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Identification of flavouring substances of genotoxic concern present in e-cigarette refills

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ABSTRACT

E-cigarettes have become very popular, a trend that has been stimulated by the wide variety of available e-liquid flavours. Considering the large number of e-liquid flavours (> 7000), there is an urgent need to establish a screening strategy to prioritize the flavouring substances of highest concern for human health. In the present study, a prioritization strategy combining analytical screening, *in silico* tools and literature data was developed to identify potentially genotoxic e-liquid flavourings. Based on the analysis of 129 e-liquids collected on the Belgian market, 60 flavourings with positive *in silico* predictions for genotoxicity were identified. By using literature data, genotoxicity was excluded for 33 of them whereas for 5, i.e. estragole, safrole, 2-furylmethylketon, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone and transhexanal, there was a clear concern for *in vivo* genotoxicity. A selection of 4 out of the remaining 22 flavourings was tested in two *in vitro* genotoxicity assays. Three out of the four tested flavourings induced gene mutations and chromosome damage *in vitro,* whereas equivocal results were obtained for the fourth compound. Thus, although there is a legislative framework which excludes the use of CMR compounds in e-liquids, flavourings of genotoxic concern are present and might pose a health risk for e-cigarette users.

1 INTRODUCTION

At present, the e-cigarette is the most popular alternative to tobacco smoking [1]. Unlike the traditional nicotine replacement therapy, e-cigarettes are available in many different flavours which is one of the main reasons for their popularity [2]. Today, more than 7000 different flavoured e-liquids are sold worldwide [3]. To obtain these flavours, synthetic chemicals, tobacco extracts or other herbal extracts are added to the e-liquids, collectively referred to as 'flavourings' [4].

Flavourings are also used in some conventional tobacco products. In the US, the tobacco industry uses more than 500 different additives, accounting for 10% of the total cigarette content, to improve the taste of tobacco cigarettes [5]. However, in Europe, the use of flavourings and other additives (vitamins, caffeine, certain ammonia compounds,…) in traditional tobacco cigarettes is banned as they might further encourage the use of tobacco cigarettes and maintain the nicotine addiction of the user. A similar concern has been raised regarding the use of flavourings in e-cigarettes as the large variety of 'trendy' flavours makes e-cigarettes more attractive, especially amongst minors and young adults [6]. Consequently, e-cigarettes containing nicotine may initiate nicotine addiction and function as a gateway to tobacco cigarettes in this vulnerable group. Another concern of e-cigarette use relates to the potential toxicity following inhalation of flavourings. In tobacco cigarettes, the toxicity of additives is considered of minor significance compared to the toxicity induced by the tobacco-associated components [5]. However, when used in e-cigarettes, flavourings may be the main contributor to adverse human health effects. Many of the flavourings present in e-cigarettes are food grade or fragrances used in cosmetics. However, their toxicological profile is often poorly characterized, especially upon inhalatory exposure. Some flavourings commonly used in e-cigarettes, such as diacetyl and acetylpropionyl, are known to cause a local inflammatory lung disease, i.e. bronchiolitis obliterans, when repeatedly used [7]. Additionally, some specific strawberry flavourings used in e-cigarettes have been reported to induce significant toxicity *in*

vitro such as a decrease in cell viability, metabolic activity and release of inflammatory mediators (cytokines) in H292 human bronchial epithelial cells [8]. Furthermore, flavourings present in e-liquids might undergo chemical reactions in the e-liquid mixtures or during the heating process, resulting in potential harmful reaction products such as flavorant–propylene glycol adducts and the formation of toxic aldehydes [9,10].

While regulations for tobacco cigarettes mainly focus on reducing the appeal and the addictiveness, those for e-cigarettes are aimed at regulating the ingredients of the products themselves to assure consumer safety. To this extent, the EU Member States have revised the Tobacco Product Directive (2014/40/EU) (TPD) and adopted Article 20 herein that specifically relates to electronic nicotine delivery devices. In this Article 20, the minimum general requirements for e-liquid ingredients are included [11]. As a basic safety precaution, ingredients with Carcinogenic, Mutagenic or Reprotoxic (CMR) properties are banned in e-liquids.

Yet, another more general TPD requirement stipulates that '*flavourings, like other e-cigarette ingredients, are only allowed if they do not pose a risk to human health in heated or unheated form'*. In this context, Girvalki et al. verified the health hazard statements defined by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) for the most frequently found flavourings in e-liquids [12]. As such, 11 flavourings were identified to display hazards related to reproductive toxicity, organ toxicity upon single (benzyl acetate) or repeated (banana oil) exposure, acute oral/dermal/inhalation toxicity (allyl hexanoate) and severe skin burns (phenol, 3,5-bis(1,1-dimethylethyl)-) and eye damage (geraniol). It should, however, be noted that these are hazard statements and do not necessarily reflect a risk.

Furthermore, the TPD states that impurities in e-liquids are only allowed if they are technically unavoidable during manufacture and kept at trace levels. Hence, to minimize potential risks from

contaminants such as volatile organic components (VOCs), the use of high purity flavouring ingredients is recommended [13].

Besides specific requirements for certain ingredients, the manufacturers of e-liquids are also obliged to notify their products before they are placed on the EU market (EU 2015/2183). In this notification, a list of ingredients should be provided with toxicological information on all ingredients [14]. However, the listing highly depends on the goodwill of the manufacturers. Some manufacturers are not eager to provide the required information, because of confidentiality issues. In the Decision (EU) 2015/2183, it is stated that ingredients present at a level below 0.1% in the final product formulation may be deemed confidential or a trade secret. Consequently, these ingredients are often described collectively in the notification by an umbrella term such as e.g. 'strawberry flavouring'. In most cases, the complete composition of the e-liquid thus remains unclear to the authorities (and even to the manufacturers), especially when natural extracts are used (tobacco extracts, essential oils, herbal extracts, or non-chemically synthetized flavourings), as their composition is not always known and may vary from batch to batch depending on biological and geographical origins [15]. It is thus highly likely that e-liquids contain substances with unknown toxicological properties or known toxic substances that exceed certain safety limits. In those cases, the use of e-liquids might cause adverse human health effects.

Although their presence is legally not allowed, previous studies have shown that (potential) CMR substances occur in certain e-liquids. More specifically, VOCs such as benzene, toluene, etc. have been found to be present as residual solvents in tobacco extracts [16,17]. Also, for many of the flavourings in e-liquids, toxicological data are limited or even absent and their CMR properties remain thus unknown. Ideally, full characterization of all ingredients used in e-liquids is a first important requisite. Next, the toxicological properties of the ingredients and/or the whole e-liquid need to be identified to be able to assess their safety and evaluate possible risks associated with e-cigarette usage. Considering that there

are more than 7000 different flavoured e-liquids on the market, we developed a prioritization strategy to identify potentially genotoxic substances used as e-liquid flavouring. The strategy followed only uses non-animal methods and is based on a similar approach that has recently been applied to identify genotoxic compounds used in printed paper and board food contact materials [18]. As such, the prioritization strategy consists of four steps: (i) Identification of the substances present in the e-liquids via GC-MS screening; (ii) Prediction of the genotoxic potential of the substances using three (quantitative) structure-activity relationship (or (Q)SAR) *in silico* models; (iii) Collection of existing *in vitro* and *in vivo* genotoxicity data from public literature sources; and (iv) *In vitro* genotoxicity testing on a selection of commercially available flavourings. Based on all collected information, flavourings of high concern were identified.

2 MATERIALS AND METHOD

2.1 Chemicals

Dimethylsulfoxide (DMSO) and the positive control substances for the genotoxicity assays i.e. benzo[a]pyrene (BaP), sodium azide, 2-aminoanthrancene and 4-nitroquinoline 1-oxide (4-NQO) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). An overview of the substances tested *in vitro* and reference standards used for chemical confirmation is presented in Table 1.

Table 1 Overview of the reference standards used for chemical confirmation and the test compounds included in the *in vitro* genotoxicity study.

Name	CAS number	Provider
safrole	94-59-7	Sigma Alrich
estragole	140-67-0	Sigma Alrich
furylmethylketon	1192-62-7	Sigma Alrich
trans-hexenal	6728-26-3	Sigma Alrich
2,5-dimethyl-4-hydroxy-3(2H)-furanone	3658-77-3	Sigma Alrich

β-phellandrene	555-10-2	TRC Chemicals
isoledene	95910-36-4	Sigma Aldrich
2,3-butanedione	431-03-8	Sigma Aldrich
2,3-pentanedione	600-14-6	Sigma Aldrich
2-ethyl-4-methylthiazole	15679-12-6	Sigma Aldrich
3-hexen-2-one, 5-methyl	5166-53-0	Sigma Aldrich
p-methylbenzyl acetate	2216-45-7	Sigma Aldrich
2H-pyran-2-one, 4-hydroxy-3,6-dimethyl-	5192-62-1	Sigma Aldrich

2.2 Screening of e-liquid samples using GC-MS

A total of 129 e-liquids representative for different flavour categories [19], present on the Belgian market, were collected and screened analytically. The samples were either obtained upon inspections of different vaping shops in Belgium or were seized postal packages ordered by individuals through the internet between 2016 and 2018. The identification of the substances present in the e-liquids was done by using the National Institute of Standards and Technology (NIST) research library. A library spectral match quality of 70% was considered as a positive identification. A peak area threshold of 0.1% of the main peak was set in order to exclude contaminations from carry-over in the GC-capillary column (polysiloxanes). Two different GC-MS screening methods were used to cover the wide variability of volatility among the different compounds.

