and OECD 443 guideline studies). The target substances are those remaining. The studies listed in the white boxes (Figure 2) will not be performed on these substances but the results obtained from the source substances will be read across to the target substances.

1.2 UVCBs

This read-across approach is relatively straight forward, and guidelines are clear, when it is applied to well-defined (single molecule) chemicals. However, as discussed above, UVCBs contain a large number of variable & partly unknown constituents. These properties of UVCBs complicate the application of read-across under REACH for these substances, mainly because standard requirements (as those laid out in REACH Annex XI and the guidelines of the ECHA RAAF) to prove similarity are difficult, if not impossible, to implement. Part of the REACH regulation requires the precise composition of registered substances to be known. While this can be done for a given batch of product or substance produced, different Registrants, using different feedstock, in different refineries on different days will produce batches of product substance with subtly different constituent composition.

[4] https://echa.europa.eu/regulations/reach/understanding-reach

[5] Read-Across Assessment Framework (RAAF) - considerations on multi-constituent substances and UVCBs (2017). European Chemicals Agency; DoI: 10.2823/794394

To circumvent this, each category of substances is defined by a broad range of parameters covering groups of constituents as well as certain specific marker molecules and a boundary of tolerated concentrations of constituents allowed within any given substance that resides in the category. An example of the boundary composition and Category Information Profile (CIP) of the Resin Oils and Cyclic Dienes category is available in Appendix 1. This CIP shows, for example, that one of the known constituents that may be present in any of the substances in the category is dicyclopentadiene (DCPD)[6], which is allowed in any substance within this category, but it must be <80 % by weight. Toluene is also permitted but it must be ≤ 20% by weight and so on. The concentration ranges permit a wide variability of the various constituents, and this is required for different steam cracking plants, using similar or different feedstock, to produce a chemical product meeting product specifications (e.g. Distillates (petroleum), steam-cracked, C5-12 fraction) which make it suitable for use in downstream chemical processes despite the fact that the chemical composition can be variable depending on processing plant and feedstock used.

1.3 Category Registrations

The data used in the approach outlined in section 1.1 in combination with the variable nature of UVCBs (discussed in Section 1.2) still might not be sufficient to prove substance similarity. In addition, once substance similarity is proven and grouping acceptable, the questions of what to test (source) and what to read-across to (target) remain. Due to inherent variability, testing every substance would be impractical. Therefore, LOA have proposed to test a range of substances in the higher tier tests mandated by REACH (Figure 2), that span the boundary of the category as defined by the CIP (Appendix 1). This way, if a company produces a substance that fits within the category, its substances would be covered by the category registration in REACH. To support this strategy, further proof is needed to underpin the similarity principle beyond substance analytical composition data. Here, metabolomics has been chosen as the tool to determine if the substances within the Resin Oils and Cyclic Dienes category would elicit a similar biological response upon exposure (discussed further in 1.4). Metabolomics, combined with the available physical/chemical and analytical chemistry composition data, would prove chemical-biological similarity between the substances in the category. In the Resin Oils and Cyclic Dienes category, an analytical programme performed by LOA on all registered substances revealed that the category could be further sub-divided into four sub-categories. The proposed testing strategy in Figure 2 intends to test one substance from each sub-category, thereby covering the full range of substances within the category, however a final selection of substances for higher tier testing may be revised if needed, following integrated analysis of all the available data (analytical composition, metabolomics and OECD 422 studies) (Figure 2).

[6] DCPD: 3a,4,5,6,7,7a-hexahydro-4,7-methano-1H-indene (CAS 4488-57-7)

Figure 1. LOA non-CMR UVCB Category Testing Strategy

CMR: Carcinogenic, Mutagenic, Reproductive toxicants. The LOA approach to UVCB category read-across is outlined. This approach is applied to all substances in the Resin Oils and Cyclic Dienes Category. The data generated in box 1 (analytical composition) has been confirmed. In box 2, supporting genotoxicity data has confirmed that the streams in the Resin Oils and Cyclic Dienes Category was not genotoxic. The remaining data from box 2 (14-day repeat dose toxicity with metabolomics) and box 3 (combined repeated dose toxicity study with reproductive/developmental toxicity screening test (OECD 422), represent the 3rd and 4th column in Figure 2 (shaded green) The information in box 4 is represented in columns 4-7 of Figure 2, however, these studies will not be commenced until a full integrated analysis of all preceding data has been performed.

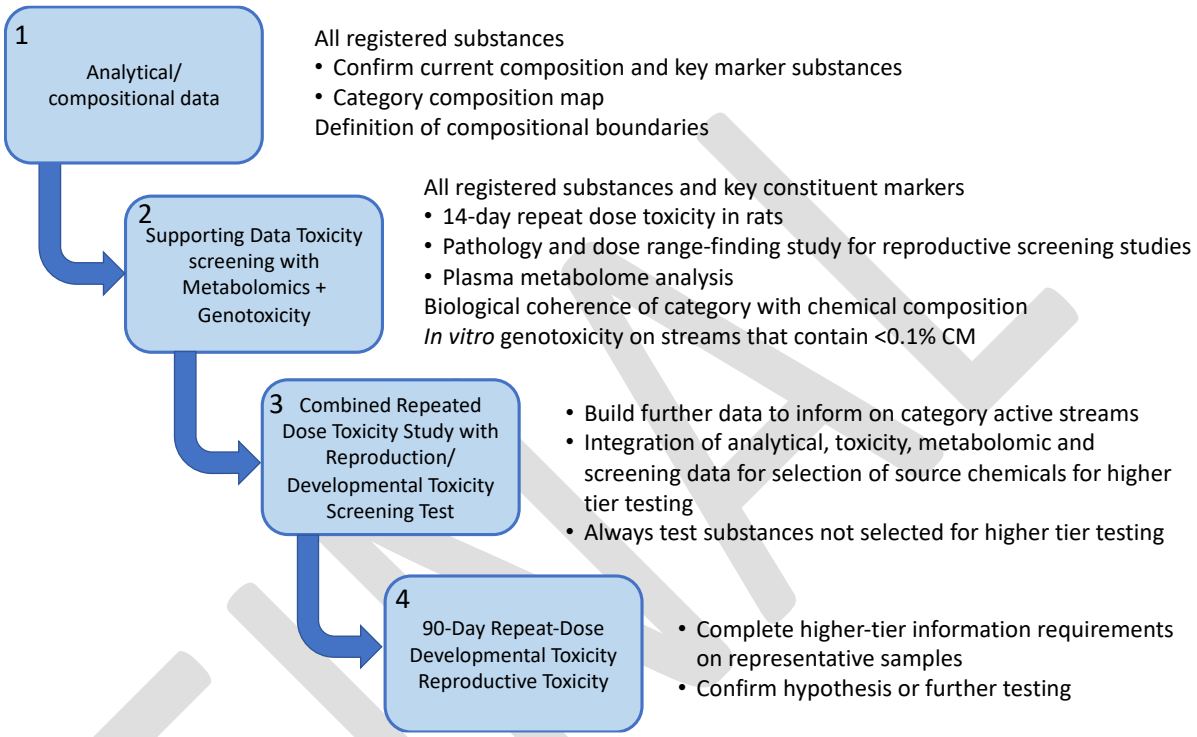


Figure 2.Testing Strategy proposed for Resin Oils and Cyclic Dienes Category.

