Scientific Committee on Health, Environmental and Emerging Risks

SCHEER

Preliminary Opinion on

Additives used in tobacco products

(Opinion 2)

Tobacco Additives II

The SCHEER approved this Opinion for public consultation at the 2nd Plenary on 6 July 2016

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http://ec.europa.eu/health/scientific_committees/emerging/members_wg/index_en.htm

ABSTRACT

The Commission has established a priority list of 15 additives contained in cigarettes and roll-your-own tobacco subject to enhanced reporting obligations. The EU Tobacco Product Directive (TPD) prescribes that Member States shall require manufacturers and importers of tobacco products to carry out comprehensive studies on these additives. The SCHEER was asked to provide guidance on the type and criteria for these comprehensive studies, and the most suitable methodologies to be used. In answer to this request, the current Opinion will guide comprehensive studies for the first list of 15priority additives, and for additives on future updated lists; it also provides a reporting template. As tobacco additives have no benefits for health or otherwise, but rather promote use and addiction to an extremely toxic product, a risk-benefit analysis is not the appropriate paradigm for assessing the additive. Here, the precautionary principle should come into full force.

In the first part, SCHEER proposes a step-wise strategy, as the most pragmatic and efficient way to proceed in the assessment of the toxic and addictive effects as well as characterising flavour properties, as contributing to attractive effects of tobacco additives. The proposed strategy ensures that testing is minimised. In step 1, an evaluation of the literature available on toxicity, addictiveness and characterising flavour (contributing to attractiveness) for the additive needs to be carried out (step 1). In step 2, this evaluation is extended to the additive's pyrolysis products; if no data are available on the identity of the pyrolysis products, they need to be generated using relevant test conditions. Here, it is important to note that no validated methods exist for the determination of pyrolysis products from tobacco additives, but some indications are given in the Opinion.

In case data recieved in Step 1 and 2 are not sufficient or robust enough to make the evaluation possible, non-testing methods such as quantitative structure–activity relationship (QSAR) and read across are proposed, followed by *in vitro* approaches addressing the different endpoints to be considered, all of which could be done inStep 3. Regarding types of effects, unless the previous step highlighted some concern for a specific end-point, toxicity should be assessed first, as accepted methods and evaluation frameworks are available, followed by assessing whether a product contains a characterising flavour. Next, addictiveness should be assessed, an effect for which no validated tests are available, although mechanisms underlying addictiveness are known. The issue related to interaction of the additive with other additives/ingredients is also considered.

In addition to proposing specific steps and tests to be considered by industry, some general criteria were also identified. Most importantly, the test outcomes should be relevant for tobacco smoking. This implies that they should be related to actual human exposure to tobacco smoke and to tobacco-induced diseases. Furthermore, comparative toxicity testing strategies, where differences in the effect of the tobacco product with and without the additive are evaluated, are not considered suitable with the currently available methodology. These studies lack discriminative power, and their results cannot be generalised to all products and brands, having a different composition with respect to tobacco type, blend and additives. Comparative studies are also not endorsed to study the effect of additives on addictiveness and inhalation facilitation, for the same reasons. Instead, the effects of the pure additive, and its pyrolysis products, must be considered. For ethical reasons, animal studies are not endorsed to assess the safety of a tobacco additive. Therefore, as a principle, only *in silico* and *in vitro* studies should be

considered, following the EU policy to ban animal studies for chemicals to be used in voluntary products. Human studies are discouraged. These may be used in case of flavour assessment, but only if the study subjects are not exposed to the harmful smoke emissions of tobacco products.

The major data gaps already identified in Tobacco Opinion 1 for the 15 additives included in the EU Commission priority list have been analysed. Based on this analysis, the activities to be performed upfront have been described. In general, important data gaps for the 15 priority additives are information on addictiveness and attractiveness, as well as on the identity of the pyrolysis products.

Keywords: tobacco, additives, combustion products, cigarettes, roll-your-own, smoking, toxicity, addictiveness, attractiveness, characterising flavour, facilitated inhalation.