2.2.1 Method A: high volatiles

The screening was performed on an Agilent 6890 N gas chromatograph coupled to an Agilent 5973N single quadrupole mass spectrometer and equipped with a G188A static headspace sampler (Agilent Technologies, Palo Alto, USA). The samples were diluted by dissolving 1 g e-liquid sample in 10 ml water of which 300 µl was transferred to a 10 ml sealed vial, placed in the autosampler oven to be heated and agitated in order to generate a gas phase. The incubation temperature was maintained at 85°C with an

equilibration time of 15 min. The injector port was kept at 160°C, in split injection mode (split ratio 15:1), while the temperatures of the headspace loop and the transfer line were maintained at 100 and 120°C, respectively. The substances were separated on a VF-5 ms (5% phenyl-95% methylpolysiloxane) capillary column of 60 m with Ø 0.25 mm and film thickness of 0.25 µm and an integrated guard column of 10 m (#CP9013, Factor four, Agilent, California, USA). Helium carrier gas was used at a constant flow of 1.0 ml/min. The initial oven temperature of 45°C was maintained for 10 min, followed by a temperature ramp of 40°C/min to a final temperature of 250°C. The total run time was 18 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV. Temperatures of the ion source, the quadrupole, and the interface were set at 230, 150 and 280°C, respectively. The identification was performed in full scan mode from 25 to 400 m/z.

2.2.2 Methods B: semi-volatiles

The second screening method was applied to detect the semi-volatile substances in e-liquids. Thus, the incubation temperature was maintained at 145°C with an equilibration time of 15 min to obtain full evaporation mode. The injector port was kept at 160 °C, in split injection mode (split ratio 15:1), while the temperatures of the headspace loop and the transfer line were maintained at 150 and 155°C, respectively. To minimize the interference of the matrix components propylene glycol and glycerol, a liquid-liquid extraction was applied as sample preparation step. The substances were extracted with hexane followed by separation through a "freeze-and-pour" technique. For each sample extraction, 0.3 g of the e-liquid was weighted in a glass vial of 20 ml, mixed with 3 ml hexane and covered with a Teflon seal. During the first extraction step, vials were vortexed for 10 s and then sonicated for 3 min at 50°C. Afterwards, vials were transferred to a cooling bath of −78°C (dry ice dissolved in acetone) for 2 min, followed by 1 min centrifugation at 860 g. Three quarters of the supernatants was transferred to a glass vial. These extraction steps were repeated again by adding another 3 ml of hexane. After extraction, 300 µl of the extracted solution was transferred to a headspace vial of 10 ml. The GC-MS conditions were similar to

those of the method for high volatiles, except for the temperature gradient. The temperature gradient started at 65°C (held for 3 min) and raised with 5°C/min to reach 90°C. The temperature gradient continued at 20°C/min, until 185°C, followed by another fast decrease in temperature to 100°C by 30°C/min, that finally increased with 35°C/min until 290°C (held for 3 min). The total runtime of the method was 24 min.

2.3 *In silico* prediction of genotoxicity

The genotoxic potential of the compounds identified through the analytical screening of the e-liquids was investigated *in silico* using three (Q)SAR models. Predictions were only made for the endpoint 'mutagenicity *in vitro*', specifically bacterial mutagenicity, as (Q)SAR models for the other genotoxic endpoints are less developed. The three models were complementary both with respect to their prediction method (SAR/QSAR) and their availability (free/commercial). Two of the models, i.e. Derek Nexus™ v 6.0 and Sarah Nexus™ v 3.0 are integrated within the commercially available Nexus platform (version 2.2) provided by Lhasa Limited. Derek Nexus is a SAR tool that runs predictions for, among others, *in vitro* mutagenicity through expert-based rules whereas Sarah Nexus is QSAR-based Ames mutagenicity model. The third model, i.e. the VEGA Consensus model for mutagenicity (v 1.1.5 36), is part of the open access VEGA hub (https://www.vegahub.eu/). The latter is a library of QSAR models predicting physico-chemical, fate and (eco)toxicological parameters of chemicals developed by the Istituto di Ricerche Farmacologiche Mario Negri IRCCS (IRFMN). Based on their Chemical Abstract Service (CAS) number, the simplified molecular input line entry system (SMILES) representation of each substance was extracted via PubChem (National Institutes of Health) [21] and ChemSpider (Royal Society of Chemistry) [22]. For Derek Nexus, predictions for *in vitro* mutagenicity were run with the setting species = bacterium. Because Derek Nexus is a rule-based SAR model developed with open literature and confidential data, there is no defined training set nor applicability domain available. When no alert is found, the software labels the compound

as inactive (i.e. negative). In the Sarah Nexus model, the query compound is fragmented, after which the fragments are reviewed for activity versus inactivity. After generating a network of hypotheses by arranging meaningful fragments, relevant hypotheses are applied to inform an overall mutagenicity prediction. A confidence score and applicability domain check are also performed to arrive to the final conclusion. For Sarah Nexus, substances were considered positive if the prediction outcome was '*positive*' or '*equivocal*'. This is a conservative interpretation of '*equivocal*' in Sarah Nexus used by the authors; Lhasa Limited intend this result to be '*the confidence level below which a prediction of positive or negative is unable to be made*'.

The VEGA Consensus mutagenicity model combines the information of four global models to evaluate bacterial mutagenicity:

- The CAESAR mutagenicity model, a (Q)SAR hybrid model combining machine-learning algorithm with two sets of SAs;
- The Istituto Superioire di Sanità (ISS) mutagenicity model, which also contains SAs, but extracted from another *in silico* tool, namely Toxtree, and is a rule-based SAR model;
- The SARpy mutagenicity model, a QSAR model that determines if the test compounds are mutagenic or non-mutagenic based on the presence of SAs [23];
- KNN, short for K-nearest neighbor, a read-across model in which the software identifies chemicals which are more similar to target compounds [24].

The separate results of the four models are combined into one final 'VEGA Consensus' result. The weighted consensus result is obtained by taking into account the result of each individual model and its associated compound-specific applicability domain index (ADI). For VEGA, substances were considered positive if the prediction outcome was 'Mutagenic'.

2.4 Genotoxicity data collected from EU databases

For the flavouring substances with a positive prediction outcome for '*in vitro* bacterial mutagenicity' in at least one of the three (Q)SAR models, genotoxicity data was collected from previous safety evaluations by European Authorities from different regulatory domains using the strategy previously proposed by Van Bossuyt et al [18]. In the first step, the genotoxic potential of the compound was verified in the harmonized classification according to the Classification, Labelling and Packaging (CLP) regulation [25]. If there was no harmonized CLP classification available, genotoxicity data were collected from evaluations by EU authorities i.e. Opinions issued by the European Food Safety Authority (EFSA) via the Open Food Tox database [26] and by the European Medicines Agency (EMA) and the Scientific Committee on Consumer Safety (SCCS). In case no evaluation of the genotoxic potential was available in these Opinions, the European Chemicals Agency (ECHA) database was consulted [27]. The ECHA database has been constructed under the framework of the Registration, Evaluation, Authorization And Restriction Of Chemicals (REACH) regulation, which establishes procedures for collecting and assessing hazards of substances [28]. Chemical manufacturers need to register their substances (if manufactured or imported in the quantity of 1 ton or more per year) and provide amongst others information on toxicological data. The approach described by Mertens et al. was used to retrieve genotoxicity data from the ECHA database [29].

2.5 *In vitro* genotoxicity testing

A selection of substances with a positive prediction in at least one of the three (Q)SAR models and for which no *in vitro* genotoxicity data was found and that were commercially available, were tested *in vitro* using a battery of two tests i.e. the Ames test and the *in vitro* micronucleus test. The former detects gene mutations [30], whereas the latter picks up structural and numerical chromosomal aberrations [31].

2.5.1 Ames test

The test was performed according to the OECD Guideline for testing of chemicals, Test No. 471: Bacterial reverse mutation test [32] with slight modifications. Normally the OECD recommends to use five bacterial tester strains to cover the full range of mutagenicity. In the present screening study, only the strains *Salmonella typhimurium* TA98 and TA100 were used. TA100 is able to detect 83% of the mutagens whereas TA98 detects 67%. When used in combination, TA98 and TA100 are able to pick up approximately 93% of all 224 mutagens tested by the National Toxicology Program [33].

Salmonella typhimurium bacteria (TA98 or TA100) (Moltox, Boone, USA) were grown overnight and 100 µl of the bacterial suspension was mixed with 100 µl of the test solution, 500 µl sodium phosphate buffer pH 7.4, and 2 ml overlay agar enriched with a histidine-biotine solution. To test the substance in its metabolized form, the buffer was replaced by a 5% S9 metabolization mix (prepared from lyophilized rat liver S9 mixed with nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system – both from Moltox). The resulting mixture was poured onto a minimal glucose agar plate (E&O Laboratories Ltd., Bonnybridge, United Kingdom). Triplicate plates were poured for each test condition. All substances were tested in at least five concentrations as prescribed by OECD test guideline 471 [32]. Before the start of the experiment, a dilution range of the test compound was made. DMSO was used as a solvent for the test compounds. Positive, negative and solvent control plates were prepared in parallel with the test substance plates. As positive controls, 4-nitroquinoline-N-oxide (4NQO; 2 µg/ml; TA98 without S9), sodium azide (20 µg/ml; TA100 without S9) and 2-aminoanthracene (10 µg/ml; TA98 and TA100 with S9) were used. After incubation during 48 hours at 37°C (Binder, Tuttlingen, Germany), the amount of revertant colonies was counted and compared to the amount of revertants present in the solvent control. A compound was considered mutagenic *in vitro* in case the amount of revertants had doubled compared to the solvent control. The effect also needed to be concentration-dependent.