Inside the Resin Oils and Cyclic Dienes category, there are 4 sub-categories identified based on analytical composition data. The category substances have been coded L-01 to L-12. The 14-day repeat dose toxicity with metabolomics study in rats would not normally be required in REACH, however it is being used as supporting information to demonstrate that the category substances produce a similar biological response (i.e., their metabolomic fingerprint is similar). This test was performed on all substances within the category. In addition, the OECD 422 (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test) bridging studies are currently on-going and performed on all substances within the category. Combined, they add multiple layers of biological evidence which when integrated with the available analytical data, will underpin the proposed testing strategy. This approach combines targeted higher tier testing on selected substances covering the boundary of the category with read-across to the remaining substances in the category. For information, the OECD guidelines listed in the table refer to the following human health endpoints: in addition to OECD 422, the OECD 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents), the OECD 414 (Prenatal Developmental Toxicity Study) which is required in two species, typically rat and rabbit, and the OECD 443 (Extended One-Generation Reproductive Toxicity Study). Full description of all studies can be found on the OECD website (https://www.oecd-ilibrary.org).

Group	Representative Sample ID	14 day + Metabolomics	OECD 422	OECD 408	OECD 414 1st species	OECD 414 2nd Species	OECD 443
High Bicyclic olefin content with low to medium aromatics content	L-09						
	L-04			Read Across if Category Hypothesis shown			
	L-10						
	L-08						
Intermediate Bicyclic olefin content	L-07						
Moderate High Bicyclic olefin content	L-01			Read Across if Category Hypothesis shown			
Moderate High Bicyclic olefin content	L-02						
	L-05			Read Across if Category Hypothesis shown			
High Aromatic Cyclic Olefins diolefins and alkanes	L-11						
	L-03			Read Across if Category Hypothesis shown			
	L-06						
	L-12						

1.4 Metabolomics

Metabolomics is the large-scale study of small molecules, also known as metabolites, within cells, tissues, biofluids (e.g. plasma) or organisms. It does not study the metabolism of a substance administered to an animal or cell type, but studies the changes to the endogenous metabolites, normally found within a system (cell, plant, animal). Collectively these small molecules or metabolites and their interaction with the system is called the metabolome, and it is a good marker for the biological response in a test organism to its environment – i.e., it's phenotype as illustrated in Figure 3[7]. A schematic of the study design used in the metabolomic programme can be viewed in Figure 3.

[7] VIANT, M. R., EBBELS, T. M. D., BEGER, R. D., EKMAN, D. R., EPPS, D. J. T., KAMP, H., LEONARDS, P. E. G., LOIZOU, G. D., MACRAE, J. I., VAN RAVENZWAAY, B., ROCCA-SERRA, P., SALEK, R. M., WALK, T. & WEBER, R. J. M. 2019. Use cases, best practice and reporting standards for metabolomics in regulatory toxicology. Nat Commun, 10, 3041. DOI: 10.1038/s41467-019-10900-y

Figure 3. Relationship between the metabolome and biochemical and molecular biological activity

These metabolomic phenotype can be determined by measuring small molecules (e.g. glucose, cholesterol, amino acids etc) which can be extracted from a biofluid or tissue such as plasma and analysed using very sensitive and specific techniques like mass-spectrometry. Exogenous as well as endogenous molecules can impact on the metabolome by affecting gene expression, resulting in biochemical changes in protein function ultimately affecting the balance of metabolites measured in the system. Certain effects have established patterns of metabolite (metabolome) changes that can be used to infer or predict the ultimate outcome or phenotype.

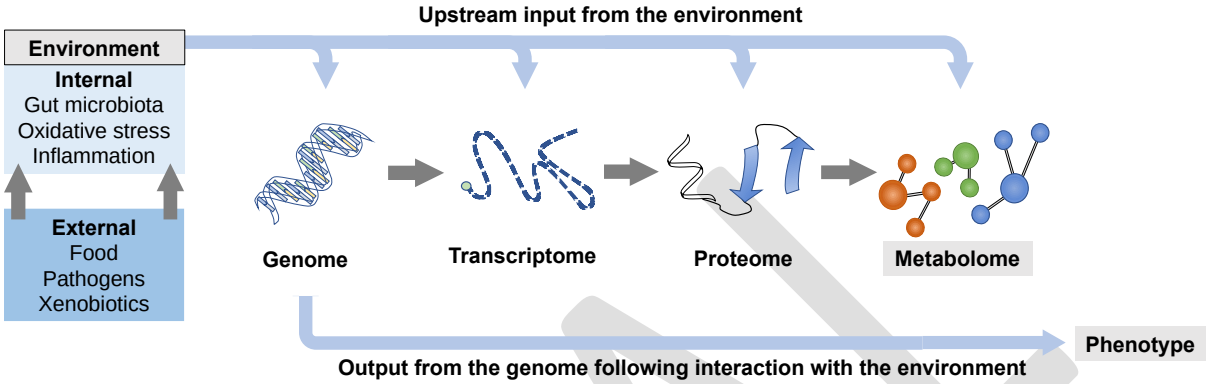
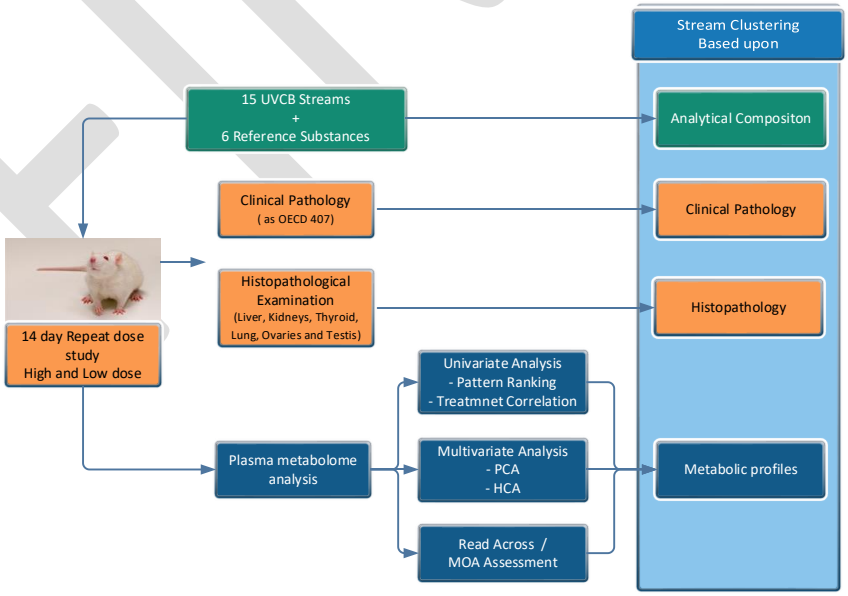