Opinion to be cited as:

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1 MANDATE

1.1 Background

The new Tobacco Products Directive 2014/40/EU strengthens the rules regarding the reporting and composition of tobacco products. In addition to tightening the obligations of manufacturers to report on ingredients[1] contained in tobacco products, the Directive regulates permissible additives (or levels thereof) in order to improve the functioning of the internal market whilst guaranteeing a high level of public health.

A) Article 7 of Directive 2014/40/EU foresees in particular the prohibition of the following:

1) tobacco products with a characterising flavour (Art 7(1))
2) tobacco products containing the following additives[2] (Art 7(6)):
   a) vitamins or other additives that create the impression that a tobacco product has a health benefit or presents reduced health risks;
   b) caffeine or taurine or other additives and stimulant compounds that are associated with energy and vitality;
   c) additives having colouring properties for emissions;
   d) for tobacco products for smoking, additives that facilitate inhalation or nicotine uptake; and
   e) additives that have CMR[3] properties in unburnt form.
3) tobacco products containing flavourings in any of their components such as filters, papers, packages, capsules or any technical features allowing modification of the smell or taste of the tobacco products concerned or their smoke intensity. Filters, papers and capsules shall not contain tobacco or nicotine. (Art 7(7))
4) tobacco products containing additives in quantities that increase the toxic or addictive effect, or the CMR properties of a tobacco product at the stage of consumption to a significant or measureable degree. (Art 7(9))

The provisions outlined above shall apply in the first stage to cigarettes and roll-your-own tobacco. The exemption for other product categories may be removed under certain conditions.

B) Moreover, in line with Article 6 the Commission has to develop and update a **priority list of at least 15 additives** contained in cigarettes and roll your own tobacco by May 2016. This list shall contain additives

1) for which initial indications, research, or regulation in other jurisdictions exist suggesting that they have one of the following properties:
   a) contributes to the toxicity or addictiveness of the products concerned / increases the toxicity or addictiveness of any of the products concerned to a significant or measurable degree;
   b) results in a characterising flavour[4];

[1] 'ingredient' means tobacco, an additive, as well as any substance or element present in a finished tobacco product or related products, including paper, filter, ink, capsules and adhesives (TPD 2014/40/EU)

[2] 'additive' means a substance, other than tobacco, that is added to a tobacco product, a unit packet or to any outside packaging (TPD 2014/40/EU)

[3] CMR - carcinogenic, mutagenic or toxic for reproduction

c) facilitates inhalation or nicotine uptake; or
d) leads to the formation of substances that have CMR properties / increases the CMR properties in any of the products concerned to a significant or measurable degree; and

2) which are amongst the most commonly used additives by weight or number according to the reporting of ingredients.

For these priority additives, enhanced reporting obligations will apply in the form of comprehensive studies which shall examine for each additive whether it has any of the properties 1 a) to d) specified above. Those studies shall take into account the intended use of the products concerned and examine in particular the emissions resulting from the combustion process involving the additive concerned. The studies shall also examine the interaction of that additive with other ingredients contained in the products concerned. The results of these studies shall assist Member States and the Commission in their enforcement efforts regarding Art. 7.

The SCENIHR published a scientific Opinion on the attractiveness and addictiveness of additives in 2010[5]. In light of the time that has passed since then and the need to address the current regulatory requirements, the SCENIHR has been asked to address the questions outlined in the Terms of Reference below.

[4] 'characterising flavour' means a clearly noticeable smell or taste other than one of tobacco, resulting from an additive or a combination of additives, including, but not limited to, fruit, spice, herbs, alcohol, candy, menthol or vanilla, which is noticeable before or during the consumption of the tobacco product (TPD 2014/40/EU)

[5] http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_031.pdf

1.2 Terms of reference

The main purpose of the requested scientific Opinion is to assist the Commission in identifying the additives that should be put on the priority list. The scientific Opinion can, however, also provide useful input for Member States and the Commission in their broader regulatory/enforcement activities (e.g. setting thresholds/banning of additives), in particular in areas where the knowledge base may currently still be limited. In particular, the Committee is asked the following:

Opinion 1:

1. Based on scientific evidence (including a review of relevant scientific data) and other relevant information currently available (initial indications, regulation in other jurisdictions), the Committee is asked to identify - for each category separately - those additives that fall/are suspected to fall within the scope of the following categories:

a. Contributing to the toxicity or addictiveness of the products concerned / increasing the toxicity or addictiveness of any of the products concerned to a significant or measurable degree;
b. Resulting in a characterising flavour;
c. Facilitating inhalation or nicotine uptake;
d. Leading to the formation of substances that have CMR properties / increasing the CMR properties in any of the products concerned (cigarettes/roll-your-own) to a significant or measurable degree;[6]

The assessment should include for each of the additives identified a comprehensive description of the type of information supporting its identification as well as a description and quantification of the strength of the observed characteristic and the strength of the available evidence supporting this finding[7]. If the Committee identifies more than 20 additives for a category, the Committee is entitled to prioritise in the light of the criteria set out in this section. In this case, the description is limited to the top 20 additives per category, whilst the other additives can be listed without description.

The Committee is asked to also consider in its assessment the interaction with other ingredients contained in the products concerned and the emissions resulting from the combustion process involving the additive concerned as well as the intended use of the products. Relevant knowledge gaps should be identified.

As far as relevant information is available, the Scientific Committee is asked to identify within its assessment the most commonly used additives by weight or number. If additives belong to a single group of substances with identical or very similar properties, both the group of substances and the list of substances falling into that group shall be presented and the most relevant substance(s) within that group identified.

6 If an additive is included in Annex VI of Regulation (EC) No 1272/2008, its CMR-classification should be provided and considered as appropriate. Additives that have CMR properties in unburnt form should be identified/listed, but do not require a comprehensive description.

7 Registrations/assessments of relevant substances under Regulation (EC) No 1907/2006 should be provided and considered as appropriate.

When examining the composition of tobacco products and the use of individual substances, the Scientific Committee is invited to consult the data on additives reported by the tobacco industry under the Tobacco Products Directive 2001/37/EC, but may also consider additional data sources. Furthermore, the Committee is invited to consider during their assessment the lists of additives permitted/prohibited for use in tobacco products as implemented by certain Member States.

2. Based on its assessment in point 1, the Committee is asked to establish a list of a minimum of 20 and maximum of 30 additives that are suitable/recommended to be added to the priority list of additives in line with Article 6 of TPD 2014/40/EU. When establishing the list, the Committee shall consider the public health risks associated with the additives (actual or suspected), strength of the available evidence and to the extent possible, the frequency of use of the additives in tobacco products. The Committee should indicate as far as possible rankings of additives in light of the above and provide an explanation for its ranking[8].

Opinion 2:

3. Furthermore, the Committee is asked to advise the Commission on the type and criteria for comprehensive studies that should be requested from manufacturers to assess the relevance of the individual additives, considering inter alia the knowledge gaps identified in point 1 above and the interaction of the additive with other additives/ingredients. Advice is also sought on the most suitable methodologies to be used (including a structure of the reports that can be peer reviewed).

[8]Substances belonging to the same group of identical/very similar substances should be considered jointly.

2 SCIENTIFIC RATIONAL

2.1 Introduction

In response to the Commission's requests, the SCENIHR adopted Opinion 1 (Tobacco Additives I), in which 48 single chemicals were listed as priority additives, which met the 30 entries maximum limit because some chemicals with very similar structures (i.e. aliphatic gamma-lactones, including 8 chemicals) and/or properties (e.g. weak acids, including 8 group members) were grouped together. They were selected on the basis of two initial criteria: the frequency of use in different brands and the amounts used in cigarettes, then further screened based on their hazardous properties, because they have or are suspected to have one or more of the following properties:

a. Contributing to the toxicity or addictiveness of the products concerned / increasing the toxicity or addictiveness of any of the products concerned to a significant or measurable degree;
b. Resulting in a characterising flavour;
c. Facilitating inhalation or nicotine uptake;
d. Leading to the formation of substances that have CMR properties / increasing the CMR properties in any of the products concerned (cigarettes/roll-your-own) to a significant or measurable degree.