2.5.2 *In vitro* micronucleus assay

The *in vitro* micronucleus test was carried out following the OECD guideline 487, with some modifications [34]. CHO-K1 cells were seeded at a density of 2.0×10^5 cell/ml (with S9) or 1.0×10^5 cell/ml (without S9) and exposed to five different concentrations of the test substance in the absence (24 h) or presence of S9 (4 h) fraction. The test concentrations were selected based on the results obtained in cytotoxicity assays. The phosphate buffer saline (PBS) medium with DMSO was used as negative control; 15 µg/ml methyl methanesulphonate (MMS) (without S9 fraction) and 25 µg/ml benzo(a)pyrene (BaP) (with S9 fraction) as positive controls. After exposure to the test compound, cells were incubated for 21 h with cythochalasin B (Cyt-B) (3 µg/ml) to block cytokinesis and to obtain binucleated cells. Afterwards, cells received a hypotonic treatment with potassium chloride followed by fixation. Next, cells were smeared onto clean microscopic glass slides. The slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and evaluated for the presence of micronucleated binuclear cells using a Zeiss Axiovert 40 microscope with MetaFer4 version 3.13.0 using MNScoreX software for analysis of micronuclei. Two slides were analyzed per test condition and 1100 cells were scored per slide, resulting in at least 2000 evaluated cells per test condition.

After analysis for the presence of micronuclei, cells were stained with acridine orange (AO) (33.3 µg/ml) to evaluate cytotoxicity. Slides were rinsed with Sörensen buffer to eliminate excess of staining solution and for approximately 500 cells per test condition, the number of mono-, bi- and multinuclear cells was determined manually. The level of cytotoxicity was evaluated by calculating the cytokinesis-block proliferations index (CBPI) which is defined as the ratio of:

$$\frac{mononuclears + 2 \times binuclears + 3 \times trinuclears + 4 \times tetra\ (and\ higher) nuclears}{total\ viable\ cells}$$

Results were summarized and analysed with GraphPad Prism version 7.01. A Fisher's exact test was performed in GraphPad to evaluate whether there was a statistically significant difference between a test

condition and the negative control ($p < 0.05$). The chi-square test was used to evaluate whether the test compound induced a dose-dependent effect ($p < 0.05$).

3 RESULTS

An overview of the results obtained in the different steps of the prioritization strategy to identify genotoxic flavourings in e-liquids is given in Figure 1.

3.1 Screening of e-liquid samples using GC-MS

After screening 129 e-liquids, 807 individual substances were identified including nicotine, nicotine-impurities, VOCs impurities, additives (diacetin) and flavouring substances (incl. synthetic, components from essential oils or other herbal extracts). The NIST provided a CAS number for each identified component which was also used to retrieve the SMILES formula in PubChem or ChemSpider [21,22].

3.2 Genotoxicity prediction using *in silico* tools

Out of the 807 substances analyzed, 103 showed a positive prediction outcome for *in vitro* bacterial mutagenicity in at least one of the three (Q)SAR models. Consequently, 87% of the screened substances were concluded to be negative for *in vitro* bacterial mutagenicity in the applied *in silico* models as negative predictions for *in vitro* bacterial mutagenicity were produced by the three models. However, of these negative predictions, 8 were labeled as containing “misclassified features” and 6 “unclassified features” in Derek Nexus. When a query does not fire an alert for mutagenicity *in vitro* in Derek Nexus, the compound is compared to a reference set of Ames test data. In the case a chemical fragment, or “feature”, is unclassified, it is present in the query but not the reference set, whereas a misclassified feature is found in a reference set compound which is positive in the Ames test, but not defined as a structural alert [35]. Hence, there is a possible, but not a guaranteed, relationship between these features and mutagenic activity.

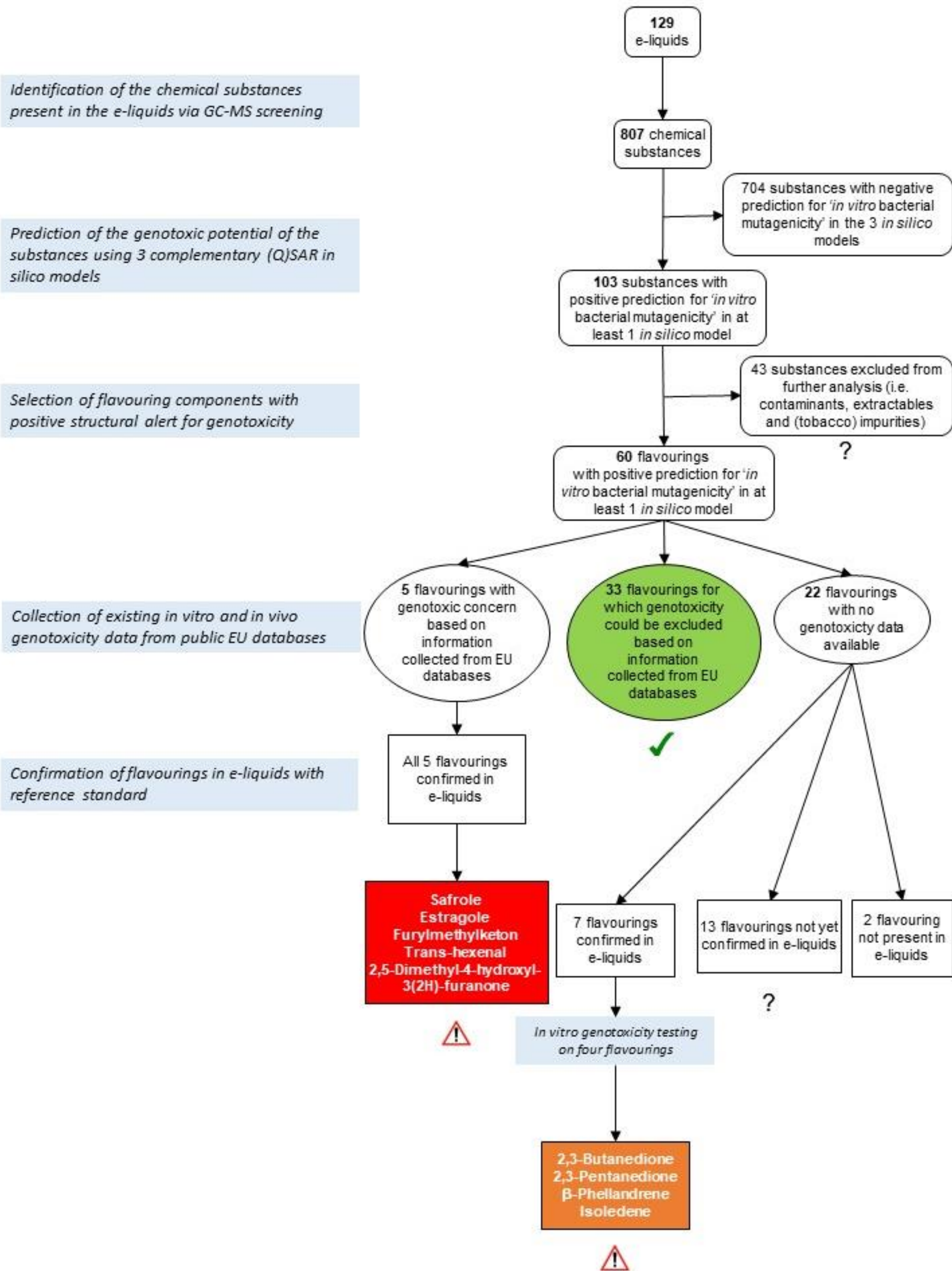


Figure 1 *Summary of the results obtained with prioritization strategy to identity genotoxic flavourings in e-liquids.*

Since the negative predictivity remains high for both, these can be regarded as a negative prediction that are flagged for expert review [35]. In our strategy, we did not include an elaborate expert reviewing process and these were thus accepted to be negative.

Overall, only 5 substances were predicted positive in all three *in silico* tools. Interestingly, most positive predictions for *in vitro* bacterial mutagenicity were obtained with the quantitative prediction model Sarah Nexus^{TM}. The *in silico* tools used are based on a different methodology and different training sets which might explain the significant difference in the number of positive prediction outcomes.

3.3 <u>Genotoxicity data collected from EU databases</u>

As the present study focused only on flavourings, contaminants/extractables and (tobacco) impurities that can also be present in e-liquids were first excluded before starting the data collection. However, manufacturers should be aware of the presence of these types of compounds and regulators need to address them explicitly in future regulatory amendments. In total, 43 compounds belonging to the group of 'contaminants/extractables and (tobacco) impurities' were identified among the 103 substances with a structural alert for mutagenicity in at least one of the three (Q)SAR models. Consequently, 60 out of the 103 substances with a positive prediction outcome for *in vitro* bacterial mutagenicity in at least one of the three (Q)SAR models were flavourings. Probably there are more 'contaminants/extractables and (tobacco) impurities' among the 807 that have been analytically identified. However, selection of the flavourings was only done after applying the *in silico* models as looking into all 807 substances would have been too time-consuming.

Based on the collection of the information from EU databases for the 60 flavourings, 5 compounds with a concern for genotoxicity could be identified, whereas genotoxicity could be excluded for 33 flavourings.