Figure 4. Metabolomic programme study design

Rats were exposed orally to either no substance or a dose-range of the substances in the Resin Oils and Cyclic Dienes category or to marker substances for 14 days. Standard clinical chemistry (according to the OECD TG 407) and histopathology evaluation (selected organs) was conducted (For details see (https://www.oecd-ilibrary.org). Levels of endogenous metabolites were measured in rat plasma, from which the molecular phenotype resulting from this exposure was determined for each substance. Statistical analysis was applied using a combination of univariate and multivariate mathematical techniques



Statistical analysis performed metabolome pattern effects in rat plasma over several years have shown that blood sampling at 14 days is at least as sensitive as a 28-day sampling time point for assessing

patterns of metabolome change associated with organ toxicity [8] [9]. In contrast, sampling following either 7- or 90-day exposure time-points has shown these are less sensitive than either 14- or 28-days. This is most likely due to not enough time having passed at 7 days to develop a compound associated toxicity changes in the plasma metabolome and in the case of 90 days, adaptive changes have occurred to the extent that the animal is again in homeostasis. Therefore, in the current study, rats were exposed orally for 14 days to a representative of each stream in the Resin Oils and Cyclic Dienes category and to marker substances as indicated in Figure 4. Studies with an exposure over 14 days are commonly conducted as so-called range-finding studies to enable the dose setting for regulatory studies, such as the OECD 422 study. Levels of endogenous metabolites were measured in rat plasma, from which the molecular phenotype resulting from this exposure was determined for each substance using a combination of pattern recognition and treatment correlation, principal component analysis and hierarchical clustering mathematical techniques (for more detail see Section 2.2.2). Substances of the Resin Oils and Cyclic Dienes category (or sub-categories (see Figure 2) that would generate a similar metabolomic phenotype, would be expected to generate similar molecular changes following exposure, if the substances were similar. This further underpins the chemical-biological grouping of these substances which will ultimately facilitate the read-across and testing strategy as outlined in Figure 1 and Figure 2. In addition, to help build the read-across hypothesis, additional mechanistic insights into the observed metabolomic fingerprint of the substances in the Resin Oils and Cyclic Dienes category, as well as those of the marker compounds, were obtained by comparing metabolomic fingerprint observed to those in the BASF MetaMap® database. BASF have developed this database over 15 years, and it includes the metabolomic signatures identified for over 110 toxicity profiles. For further detail a full introduction to metabolomics, including the MetaMap® database, is referred to the accompanying LOA webinar recording and slide deck. A summary of the data obtained can be seen in (Figure 5, Figure 6, Figure 7 and Figure 8).

2 Summary of the outcome of the metabolomics study on substances of the Resin Oils and Cyclic Dienes category

First, appropriate dose levels for all substances obtained from lead registrants in the Resin Oils and Cyclic Dienes category and most common marker substances, that make up a significant proportion of the constituents present in the substances of the Resin Oils and Cyclic Dienes category, were determined in a 7-day repeat oral dosing study in rats. These substances were then administered daily (orally) for 14 days to male and female rats in the metabolomics programme. In addition, control groups, which only received corn oil as a vehicle, were dosed in a similar manner. Marker substances were also dosed individually in the same way (Table 1). Testing marker compounds/substances is important because the

[8] VAN RAVENZWAAY, B., MONTOYA, G.A., FABIAN, E., HEROLD, M., KRENNRICH, G., LOOSER, R., MELLERT, W., PETER, E., STRAUSS, V., WALK, T., KAMP, H. (2014) The sensitivity of metabolomics versus classical regulatory toxicology from a NOAEL perspective. Toxicology Letters, 227: 20–28

[9] VAN RAVENZWAAY, B., KAMP, H., MONTOYA, G.A., STRAUSS, V., FABIAN, E., MELLERT, W., KRENNRICH, G., WALK, T., PETER, E., LOOSER, R., HEROLD, M. (2015) The development of a database for metabolomics – looking back on ten years of experience. Int. J. Biotechnology, Vol. 14, No. 1

toxicity profile of these substances is understood and, they are used to drive the hazard classification and labelling of the LOA streams depending on their concentration level in the UVCB substances. The Resin Oils and Cyclic Diene category substances contain varying amounts of these marker compounds (also referred to as mono-constituents) and the compositional analysis of the Resin Oils and Cyclic Dienes substances used in this programme has confirmed the presence or absence of the selected marker constituents and is outlined in Table 2.

Table 1 Selected substances and dose levels used in the LOA metabolomics programme

The Resin Oils and Cyclic Dienes category substances are coded as L-01 to L-12. The BASF LOA 1,2 and 3 are independent of LOA but chemically similar to substances in the Resin Oils and Cyclic Dienes category. The dose levels delivered to male and female rats orally each day are mg/kg bodyweight/day. The typical concentrations of the marker substances present as constituents in the Resin Oils and Cyclic Dienes category substances can be seen in Appendix 1 and the relative concentrations of the marker substances tested are presented in Table 2.

LOA Stream	Low Dose (mg/kg bw)	High Dose (mg/kg bw)		BASF LOA Stream	Low Dose (mg/kg bw)	High Dose (mg/kg bw)
L-01	300	1000		**BASF LOA 1**	300	1000
L-02	250	750		**BASF LOA 2**	300	1000
L-03	150	500		**BASF LOA 3**	300	1000
L-04	70	200		**Marker Compounds**	**Low Dose** (mg/kg bw)	**High Dose** (mg/kg bw)
L-05	250	750		**Indene**	100	450
L-06	300	1000		**Cyclopentane**	300	1000
L-07	200	600		**Dicyclopentadiene**	50	150
L-08	100	300		**Naphthalene**	250	600
L-09	70	200		**Xylene**	300	1000
L-10	300	1000		**Benzene**	300	1000
L-11	150	500		***Ethylbenzene****	*250*	*750*
L-12	300	1000		***Toluene****	*600*	*1250*
				Styrene*	*200*	*600*

* These marker compounds were tested separately in a 28-day repeated dose toxicity study. These data already existed at the time of the metabolomics programme and it was not considered ethical to repeat these substances for 14 days, as no new information would have been obtained.