On the basis of these criteria:

- 17 substances were identified because they fall or are suspected to fall in the category: toxic in unburnt form, among which 6 are suspected of CMR potential, which were ranked highest on the suggested list because the Tobacco Products Directive foresees the prohibition of additives that have CMR properties in unburnt form.
- 20 substances were identified because they are known or suspected of forming irritant, toxic and/or CMR chemicals after combustion including sugars, sugar-containing additives and cellulose.
- 14 substances were identified because they are suspected of facilitating inhalation or of increasing nicotine uptake.
- 19 substances were identified because they show a characterising flavour, a factor potentially contributing to attractiveness.

Since SCENIHR was asked to prioritize the selected chemicals to the best of its ability, three groups were identified. In addition to the 6 chemicals suspected of CMR potential, menthol was included in the 'highest priority group'.

A second group was identified based on the possibility of forming CMR compounds after combustion.

All the remaining identified additives are categorised in the third group, although it was not possible to rank them on the basis of their specific hazard profile and the only possibility was to use content/frequency ranking as a possible criteria for prioritisation or a combination of more than one of four characteristics provided for in Article 6.

On May 18, 2016, the Commission adopted the Commission Implementing Decision (EU) 2016/787 laying down a priority list of additives contained in cigarettes and roll-your-

own tobacco subject to enhanced reporting obligations[9], identifying 15 chemicals among those listed in the SCENIHR Opinion (Additives used in tobacco products; Tobacco Opinion 1) adopted in January 2016.

In this Opinion 2, on the basis of the knowledge gaps mentioned in the next section, and after revising the available open literature and approaches taken by International Agencies, SCHEER provides advice to the Commission on the type and criteria for comprehensive studies that should be requested from manufacturers to assess the relevance of the individual additives, proposing a step-wise strategy (Section 3.4). The issue related to interaction of the additive with other additives/ingredients is also considered.

It should be noted that, by contrast to adding them to medicines or food, for example, additives in tobacco products have no health or other benefits for the consumer. On the contrary, by making smoking more attractive, they promote an extremely unhealthy behaviour. Therefore, a risk-benefit analysis is not the appropriate paradigm for assessing the additive and as such the level of proof of safety must be set much higher than for other products. Considering that for many additives the toxicological information relevant to inhalation is often scant, it is a SCHEER recommendation that the precautionary principle as a quintessential element of preventive toxicology should come into full force (Reichl and Schwenk, 2004). It stipulates that a reasonable suspicion of toxicity is sufficient to deny approval of such a substance (DKFZ, 2010). The same reasoning applies to the addictive and attractive effects of tobacco additives, as they will indirectly lead to adverse health consequences by increasing consumption of the product.

In addition to the general strategy, the major data gaps already identified in Tobacco Opinion 1 have been analysed to determine the most appropriate steps (and end-points) to be carried out and then used for the evaluation (Section 3.5), in order to speed up the process, making possible testing feasible within the 18-month time-frame. To give an example, for the 6 chemicals for which a genotoxic potential could not be ruled out for the unburnt form, the first step will be to evaluate their genotoxicity: in case of positive results, no other testing will be necessary, since according to the TPD they will automatically be banned for use as tobacco additives. In case of negative results, they will enter the general strategy of testing and be considered as would any other compound.

[9] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016D0787&from=EN

2.2 Knowledge gaps identified in Opinion 1

There was generally scant toxicological information regarding tobacco additives analysed for Opinion 1, and the available information was often limited to the oral route of exposure, especially for flavouring substances that are used by the food industry, or, to a lesser degree, to the dermal route, for substances that are also commonly used in cosmetic products. Data on the effects of additives in tobacco following inhalation is generally not available, although this is the most relevant exposure route. Indeed, the additives are either transferred to inhaled smoke in pure form, or are combusted and converted via pyrolysis into potentially toxic products. Because there was also little data on their kinetic behaviour, it was difficult, if not impossible, to make route-to-route extrapolation for additives.