However, for 22 flavourings no genotoxicity data was available according to the consulted literature and thus genotoxic potential could not be excluded (Table 2.).

Flavourings of genotoxic concern

For 1 out of the 60 flavouring substances, a harmonized CLP classification for mutagenicity has been established. Indeed, **safrole** has been classified as a Mutagen category 2 and a Carcinogen category 1B. According to an opinion of the Scientific Committee on Food [36], safrole may not be used as a flavour in food due to these CMR classifications, but it is often found in essential oils. Essential oils containing safrole should not be used at a level such that the total concentration of safrole exceeds 0.01% in consumer products. Examples of essential oils with a high safrole content are Sassafras oil, Ocotea Cymbarum oil and certain qualities of Camphor oils. These recommendations are based on the conclusions of the Scientific Committee on Cosmetology of the EEC on safrole [37].

For another substance, **estragole**, the (*in vivo*) genotoxicity has been generally acknowledged in a previous evaluation by EMA [38]. Estragole is mostly found in tobacco flavours to add a herbal anise aroma, but it can also be part of various natural extracts. A large number of plants and their preparations have been reported to contain estragole, sometimes in very high amounts such as *Foeniculum vulgare Mill.* (both fruit and essential oil) and Pimpinella anisum L. (fruit).

2,5-Dimethyl-4-hydroxy-3(2H)-furanone has previously been evaluated by EFSA and was considered to be genotoxic *in vivo*. It is also called strawberry furanone because it is used to add a strawberry aroma to food. Several *in vitro* and *in vivo* genotoxicity studies indicate that the substance induces mutagenic responses [39]. In-depth investigation shows that the observed positive results are due to the production of reactive oxygen species, potentiated by the presence of metals in the cell medium. The resulting DNA damage is only observed once the cell antioxidant capacity has been exhausted. EFSA stated that this effect is unlikely to occur at the low levels used for flavourings in foods. Yet, available data for this

substance clearly illustrate that a separate risk assessment is needed for exposure to these concentrations through inhalation of e-cigarettes.

For two compounds, **trans-hexenal** and **2-furylmethylketon,** an evaluation was done by EFSA, but no conclusion on the *in vivo* genotoxic potential of the compounds could be made and therefore further data was requested [40,41]. 2-Furylmethylketon is used as a food flavouring and fragrance and can also be found in tobacco. The EFSA concluded that the genotoxic potential of furylmethylketon could not be excluded. Based on the available experimental information the substance may give rise to DNA damage, possibly resulting in chromosomal aberrations rather than gene mutations [40]. Trans-hexenal was found in e-liquids with a green fruit flavor. Also for this compound, EFSA could not exclude a genotoxic concern. Both gene mutations in *Salmonella typhimurium* TA100, and chromosomal aberrations in mammalian cells were observed *in vitro.* However, available experimental data from animals did not show an induction of gene mutations by trans-hexenal [41]. In contrast, the available data were insufficient to assess the clastogenic potential of this compound at the first site of contact and in the liver *in vivo*. Therefore, the genotoxicity concern could not be ruled out by EFSA and additional information was required.

The presence of the 5 substances mentioned with a concern for genotoxicity in the e-liquids was confirmed with reference standards used in GC-MS in full scan mode if the library spectral match quality was < 90. All substances for which the matching score was higher than 90 were considered to be present without additional confirmation.

<u>Flavourings for which genotoxic concern could be excluded</u>

In contrast, for 33 of the flavourings with a positive prediction for *in vitro* bacterial mutagenicity in at least one of the (Q)SAR models, a genotoxic concern could be excluded based on the information collected from EU databases (Table 2). These included substances for which results were negative either in all *in vitro* tests or in the *in vivo* follow-up genotoxicity tests. For 14 of those substances, the genotoxic concern

was excluded based on read-across with genotoxicity data from substances with comparable structures. As these 33 are not considered to be of genotoxic concern, no additional analysis was performed to confirm their presence in the e-liquids.

Flavourings for which more genotoxity data is needed

For the remaining 22 substances (Table 3), no genotoxicity data was available in the consulted literature. As these substances are of potential concern, confirmation of the presence of these substances in the e-liquid was needed if the matching score was < 90. For 2-acetylthiazole, the presence in the e-liquid did not need to be no confirmed as the matching score was > 90 (93.89). Confirmation of the other flavourings required the (commercial) availability of a reference standard for a reasonable price which was not the case for 13 out of the 21 substances. This can be explained by the fact that most of these substances are tobacco or natural extracts. For 6 out of the 8 substances for which a standard was commercially available, their presence in the e-liquid could be confirmed with GC-MS using full scan mode. The other 2 substances were not detected in the e-liquid when using a reference standard-based methodology. The absence of these compounds in e-liquid is probably due to a mismatch with the NIST-library. As these two components were not present, they should not be further explored.

Table 2 Conclusion of the EU evaluations for genetic toxicity of the substances with a positive *in silico* prediction for *in vitro* bacterial mutagenicity. Results are expressed as (+) positive, (-) negative or (±) inconclusive if genotoxicity could not be excluded and additional information is requested. European Medicine Agency (EMA), European Food Safety Authority (EFSA).

Name	CAS	EU evaluation		CONCLUSION
1,3-cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-	116-26-7	EFSA		-
1-hexen-3-one	1629-60-3	EFSA	based on read across data	-
2,4,6-octatriene, 2,6-dimethyl-	673-84-7	EFSA	based on read across data	-
2,4,6-octatriene, 2,6-dimethyl-, (E,Z)-	7216-56-0	EFSA	based on read across data	-
2,5-dimethyl-4-hydroxy-3(2H)-furanone	3658-77-3	EFSA		+
2,6-dimethyl pyrazine	108-50-9	EFSA		-
2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	23696-85-7	EFSA	based on read across data	-
2-furancarboxaldehyde, 5-methyl-	620-02-0	EFSA		-
2-hexenal, (E)-	6728-26-3	EFSA		±
2-phenyl-1,3-dioxan-5-ol	1319-88-6	EFSA	based on read across data	-
2-propenal, 3-(2-methoxyphenyl)-	1504-74-1	EFSA	based on read across data	-
benzaldehyde	100-52-7	EFSA		-
benzaldehyde propylene glycol acetal	2568-25-4	EFSA	based on read across data	-
benzaldehyde, 2-methoxy-	135-02-4	EFSA	based on read across data	-
benzaldehyde, 4-methoxy-	123-11-5	EFSA		-
benzene, 1,1'-[oxybis(methylene)]bis-	103-50-4	EFSA		-
benzene, 1,4-dimethoxy-	150-78-7	EFSA		-
caryophyllene oxide	1139-30-6	EFSA		-
cinnamaldehyde, (E)-	14371-10-9	EFSA		-
cinnamyl cinnamate	122-69-0	EFSA		-
Courmarine	91-64-5	EFSA		-
estragole	140-67-0	EMA		+
ethanone, 1-(2-furanyl)-	1192-62-7	EFSA		±
ethanone, 1-(3-pyridinyl)-	350-03-8	EFSA	based on read across data	-
ethylmaltol	4940-11-8	EFSA		-
furfural	98-01-1	EFSA		-
isomenthone	1196-31-2	EFSA	based on read across data	-
maltol	118-71-8	EFSA		-
methyl salicylate	119-36-8	EFSA		-
phenol, 2-methoxy-	90-05-1	EFSA		-
piperidine	110-89-4	EFSA	based on read across data	-

piperonal	120-57-0	EFSA		-
propylene glycol acetone ketal	1193-11-9	EFSA	based on read across data	-
pyridine, 3-ethyl-	536-78-7	EFSA		-
safrole	94-59-7	Harmonized CLP		+
thiazole, 4-methyl-2-(1-methylethyl)-	15679-13-7	EFSA	based on read across data	-
α -phellandrene	99-83-2	EFSA	based on read across data	-
γ-terpinene	99-85-4	EFSA		-

Table 3 Overview of the substances with a positive alert for *in vitro* bacterial mutagenicity and for which no information on genetic toxicity was available from EU authorities.

Name	CAS	Commercially available	Confirmed in e-liquid	CATEGORY
Potentially high concern				
β-phellandrene	555-10-2	YES	YES	flavour and fragrance use
isoledene	95910-36-4	YES	YES	natural extract
2,3-butanedione	431-03-8	YES	YES	flavour and fragrance use
2,3-pentanedione	600-14-6	YES	YES	flavour and fragrance use
2-ethyl-4-methylthiazole	15679-12-6	YES	YES	flavour and fragrance use
2-acetylthiazole	24295-03-2	NA	> 90	flavour and fragrance use
3-hexen-2-one, 5-methyl-	5166-53-0	YES	YES	flavour and fragrance use
Needs confirmation				
naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1s-cis)-	483-77-2	YES	NOT TESTED	natural extract
α-calacorene	21391-99-1	YES	NOT TESTED	natural extract
1,3,5-cycloheptatriene, 1-methoxy-	1728-32-1	YES	NOT TESTED	natural extract
α -methyl- α -[4-methyl-3-pentenyl]oxiranemethanol	1000132-13-0	NO	NOT TESTED	natural extract
1,3-dioxane, 2-methyl-	626-68-6	NO	NOT TESTED	natural extract
1H-azepin-1-amine, N-ethylidenehexahydro-	75268-01-8	NO	NOT TESTED	natural extract
bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	28634-89-1	NO	NOT TESTED	natural extract
cyclohexene, 4-methylene-1-(1-methylethyl)-	99-84-3	NO	NOT TESTED	natural extract
naphthalene, 1,2-dihydro-2,5,8-trimethyl-	30316-23-5	NO	NOT TESTED	natural extract
cyclohex-3-enecarboxaldehyde, 2,4,6-trimethyl-, oxime	1000294-92-2	NO	NOT TESTED	flavour
anisaldehyde propylene glycol acetal	6414-32-0	YES	NOT TESTED	flavour and fragrance use
alpha,alpha,4-trimethylbenzyl carbanilate	7366-54-3	NO	NOT TESTED	natural extract
(+-)-4-amino-4,5-dihydro-2(3H)-furanone	16504-58-8	NO	NOT TESTED	
Not detected in e-liquid samples				
p-methylbenzyl acetate	2216-45-7	YES	NO	flavour and fragrance use
2H-pyran-2-one, 4-hydroxy-3,6-dimethyl-	5192-62-1	YES	NO	flavour use

3.4 *In vitro* genotoxicity testing

Next, the Ames test and the *in vitro* micronucleus test were performed for a selection of 4 out of the 7 confirmed flavourings with a positive prediction outcome in at least one of the (Q)SAR models and for which genotoxicity data is lacking. These substances included β-phellandrene, isoledene, 2,3-pentadione and 2,3-butanedione. An overview of the *in vitro* genotoxicity test results is given in Table 4.