Table 2 Content (% by weight) of the marker compounds in the tested Resin Oils and Cyclic Dienes substances (the intensity of the red colour denotes the relative composition of the marker compound across the substances tested)

Marker Substances	L-01	L-02	L-03	L-04	L-05	L-06	L-07	L-08	L-09	L-10	L-11	L-12	BASF-1	BASF-2	BASF-3
Benzene	0.23	0.00	0.00	0.00	0.00	0.00	0.00	15.00	0.57	0.00	0.00	0.00	0.05	0.02	0.08
Cyclopentane	9.99	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.60	0.00	0.00	0.00	0.00	0.00
Dicyclopentadiene	0.68	22.65	0.00	73.42	19.57	0.00	25.37	37.99	57.57	6.30	0.54	0.00	0.11	0.07	0.03
Ethylbenzene	0.12	3.51	0.17	0.00	3.35	0.00	0.00	0.11	0.00	0.00	0.00	0.00	4.65	0.03	0.00
Indene	0.00	0.00	56.32	0.00	3.61	2.32	14.19	0.00	0.31	0.00	66.21	4.29	1.78	23.19	24.06
Naphthalene	2.15	0.22	0.00	0.00	1.03	28.16	8.24	0.00	0.27	0.00	1.13	8.96	11.41	1.27	6.65
Styrene	0.00	18.72	0.53	0.00	15.62	0.00	1.31	0.00	0.00	0.00	0.92	1.30	0.25	1.57	2.27
Toluene	0.47	0.75	0.00	0.00	1.55	0.00	0.00	5.23	0.19	0.00	0.00	0.00	0.37	0.03	0.06
Xylene	0.32	13.41	0.92	6.59	12.94	0.00	0.20	0.00	0.00	0.00	0.89	0.00	6.31	0.44	0.70

The intensity of the red color denotes the relative composition of the marker substance across streams.

A detailed discussion of the results of the metabolomics study will not be conducted in this paper. The reader is referred to the accompanying LOA metabolomics webinar recording and slide deck for a detailed description of the outcomes of this work. The important conclusions are summarised below.

2.1 Classical toxicology data

In addition to metabolomic analysis, classical toxicological parameters (food consumption, body weights, clinical observations, organ weights and histopathology) were measured in the animals. Clinical symptoms, food consumption, bodyweight effects were as expected from all substances. Consistent with the expected effects of the marker substances, clinical chemistry and haematology showed effects on liver and the red blood cell systems. Organ weights mainly affected include the liver, thyroid (males and females) and kidneys (males only). This was accompanied by histopathological effects observed in the thyroid of both sexes (i.e. follicular cell hypertrophy), a mechanism consistent with the classical mechanism driven in rodents by induction of liver enzyme activity seen with these substances. This mechanism occurs in rodents when liver enzyme induction results in greater than normal thyroid hormone metabolism and excretion. There is a feedback mechanism to the thyroid to produce greater levels of thyroid hormone to replenish that removed by metabolism, resulting in a larger thyroid developing to meet that demand. This effect is completely reversible. The kidneys of male rats also show toxicity caused by a rat-specific production of a protein called alpha2U globulin, which accumulates in the rat kidney and causes nephron toxicity. A summary of the main classical toxicology findings can be seen in Table 3.

Table 3. Summary Results of Clinical Examination, Clinical Pathology and Histopathology for high doses tested

Legend: Be. – Benzene, Xy. – Xylene, Na. – Naphthalene, Di. – Dicyclopentadiene, Cy. – Cyclopentane, In. – Indene, BL1 – BASF LOA 1, BL2 – BASF LOA 2, and BL3 – BASF LOA 3. Green= effect observed, Red= effect not observed.

	Marker Compounds						BASF LOA			LOA Streams											
	Be.	**Xy.**	**Na.**	**Di.**	**Cy.**	**In.**	**BL1**	**BL2**	**BL3**	**L-01**	**L-02**	**L-03**	**L-04**	**L-05**	**L-06**	**L-07**	**L-08**	**L-09**	**L-10**	**L-11**	**L-12**
Dose Level (mg/kg bw)	**1000**	**1000**	**600**	**150**	**1000**	**450**	**1000**	**1000**	**1000**	**1000**	**750**	**500**	**200**	**750**	**1000**	**600**	**300**	**200**	**1000**	**500**	**1000**
Clinical Examination																					
Clinical signs																					
Temporary body weight effects																					
Body weight effects on day 13																					
Clinical Pathology																					
Red blood cell system																					
Liver																					
Electrolyte imbalance																					
Histopathology																					
Kidney (males, alpha 2u)																					
Liver																					
Thyroid																					

2.2 Metabolomic data summary

2.2.1 Metabolomic pattern ranking against the BASF MetaMap® database

The BASF MetaMap® database contains the metabolomic signature for greater than 110 well defined toxicity outcomes. Comparing the metabolome pattern obtained from the substances in the Resin Oils and Cyclic Dienes category and the marker substances, with the signatures in the BASF MetaMap® database, the predominant hits suggested effects in the liver, thyroid (indirect toxicity), kidney, and blood. These results are completely consistent with the known effects of the marker substances, and importantly, no effects were observed in any of the Resin Oils and Cyclic Dienes category substances that were not observed in the marker substances (Figure 5, Figure 6, Figure 7 and Figure 8). For a full summary of the data, please see the accompanying webinar recording and slide deck.

Figure 5. Male rat toxicity overview: Ranking pattern against the BASF MetaMap® toxicity database

Boxes coloured red show a complete mismatch with the known toxicity metabolome profile listed in the first column of the chart.

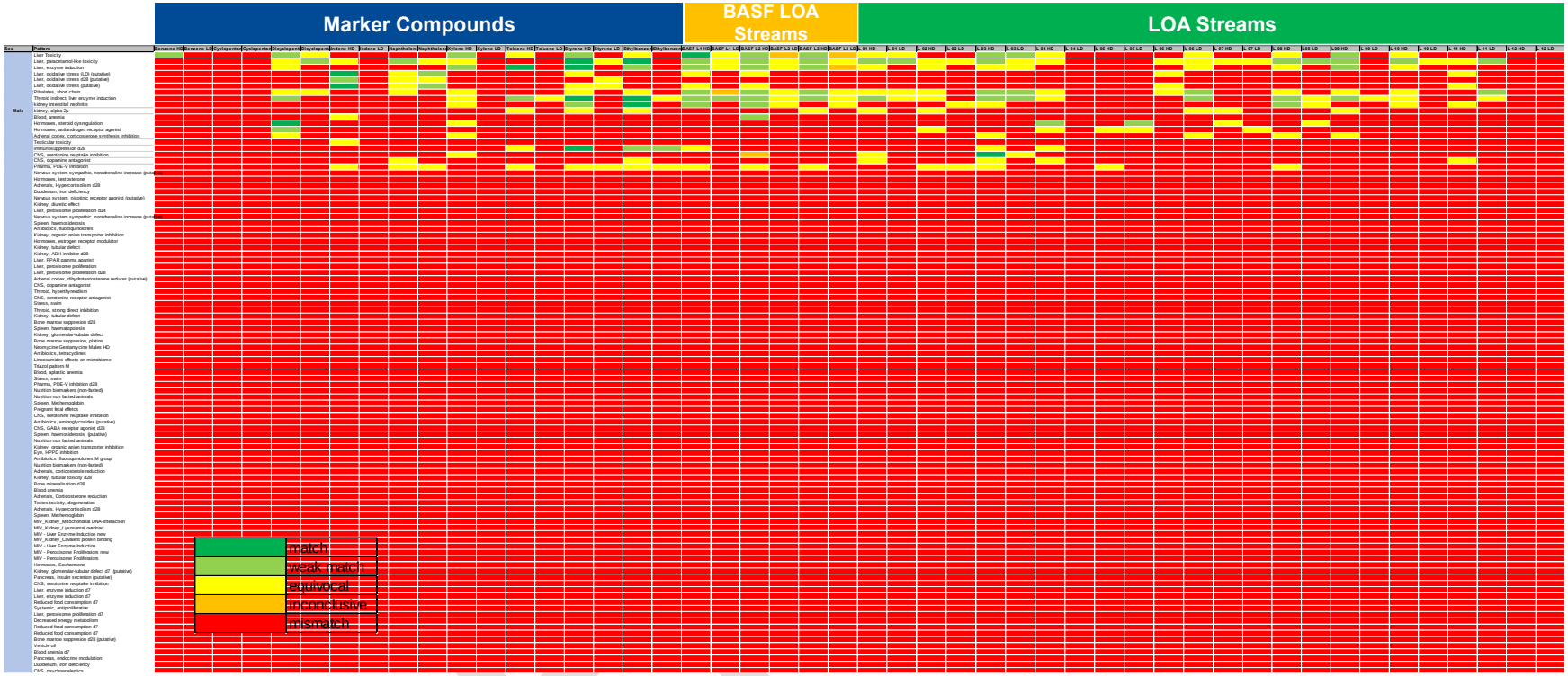


Figure 6 Male rat Toxicity overview: Ranking pattern against the BASF MetaMap® toxicity database (Exploded view of the top rows from Figure 5

The upper panel contains the metabolome pattern ranking of the marker substances and the LOA BASF substances. The lower panel contains the metabolome pattern ranking of the substances present in the Resin Oils and Cyclic Dienes category.

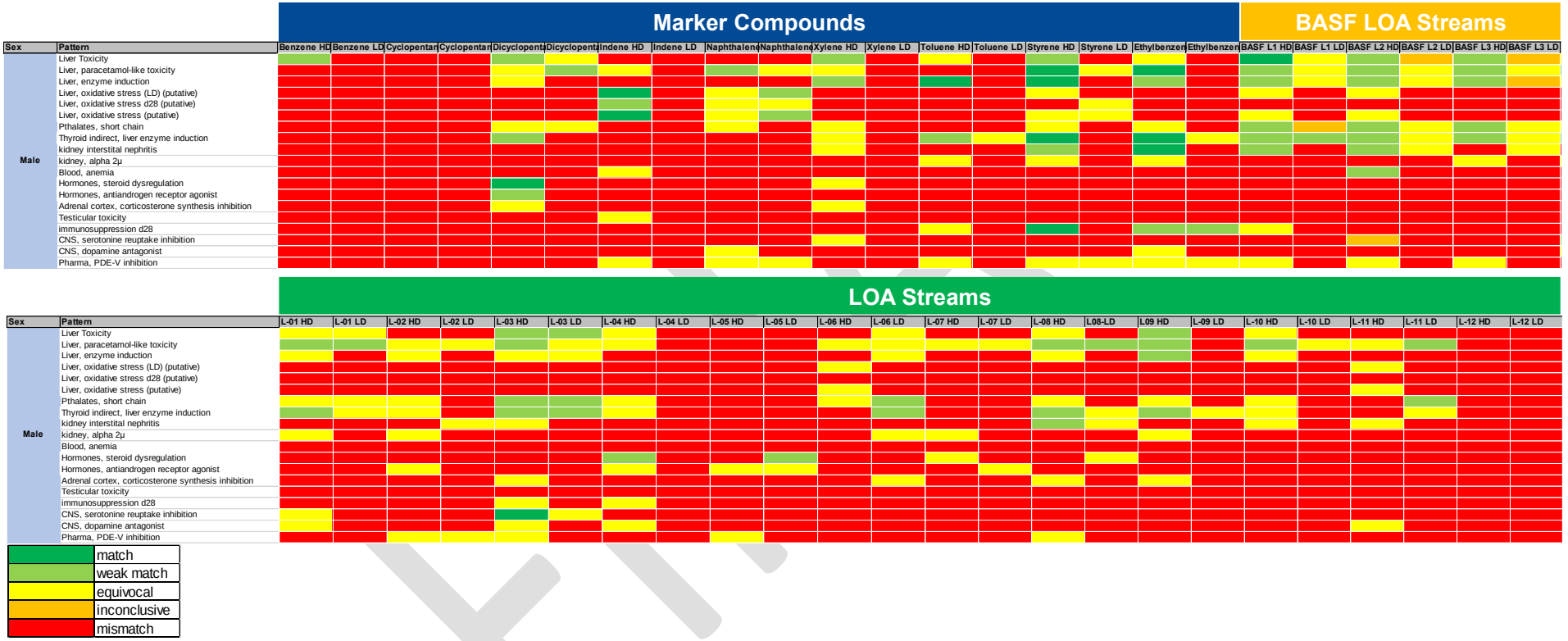


Figure 7. Female rat toxicity overview: Ranking pattern against the BASF MetaMap® toxicity database