Table 4. Overview of the results in the Ames test (TA98 and TA100 strains) and the *in vitro* micronucleus test in presence and in absence of an S9 metabolisation system for a selection of substances with a positive prediction outcome in at least one of the (Q)SAR models and for which genotoxicity data was not previously published. Results are indicated as (+) positive or (-) negative

	Ames test				***In vitro* micronucleus assay**	
	TA98		**TA100**			
	-S9	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
β-phellandrene	-	-	-	-	-	+*
isoledene	+	+	+	+	-	+
2,3-pentanedione	+	+	-	-	+	+
2,3-butanedione	+*	+	+*	+	-	+

** Only at the highest concentration tested*

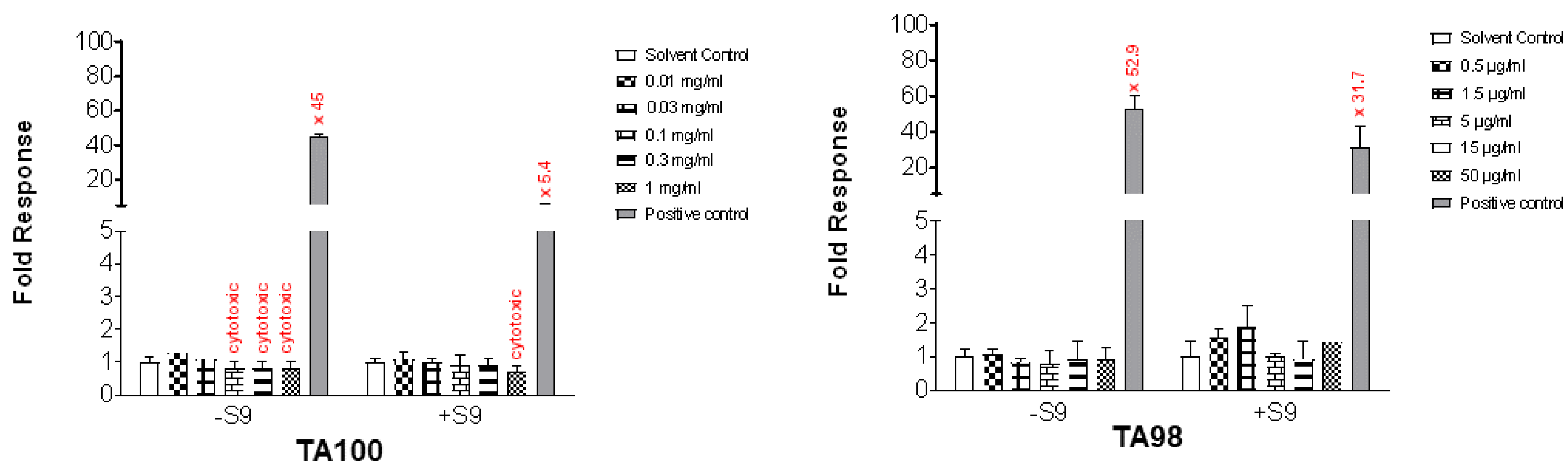
3.4.1 Ames test

β-Phellandrene was first tested in *Salmonella typhimurium* TA100 in concentrations up to 1mg/ml both in the absence and presence of S9 metabolic fraction. No increase in the number of revertants compared to the solvent control was observed for any of the test conditions. However, cytotoxicity was present at concentrations above 0.03 mg/ml. For this reason, lower concentrations (0.5 to 50 µg/ml) were tested in the *Salmonella typhimurium* TA98 strain. The background layer was intact for all the concentrations tested and no mutagenic effect could be observed in the absence nor in the presence of S9 metabolic fraction. Consequently, β-phellandrene is considered not mutagenic in TA98 and TA100 with and without S9 metabolic activation (Figure 2A). A recent study reported that β-phellandrene induced a significant increase in the number of revertants compared to the solvent control in both strain TA98 and TA100 in the absence and presence of S9 metabolic fractions [42]. However, important study details (e.g. purity of

the compound, solvent used,…) are lacking to allow adequate comparison of the outcome of this study with our results.

2,3-Pentanedione did not induce a mutagenic effect in TA100 with or without S9 metabolic fraction (Figure 2A). In TA98, a concentration-dependent increase in the number of revertant colonies was observed which was more than double compared to the controls, starting from 5 mg/ml in the absence of S9 (Figure 2A). A concentration-dependent effect was also present with a metabolic activation system, although a doubling was only observed at 50 mg/ml. A uniform background layer was present at all tested concentrations indicating that the compound was not toxic to the bacteria. Based on these results, 2,3-pentanedione is considered to have a mutagenic capacity *in vitro*. Previous Ames tests did not, however, indicate mutagenicity for 2,3-pentanedione. For example, Aeschbacher et al. reported no mutagenic effects in the TA100 strain and in TA98 at concentrations ranging from 0.009 mg/ml to 900 mg/ml [43]. Florin et al. only tested 3 µmol/plate in TA100 (with and without S9) which also did not induce a mutagenic effect [44].

In TA100, **isoledene** induced a concentration-dependent increase in the number of revertant colonies which was more than double the number of revertants present in the solvent control at 15 mg/ml (Figure 2B), both with and without metabolic activation. At the highest concentration (i.e. 50 mg/ml), cytotoxicity was observed. In TA98, a duplication of the number of colonies was observed at 50 mg/ml with a concentration-dependent increase in the range of 5 mg/ml – 50 mg/ml. Based on these results, isoledene is considered to be mutagenic *in vitro*.



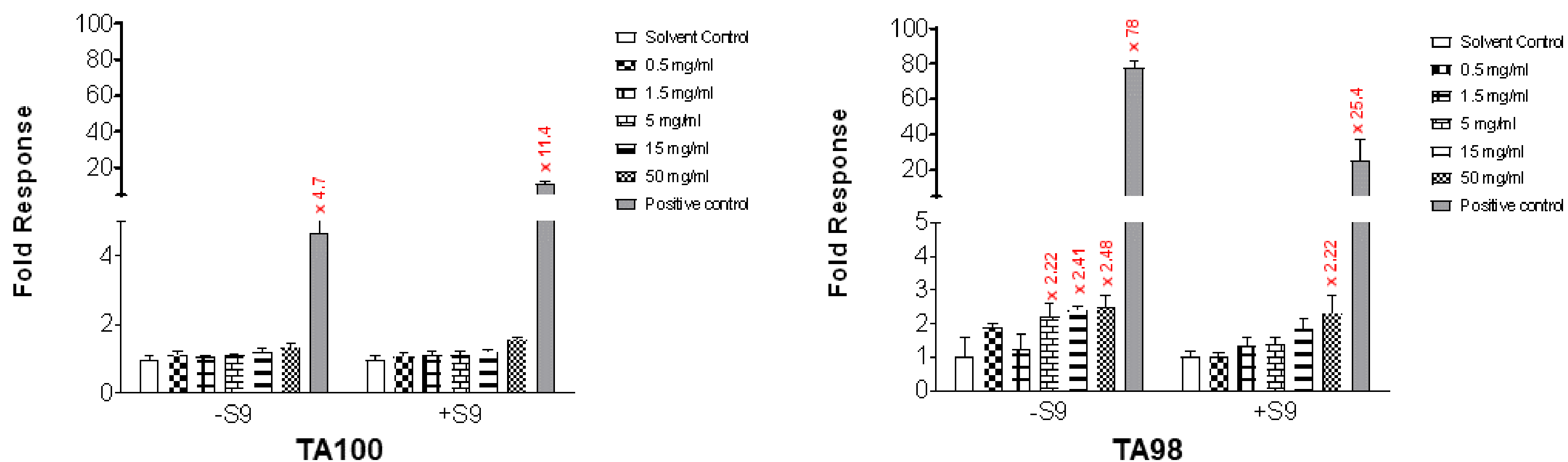
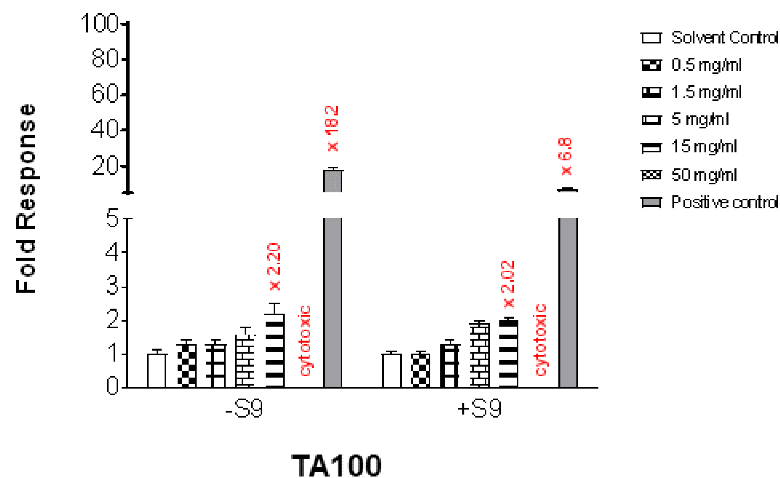
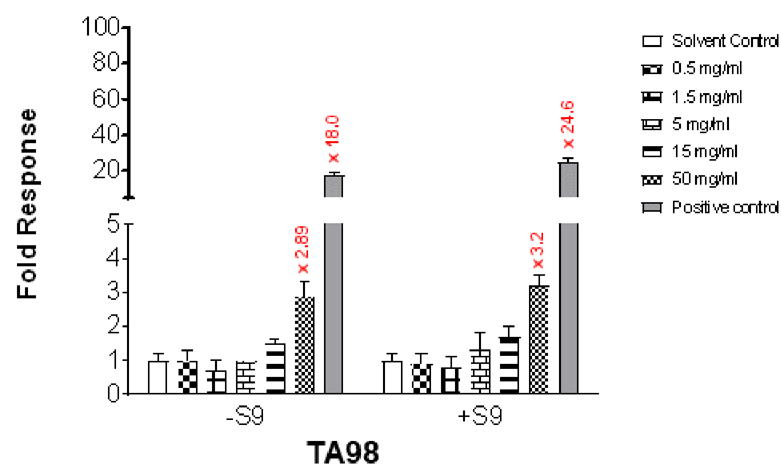