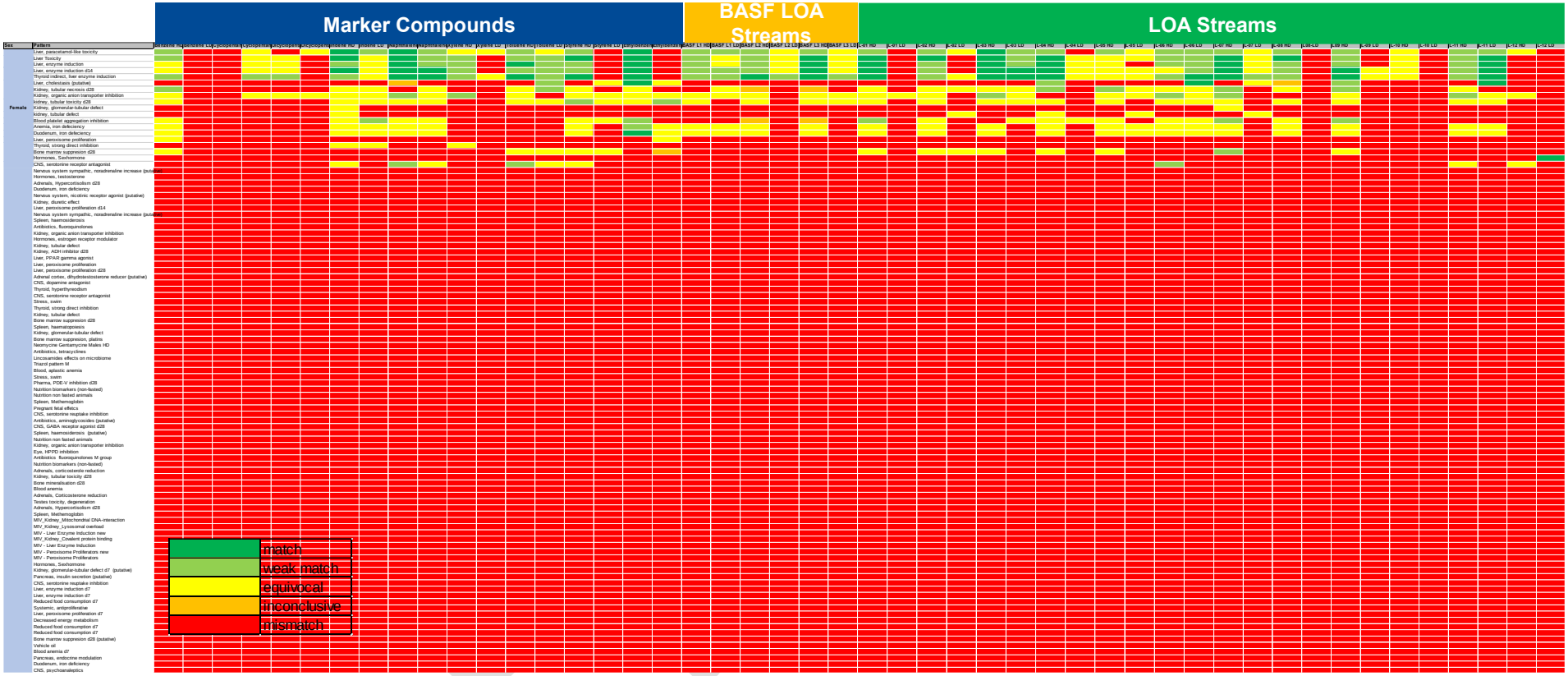
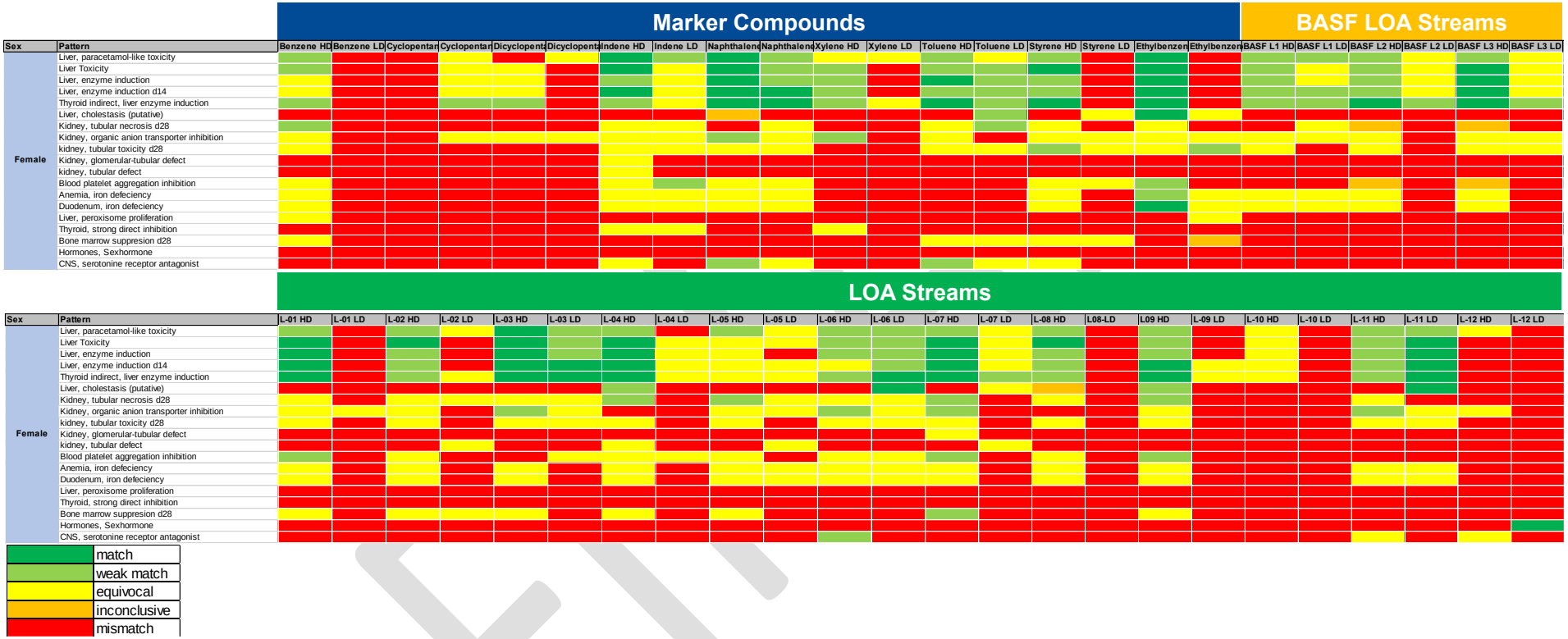


Figure 8. Female rat Toxicity overview: Ranking pattern against the BASF MetaMap® toxicity database (Exploded view of the top rows from Figure 7