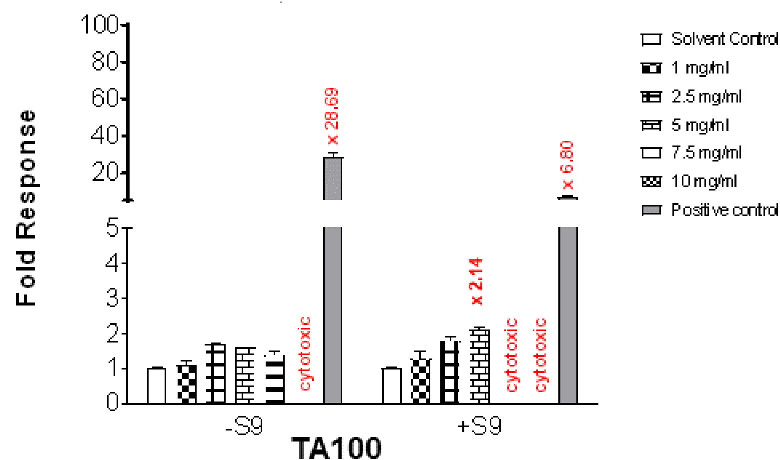
*Figure 2A Ames test results, with and without S9, of investigated substances with positive prediction in silico. Values are expressed as the mean ± SD of revertant colonies counted in triplicate plates for each tested strain (TA100 and TA98) *Statistically significant compared to solvent control (p<0.05).*

isoledene





2,3-butanedione



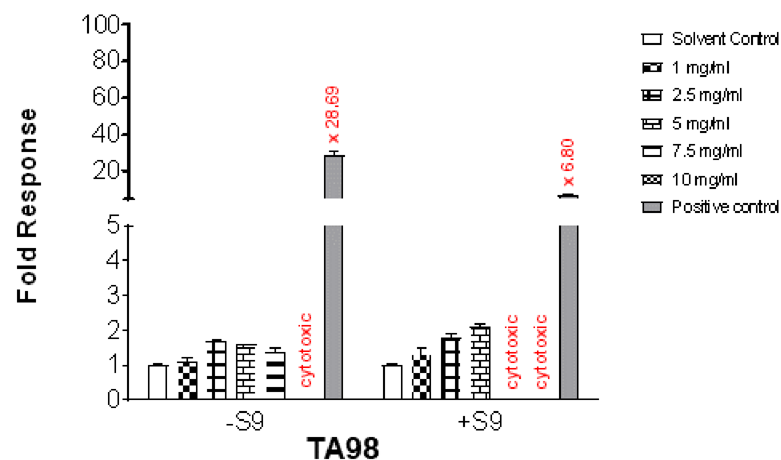


*Figure 2B Ames test results, with and without S9, of investigated substances with positive prediction in silico. Values are expressed as the mean ± SD of revertant colonies counted in triplicate plates for each tested strain (TA100 and TA98) *Statistically significant compared to solvent control ($p<0.05$).*

2,3-Butanedione was tested up to 10 mg/ml in the TA100 strain, but cytotoxicity was only observed at the two highest concentrations in the absence and presence of S9 metabolic fraction. At the lower concentrations, there was a clear concentration-dependent increase in the number of revertant colonies compared to the solvent control, both with and without S9 metabolic system. The number of revertant colonies was twice the number of the solvent control at 5 mg/ml with the S9 metabolic system. Therefore, 2,3-butanedione is considered to be mutagenic *in vitro* in TA100, both in the presence and absence of metabolic activation (Figure 2B). In TA98, 2,3-butanedione was slightly positive with and without metabolic activation at 2.5 and 5 mg/ml. At higher concentrations, significant cytotoxicity was observed, which was reflected by a decrease in the number of revertants. Aeschbacher et al. also reported a positive effect for 2,3-butanedione in TA102 (0.17 µg – 17.2 mg/plate), although in their study the compound was negative in both the TA98 and TA100 strains [43].

3.4.2 *In vitro* micronucleus test

In the *in vitro* micronucleus test without S9, no increase in the number of micronuclei in binucleated cells was observed with **β-phellandrene** in concentrations up to 0.68 mg/ml (Figure 3A). In the presence of S9, a slight but statistically significant increase in the number of micronuclei was present, but only at the highest concentration tested, i.e. 2.72 mg/ml. Limited cytotoxicity of 5% was observed under this condition.

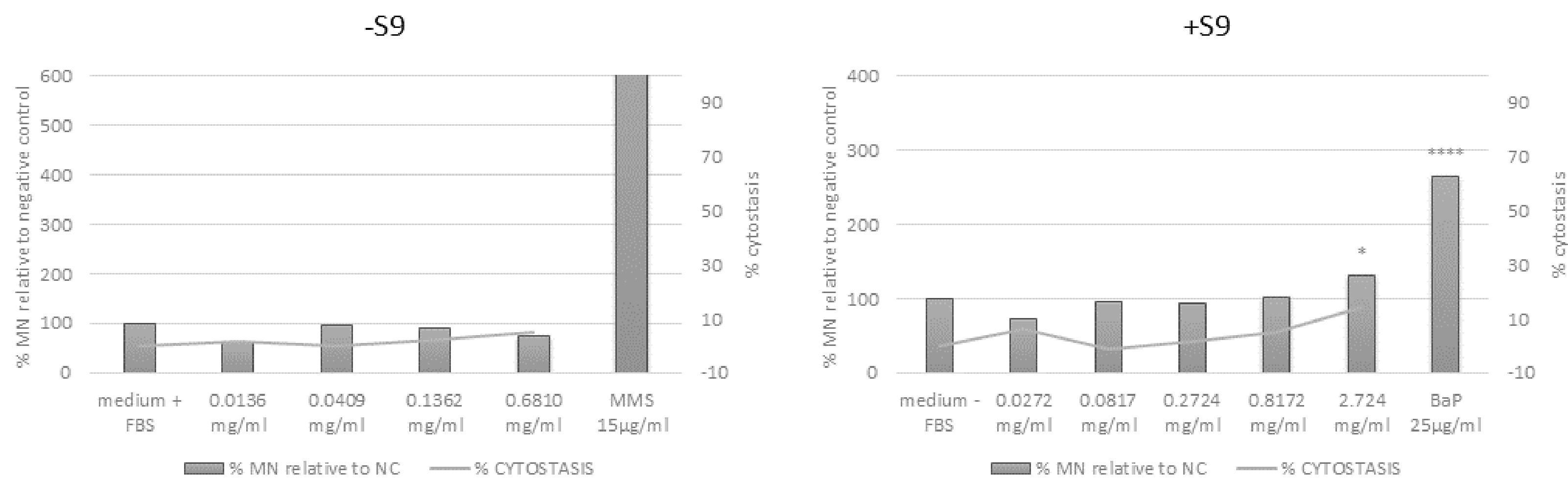
In the first experiment in the presence of S9, **isoledene** was tested up to a concentration of 1000 µg/ml. However, except for 250 µg/ml, cytotoxicity was observed in all conditions. At 250 µg/ml, an increase in the number of micronuclei in binucleated cells was present. Therefore, the test was repeated with lower concentrations of isoledene. An increased induction of micronuclei could also be seen at a concentration of 20 µg/ml (Figure 3A). In the absence of the metabolic activation system, no effect on the number of micronuclei was observed, whereas cytotoxicity was present at a concentration of 100 µg/ml. The results

of the present study indicate that isoledene causes an increase in the number of micronuclei in the presence of S9 metabolic fraction.

2,3-Pentanedione was evaluated in the *in vitro* micronucleus test at concentrations up to 50 µg/ml (without S9) or 200 µg/ml (with S9) (Figure 3B). A dose-dependent effect in the number of micronuclei was observed starting at 5 µg/ml without S9 and at 50 µg/ml with S9. However, in the presence of S9, the highest concentration tested induced no increase in the number of micronuclei because of cytotoxicity. In the absence of S9, cytotoxicity was observed at 50 µg/ml. The results of the present study indicate that 2,3-pentanedione induces chromosome damage in CHO-K1 cells.

In the first experiment in the absence of S9 metabolic fraction, **2,3-butanedione** was tested at concentrations between 10 µg/ml and 100 µg/ml. However, cytotoxicity was already observed at 25 µg/ml. At the lower concentrations, 2,3-butanedione did not induce an increase in micronucleus formation. The test was repeated with concentrations ranging from 5 µg/ml to 20 µg/ml, but there was no increase in the number of micronuclei in binucleated cells in the absence of S9 (Figure 3B). In the presence of S9, an increase in the number of micronuclei was observed at the highest concentration tested, i.e. 50 µg/ml. Above this concentration, cytotoxicity was observed.

β-phellandrene



isoledene

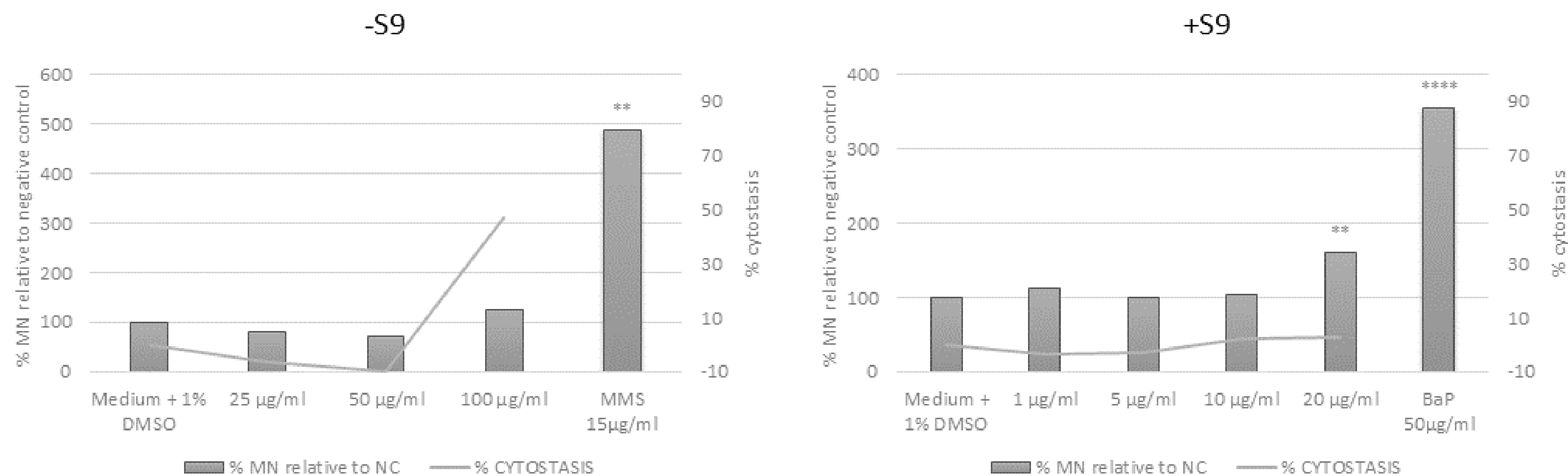
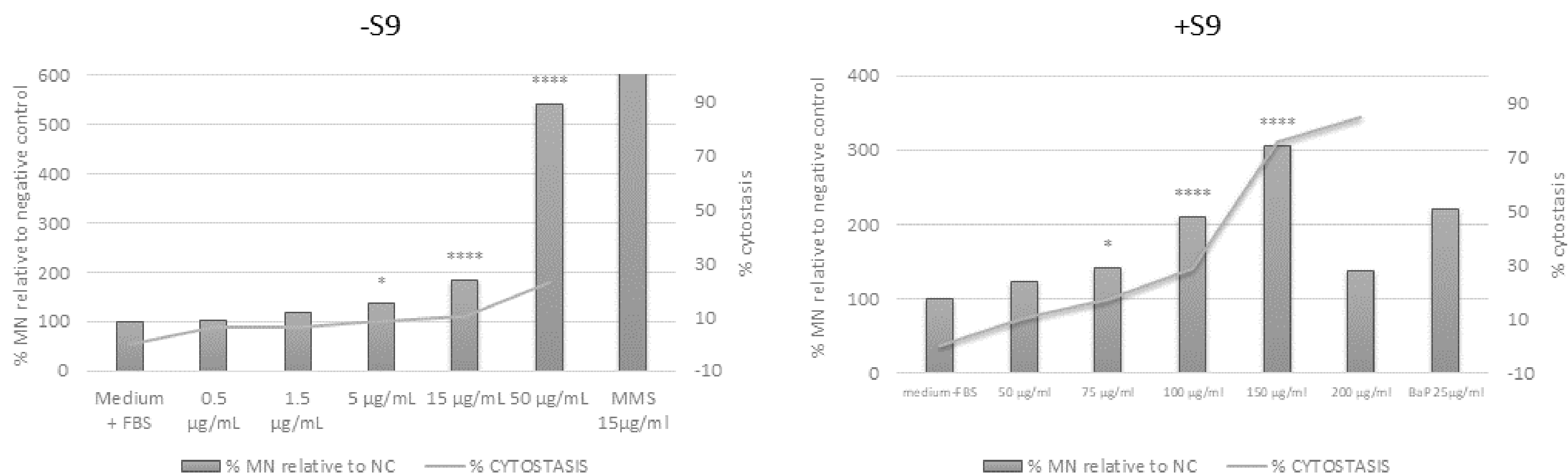


*Figure 3A In vitro micronucleus test results, with and without S9, of investigated substances with positive prediction in silico. Results are expressed as change in number MN/2000 binucleated cells (% of NC) and cytotoxicity. *Statistically significant compared to solvent control ($p<0.05$).*

2,3-pentanedione



2,3-butanedione

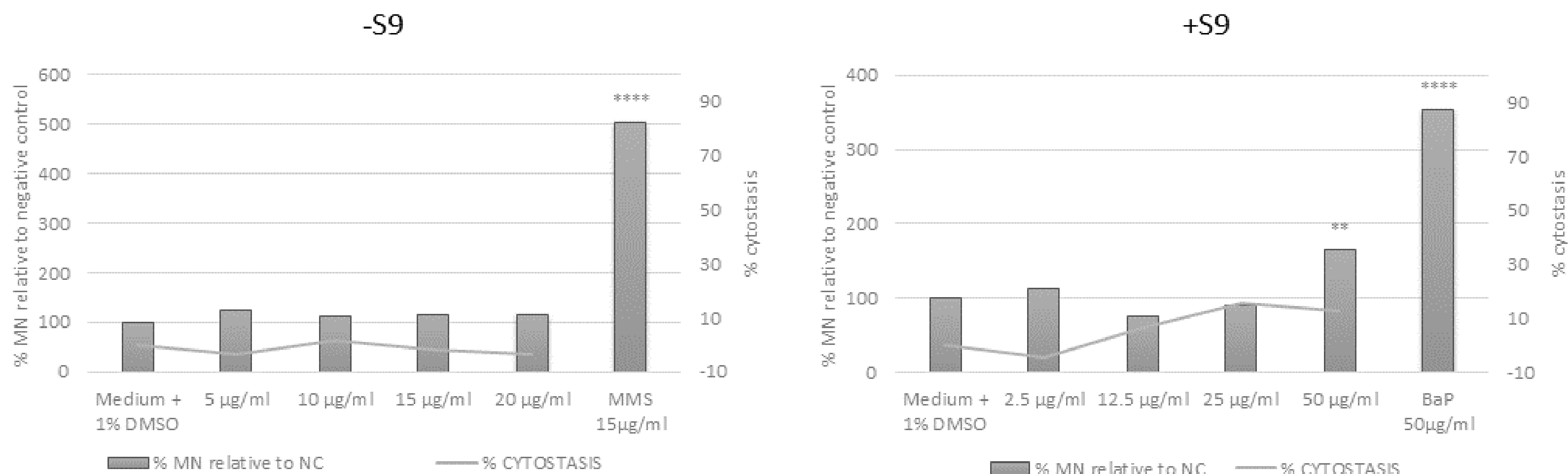


*Figure 3B In vitro micronucleus test results, with and without S9, of investigated substances with positive prediction in silico. Results are expressed as change in number MN/2000 binucleated cells (% of NC) and cytotoxicity. *Statistically significant compared to solvent control (p<0.05).*

4 DISCUSSION

In this study, 129 e-liquids containing a wide variety of flavours were analytically screened by GC-MS. As expected, many different chemicals were detected in the e-liquids, including flavourings as well as 'contaminants/extractables and (tobacco) impurities'. Besides the typical synthetic flavourings, natural extracts or even essential oils are also added as flavouring to e-liquids. These may be of concern for human health as their composition is often not well characterized. Moreover, inhalation of both essential oils and natural extracts has generally been associated with a number of adverse human health effects [45]. For essential oils, sensitization is one of the major risks after inhalatory exposure [46]. Yet, other adverse health effects have been correlated with exposure to certain substances present in essential oils and natural extracts such as are neurotoxicity [47] (convulsions, head aches), teratogenicity and abortifacient properties [48]. Next to essential oils, some perfumes and fragrances, also used as flavourings in e-liquids, are recognized as respiratory irritants that can trigger asthma [49].