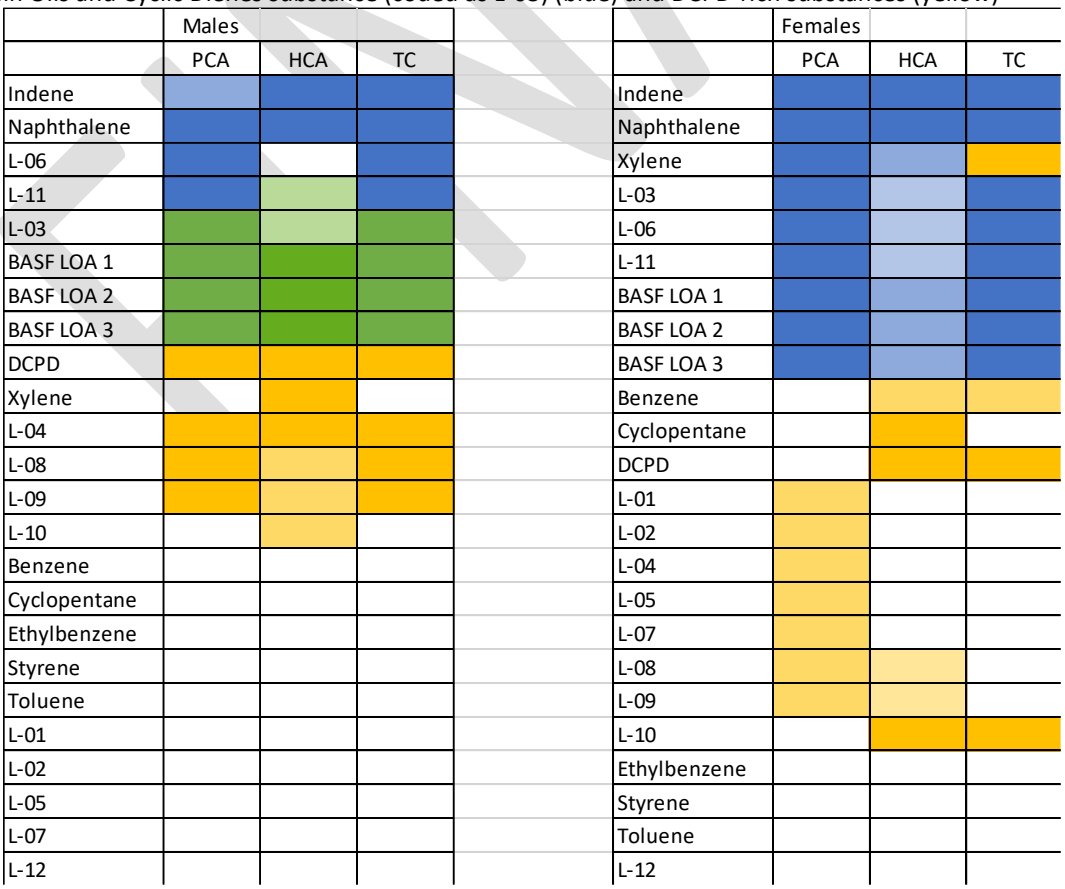


2.2.2 Clustering analysis

Following evaluation of the metabolome profile of the Resin Oils and Cyclic Dienes category against the MetaMap® database (Section 2.2.1), statistical clustering analyses were performed to look for groups with similar metabolomic profiles in the tested substances. These included treatment correlation clustering (TC), principal component analyses (PCA) and hierarchical clustering (HA). For a full review of these data, please see the accompanying webinar recording and slide deck. There was a strong dose-dependent effect observed on the plasma metabolome. The magnitude of changes observed was greater in females than males, particularly at the high dose level. In summary all three types of statistical clustering analysis revealed that each substance tested resulted in a significant change in the metabolome profile when compared to control. They also revealed a degree of sub-clustering within the Resin Oils and Cyclic Dienes category (Figure 9) that was not evident from the traditional toxicology analyses (Table 3). A comparison of the statistical clustering methods used is presented in Figure 9 and given the strong overall metabolomic response in this study, it is remarkable that a limited number of patterns were identified and that these were predominantly associated with effects in the liver.

Figure 9. Comparison of the Treatment Correlation, Principal Component Analysis and Hierarchical Clustering analysis of the Resin Oils and Cyclic Dienes, marker substances and BASF LOA substances

Colour indicates the different clusters. Three clusters evident in males, naphthalene- / indene-rich substances (blue), BASF-LOA substances and Resin Oils and Cyclic Dienes substance (coded as L-03) (green) and DCPD-rich substances (yellow). Two clusters evident in females, naphthalene- / indene-rich substances, BASF-LOA substances and Resin Oils and Cyclic Dienes substance (coded as L-03) (blue) and DCPD-rich substances (yellow)



3 Conclusion

The metabolomic programme was successful in determining appropriate doses for use in the OECD 422 studies being performed on all substances within this category (for details see Figure 2). The data show significant metabolomic changes after exposure to these substances. However, zooming out (Figure 5 and Figure 7 to a more holistic view, those findings are limited to a remarkably homogenous set of toxicity findings across all substances tested with the liver, kidney, thyroid (indirect toxicity) and red blood cell systems being affected (Figure 6 and Figure 8). These effects, known for the marker substances, were also supported in the metabolome pattern of the Resin Oils and Cyclic Dienes category. The data also reveal that the toxicity of the Resin Oils and Cyclic Dienes category is partly (although not exclusively) explained by the presence of the marker substances present in the category as the metabolome patterns observed for the UVCB substances were not identical to the individual marker substances. In addition, apart from the liver mediated thyroid changes as secondary effect, no known direct mechanisms of endocrine toxicity were observed. The overall conclusion is that these substances are biologically coherent, supporting the overall category approach.

As outlined in Figure 1 and Figure 2, the LOA category read-across strategy includes a combination of compositional analysis to confirm that substances are within the category boundaries set, a full *in vitro* genotoxicity examination demonstrating that the substances are not genotoxic and the metabolomic data to build confidence that the ultimate phenotype which develops upon exposure will be consistent across substances. In addition to the metabolomic data, each substance of the Resin Oils and Cyclic Dienes Category will also be tested in an OECD 422 (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, Reporting Q1/Q2 2022) to inform on the potential for reproductive and developmental toxicity and elucidated consistencies or difference that may exist between the streams. Once the OECD 422 studies report, LOA will conduct a full integrated analysis of all data generated to determine if the substances selected for further analysis (Figure 2), are appropriate and to re-evaluate the approach prior to conducting the remaining higher tier studies on selected streams.

It is anticipated that the approach outlined will provide a robust dataset to support the category of Resin Oils and Cyclic Dienes read-across outlined in Figure 2. In addition, this approach significantly reduces the numbers of laboratory animals required to fulfil REACH data requirements without compromising the quality of the safety assessment.

Appendix 1. Boundary composition and category information profile for Resin Oils and Cyclic Dienes

Category L Identity Profile – Resin Oils & Cyclic Dienes

This CIP represents the boundary substance compositions in the Joint Registrations for substances in **Category L** *under the EU legislation REACH[1]. It is included in the Lead Registrant dossiers that are submitted to ECHA[2]. For a valid Joint Registration, the joint registrants' own composition needs to fit within the boundaries (concentration ranges) of the constituents and purity described here. For further details, see the section on Category Identity Profile and Boundary Composition at the end of this document.*

Description of composition

The resin oils and cyclic dienes category contains unsaturated and non-hydrotreated hydrocarbon products. The physico-chemical properties associated with these types of UVCBs indicated that they comprise a category based on the range of boiling points approximately 40°C to 250°C. Members of this category will have a carbon number distribution that is predominantly C5 – C15 and may contain more than 0.6% of DCPD, and/or more than 0.1% benzene.

State / form

Liquid

Manufacturing Process

The Resin oils and cyclic dienes (DCPD rich) category covers hydrocarbons typically produced by the distillation of products from a steam cracking process. The category contains non-hydrotreated products (the Resin Oil products) and/or products that are concentrates of (1) dicyclopentadiene (DCPD) and (2) methylcyclopentadiene-cyclopentadiene codimer (MeDCPD). Member of this category will have a carbon number distribution that is predominantly C5 – C15 and may contain more than 0.1 % isoprene, and/or more than 0.6% of DCPD and/or more than 0.1% benzene. A generic description is given below.

A pyrolysis gas or naphtha starting material is steam-cracked at high heat (800 – 1000°C) and then distilled or filtered progressively at lower temperatures (approx. >200°C) to remove low carbon-number fractions (typically below C9). Further treatments, such as hydrogenation, may also be applied to streams to produce the final product, which is rich in DCPD and mono-aromatic compounds.

[1] REGULATION (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC

[2] European Chemicals Agency.

Constituents

The following constituents were selected based on the category composition data as reported by the registrants. As per the ECHA UVCB guidance, constituents which are CMR and/or PBT above 0.1% and other constituents ≥ 10% are reported in the table below.

The concentration ranges shown in this table directly reflect the boundary composition submitted in the lead registrant's dossier.