In total, more than 800 different volatile chemicals were identified in the 129 e-liquids. Due to this large number, *in silico* models were used to prioritize those substances that might be of genotoxic concern, with a focus on *in vitro* bacterial mutagenicity. In general, (Q)SAR predictions for this endpoint are accurate [50], although false negatives are still possible. However, this does not imply that the remaining substances may not induce other adverse health effects, such as pulmonary toxicity. Although in theory such toxicological effects could also be predicted *in silico*, most of the (Q)SAR models are far less developed for these endpoints [51]. It was found that 13% of the substances gave a positive prediction for mutagenicity in at least one of the applied (Q)SAR models. In the current strategy three complementary (Q)SAR models were applied. Other (Q)SAR models for mutagenicity are available. However, it is generally recommended to use a combination of rule-based SAR and statistics-based QSAR tools to assess Ames mutagenicity. For example, the ICH-M7 guideline for the assessment and control of

mutagenic impurities in pharmaceuticals, specifically requires to combine a SAR with a QSAR model as each type of model has its (dis)advantages as reviewed by Honma et al. [20].

Among the substances with a positive prediction for *in vitro* bacterial mutagenicity, there were contaminants/extractables and (tobacco) impurities. As the present study focused on flavourings, these types of substances were excluded for the next prioritization step, leaving 60 flavouring substances. For those 60 substances, literature was consulted for existing safety evaluations. Harmonized CLP classifications and EU authorities' evaluations were given higher weight than the self-classifications reported in the ECHA database by industry. Based on the information present in the existing evaluations, 3 flavourings could be identified as known and/or potential genotoxicants and for 2 flavourings the genotoxicity could not be excluded. These 5 flavourings were found in 10 out of the 129 analyzed e-liquid samples. According to the TPD, CMR substances are not allowed in e-liquids and thus these e-liquids are regarded as non-compliant with the TPD. In Table 5, more details are provided on these samples.

For 33 substances, the genotoxic concern could be excluded based on available data. Importantly, for the 22 remaining substances with a positive *in silico* prediction for *in vitro* bacterial mutagenicity, no genotoxicity data was available in the consulted EU databases. In order to know whether the respective e-liquids are compliant with the TPD, the genotoxic profile of these substances had to be evaluated further. Therefore, two *in vitro* genotoxicity tests were performed for a selection of four of the commercially available substances. It should also be noted that the Ames test was only performed in 2 bacterial tester strains. In order to conclude that a compound does not induce gene mutations in bacteria, it should be negative in all 5 strains recommended in OECD TG471.

Table 5. Overview of the e-liquids containing flavourings of genotoxic concern with associated flavour description.

Name	Number of samples	Flavour e-liquid	Flavour description	Brand
safrole	1	rum cola	sweet warm spicy woody floral sassafras anise	Sedansa Rum cola
estragole	4	tobacco, and mint	Sweet-herbaceous Anise-Fennel type	Whatafog Strong Mint/Ice ice baby Sedansa David Sedansa Deluxe tobacco E-liquid Deluxe tobacco
furylmethylketon	2	tobacco, wood, hints of spices and honey	sweet musty caramel brown bread crust, balsamic Used in Chocolate, Coffee, Roast Nut, Bread, Rum, Whiskey, Tamarind, Tea and Tobacco flavours, as a trace background note.	Mistervape Maxx blend Sedansa Chocolate
trans-hexenal	2	apple-cinnamon	fresh, green, and natural topnote in fruity floral types.	One hit wonder - Muffin Man Twelve Monkeys - Kanzi
2,5-dimethyl-4-hydroxy-3(2H)-furanone	1	strawberry	sweet cotton candy caramel strawberry sugar	Savourea Ice strawberry

β-Phellandrene was negative in the Ames test (TA98 and TA100) and slightly positive in the *in vitro* micronucleus test, whereas **isoledene**, **2,3-pentanedione and 2,3-butanedione** were positive in both tests. The latter two are found in several e-liquids and are controversial as they have shown to be responsible for developing bronchiolitis obliterans in chronic inhalation studies [7].

For 3 out of these 4 substances, the *in vitro* genotoxicity testing thus confirmed the genotoxic concern raised by the *in silico* predictions. For these compounds, additional tests are needed to assess whether the genotoxic effect will also occur *in vivo*. There were also indications in our *in vitro* experiments that β-phellandrene might be genotoxic. Therefore, further testing is required for this compound before a final conclusion on its mutagenic potential can be drawn. The other substances with a positive prediction for bacterial mutagenicity and for which no genotoxicity data were found might also have genotoxic

properties and thus represent a problem. This group of substances represent mainly substances from natural herbal and tobacco extracts. An alternative approach to evaluate the genotoxicity of these substances could be to test the extract as such or to test the e-liquid containing the substance instead of the pure compound. However, testing of extracts or e-liquids also poses different challenges such as their varying composition and possible matrix effects.

It is important to note that in this study, only the e-liquids were analytically screened and not the aerosols produced by heating of the e-liquid. Humans are not directly exposed to the e-liquid as such (except via unintentional oral or dermal contact), and consequently, from a human health point perspective, testing of aerosol emissions would provide more accurate information on the e-cigarette components to which humans are actually exposed. However, the aerosol emissions may also contain other chemicals (e.g. leaching heavy metals) and decomposition products that are not present in the e-liquid itself [9,10]. Vapourized e-liquids may therefore be more or less harmful than their liquid form. Yet, as shown in the present study in the context of priority setting and establishing a pragmatic way to check compliance with the TPD regulations, analysis of the e-liquids instead of the aerosol emissions might be more appropriate.

Some argue that by using food grade flavourings in e-cigarettes, the risks are minimized [13]. However, this is not necessarily true as the safety assessment of food flavourings is based on concentrations to which the consumer is orally exposed and these may significantly differ from the concentrations to which consumers are exposed via inhalation. The large surface area in the lungs and the absence of an epithelial barrier comparable to the gastrointestinal mucosa or the stratum corneum barrier function of the skin usually results in a higher percentage of absorption after inhalatory exposure and consequently, a higher internal dose. Also, the kinetic processes for a compound after oral exposure are different compared to those after inhalation. Hence, it is important to execute a separate risk assessment for inhalatory exposure

to flavourings present in e-liquids based on their concentration in the aerosol emissions and their toxicity both at the first site of contact and after systemic uptake via the lungs [52].

Although there is a legislative framework for e-cigarettes, monitoring the compliance of e-liquids with the TPD is very difficult. One of the basic stipulations is the prohibition of CMR substances in e-liquids. However, as illustrated in this study, some e-liquids do contain (potentially) genotoxic compounds. Secondly, manufacturers are legally obliged to list all ingredients, their concentrations and their toxicological data in the product notification to the authorities. This study illustrates that in practice, substances are used as flavourings in e-liquids without knowledge of their genotoxic potential. Hence, these flavourings are either present in concentrations below 0.1% and thus the manufacturer is not required to include that type of toxicological data in the notification. Or, alternatively, natural extract mixtures are used for which it is difficult to obtain toxicological information by the e-liquid manufacturer.

Overall, more safety measures are needed with respect to the use of flavourings in e-liquids. Some manufacturers possess the know-how to chemically screen and identify large amounts of ingredients or otherwise have the resources to demand the full chemical and toxicological characterization of the flavourings from the supplier. Authorities should monitor all notified e-liquid ingredients periodically, not only the registered ingredients in the notification dossier, but also unknown substances from extracts should be analytically screened and identified. Collaboration with other fields such as the food and flavor industry, the perfume and fragrance companies and herbal medicine producers should be promoted. Finally, the development of a list with flavourings that are allowed/permitted to be used in e-liquids is highly recommended.

5 CONCLUSION

To assure a minimal safety of flavourings present in e-cigarettes, the TPD states that the used ingredients may not have CMR properties. In this study, the 60 flavouring substances identified in 129 e-liquids available on the Belgian market were assessed for their genotoxic potential. Following a prioritization strategy based on *in silico* prediction tools and EU database consultation, we identified 5 flavouring substances of genotoxic concern (i.e. estragole, safrole, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone, furylmethylketon and trans-hexenal). On the other hand, a genotoxic concern for 33 of the 60 flavouring compounds flagged with a genotoxic alert by the *in silico* models could be excluded. Yet, for the 22 other flavouring substances, no *in vitro* nor *in vivo* data was available for the genotoxic endpoint. For 4 of the latter substances i.e. 2,3-butanedione, 2,3-pentanedione, isoledene and β-phellandrene, the performed *in vitro* tests indicate mutagenicity and/or the induction of chromosomal damage.

Overall, these results clearly raise concern regarding e-cigarette use and argue for more research to assess the safety of flavouring ingredients for which genotoxicity data is currently lacking. Meanwhile, from the precautionary principle perspective, these compounds should be restricted until more information becomes available. A list of restricted ingredients (chemical substances, but also natural extracts and essential oils) similar to e.g. Cosmetics regulations is necessary to assure the safety of the e-cigarette as an alternative and harm-reduction opportunity for tobacco smokers.

6 ACKNOWLEDGEMENTS

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