Constituent / CAS No	Typical Concentration	Concentration Range
Benzene / 71-43-2	ca. 1.0 % (w/w)	0 - ≤ 3 % (w/w)
Toluene / 108-88-3	ca. 10 % (w/w)	0 - ≤ 20 % (w/w)
Ethylbenzene / 100-41-4	ca. 5 % (w/w)	0 - ≤ 15 % (w/w)
DCPD / 77-73-6	ca. 40 % (w/w)	0 - < 80 % (w/w)
Xylenes / 1330-20-7	ca. 10 % (w/w)	0 - ≤ 20 % (w/w)
Styrene / 100-42-5	ca. 12.5 % (w/w)	0 - ≤ 25 % (w/w)
n-Hexane / 110-54-3	ca. 0 % (w/w)	0 - ≤ 0.2 % (w/w)
4-methylstyrene / 622-97-9	ca. 20 % (w/w)	0 - ≤ 40 % (w/w)
Naphthalene / 91-20-3	ca. 20 % (w/w)	0 - ≤ 40 % (w/w)
Methylnaphthalene / 90-12-0	ca. 5 % (w/w)	0 - ≤ 15 % (w/w)
1,3-Pentadiene / 504-60-9	ca. 16 % (w/w)	0 - ≤ 51 % (w/w)
2-phenylpropene / 98-83-9	ca. 5 % (w/w)	0 - ≤ 20 % (w/w)
Ethyltoluene / 25550-14-5	ca. 20 % (w/w)	0 - ≤ 40 % (w/w)
m-Ethyltoluene / 620-14-4	ca. 5 % (w/w)	0 - ≤ 13 % (w/w)
Isopropylbenzene / 98-82-8	ca. 15 % (w/w)	0 - ≤ 30 % (w/w)

Constituent / CAS No	**Typical Concentration**	**Concentration Range**
Cyclopentane / 287-92-3	ca. 25 % (w/w)	0 - ≤ 50 % (w/w)
Cyclopentene / 142-29-0	ca. 15 % (w/w)	0 - ≤ 25 % (w/w)
Methylindenes / 29036-25-7	ca. 10 % (w/w)	0 - ≤ 70 % (w/w)
Methyldicyclopentadiene / 25321-13-5	ca. 10 % (w/w)	0 - ≤ 21 % (w/w)
Indan / 496-11-7	ca. 7.5 % (w/w)	0 - ≤ 25 % (w/w)
Indene / 95-13-6	ca. 35 % (w/w)	0 - ≤ 80 % (w/w)
Trimethylbenzenes (TMB)/ 25551-13-7	ca. 20 % (w/w)	0 - ≤ 40 % (w/w)
1,3,5-Trimethylbenzene / 108-67-8	ca. 5 % (w/w)	0 - ≤ 10 % (w/w)
1,2,4-Trimethylbenzene / 95-63-6	ca. 7.5 % (w/w)	0 - ≤ 15 % (w/w)
2,3,6-Trimethyl-4-octene / 63830-65-9	ca. 20 % (w/w)	0 - ≤ 50 % (w/w)
Dihydrodicyclopentadiene / 4488-57-7	ca. 5 % (w/w)	0 - ≤ 12 % (w/w)
2-Methylstyrene / 611-15-4	ca. 7.5 % (w/w)	0 - ≤ 15 % (w/w)
3-Methylstyrene / 100-80-1	ca. 10% (w/w)	0 - ≤ 20 % (w/w)
2-methylbut-2-ene / 513-35-9	ca. 5 % (w/w)	0 - ≤ 10 % (w/w)
Vinyltoluene / 25013-15-4	ca. 30 % (w/w)	0 - ≤ 60 % (w/w)
4-ethyl-3-octene / 53966-51-1	ca. 40 % (w/w)	0 - < 80 % (w/w)
(E)-3-dodecene / 7239-23-8	ca. 5 % (w/w)	0 - ≤ 10 % (w/w)

Analytical Working Group
Technical Steering Committee
Category Identity Profile (CIP) – Category L

Category Identity Profile and Boundary Composition

This Category Identity Profile has been produced following the currently available ECHA guidance[3] by the Analytical Working Group, a Working Group of the LOA Technical Steering Committee, following consultation with Registrants.

The CIP defines the compositional boundaries of the substance that is to be (or has been) registered and consequently the substance for which intrinsic hazard data is submitted, as defined by Annexes VII-X of REACH. It is a document usually agreed between the registrants and potential registrants.

With the introduction of IUCLID 6[4] in April 2016 it became necessary for the Lead Registrant to enter the CIP details into Section 1.2 of IUCLID as the "boundary composition" of the registration prior to any new submission or spontaneous update. Failure to add it will cause rejection of the dossier when uploading into REACH IT.

The business rules of REACH IT also check that information is present which verify that minimum information is present: at least one constituent must be provided, and for each constituent, impurity and additive a reference substance with identifiers, and a concentration range must be given. For UVCB substances, a description of the starting material and process that defines the UVCB composition must be indicated. After submission, the information is displayed on the joint submission page so it can be seen by members of the Substance Information Exchange Forum (SIEF).

It is not necessary for joint registrants to report on boundary composition. However, joint registrants are required to enter their legal entity composition which is automatically checked against the substance boundary composition to ensure that the joint registrant's composition fits into the boundary composition of the CIP.

If a joint registrant is outside the CIP and boundary composition, they need to state this in their dossier - details are given in the ECHA guidance.

If you have any question on this SIP please contact sief.manager@loa-reach.com

Version:	2.1
Date:	28 July 2021
Revised by:	Jamie Dunn

[3] ECHA (2016) Guidance for identification and naming of substances under REACH and CLP June 2016 Draft Version Current link - 🕮

[4] The software used for producing dossiers for submission to ECHA under REACH. https://iuclid6.echa.europa.eu/

Constituent / CAS No	Typical Concentration	Concentration Range
1,2,4,5-tetramethylbenzene / 95-93-2	ca. 5 % (w/w)	0 - ≤ 10 % (w/w)
Propylbenzene / 103-65-1	ca. 5 % (w/w)	0 - ≤ 10 % (w/w)
4,7-Methano-1H-indene, 2,3,3a,4,7,7a-hexahydro- 19398-83-5	ca. 10 % (w/w)	0 - ≤ 20 % (w/w)
(3Z)-penta-1,3-diene / 1574-41-0	ca. 10 % (w/w)	0 - ≤ 20 % (w/w)
1,2-Dihydronaphthalene / 447-53-0	ca. 12.5 % (w/w)	0 - ≤ 25 % (w/w)
Phenol / 108-95-2	ca. 0 % (w/w)	0 - ≤ 7 % (w/w)
PIONA		
Paraffins	ca. 0.5 % (w/w)	0 - ≤ 1 % (w/w)
Isoparaffins	ca. 15 % (w/w)	0 - ≤ 25 % (w/w)
Olefins	ca. 25 % (w/w)	0 - ≤ 100 % (w/w)
Naphthenics	ca. 5 % (w/w)	0 - ≤ 10 % (w/w)
Aromatics	ca. 50 % (w/w)	0 - < 100 % (w/w)

Impurities

Not applicable to UVCBs

Additives

Not applicable to UVCBs