A general scarcity of information was observed regarding the actual level of exposure to additives both in the unburnt form in tobacco products and resulting from combustion – including data on pyrolysis. This is particularly relevant since toxic combustion products generated upon pyrolysis of additives have the potential to increase the exposure to toxic substances and thus increase the health hazard associated with cigarette smoking (National Institute for Public Health and the Environment, 2012). The fate of the additive depends on its physico-chemical properties such as its volatility and reactivity, the design of the cigarette and the smoking topography of the user. The additive may be distilled from the tobacco rod, and end up in smoke intact, or it may be (partly) combusted. In case of (partial) pyrolysis, not only the unburnt additive is relevant, as the smoker will be exposed to the pyrolysis products as well. In the tobacco matrix, either the intact additive or its pyrolysis products may react with other additives, tobacco- or smoke components (pyrosynthesis). For instance, only minor amounts of the non-volatile sugars in tobacco (approximately 0.5% of glucose and sucrose) are transferred unchanged into the mainstream smoke, whereas the major part will combust, pyrolyse or participate in pyrosynthesis processes (Talhout *et al*., 2006).

Although for most tobacco additives, direct information about their possible contribution to addictiveness and characterising flavours does not exist, information can be derived from the mode of action of the additive (e.g. addictiveness can be related to increased nicotine bioavailability or to local anaesthetic effects facilitating the inhalation of tobacco smoke).

Generally speaking, the scarcity of information on exposure and on toxic effects make risk assessment difficult, if not impossible.

2.3 Methodology

2.3.1 Development of the general approach to assess the effects of tobacco additives

Given the fact that additives in tobacco products have no health or other benefits for the consumer, but rather promote an extremely risky behaviour, risk-benefit evaluations are not appropriate. Based on evaluation of approaches for regulation of other types of components, the SCHEER concluded that a step-wise approach is the most pragmatic and efficient way to proceed in the assessment of the toxic, addictive and attractive effects of tobacco additives. The tiered approach proposed by DKFZ (DKFZ, 2010) was used as a starting point, and adapted to include the evaluation of attractive

and addictive effects of additives. The order of the steps has been proposed in such a way to minimise testing. First, an evaluation of the available literature is proposed, next, non-testing methods such as quantitative structure–activity relationship (QSAR) and read across are employed, followed by *in vitro* approaches. Regarding types of effects, toxicity is assessed first, as CMR chemicals are not allowed, and accepted methods and evaluation frameworks are available for toxicity testing, followed by characterising flavours, because accepted methods and evaluation frameworks are available. Finally, addictiveness is assessed, and since no validated tests are available here, the assessment can be guided by the knowledge of the mechanism of action.

2.3.2 Addressing the major data gaps identified in Opinion I for the priority list additives

The major data gaps already identified in Tobacco Opinion 1 for the 15 additives included in Commission Implementing Decision (EU) 2016/787 have been analysed. Based on the data gaps described in the 'Rationale for inclusion' in Opinion 1, the activities to be performed upfront have been described. Then on the basis of the obtained results, if the additive does not meet the criteria for exclusion as an additive listed in art. 7 of the TPD, it can be subject to the general evaluation step-wise procedure described in the Opinion.

2.3.3 Information collection

Information on guidance for the data collection and tests to be performed in the different steps of the step-wise approach was collected on available open literature/websites and from documents by other Committees/International Organisations (e.g. WHO, EPA, EFSA, JECFA).

2.3.4 Information evaluation

For this Opinion on tobacco additives, the available information was analysed to identify tests and testing structures that are appropriate for the assessment of the toxic, addictive and attractive effects of tobacco additives.

2.4 Step-wise approach to assess the toxic, addictive and attractive effects of tobacco additives

A pragmatic and efficient step-wise approach is suggested, in order to assess the toxic, addictive and attractive effects of tobacco additives. Tobacco industry has the burden of proof that an additive does not fall within the scope of the four categories mentioned in the terms of reference and it is tobacco industry's responsibility to deliver data. The data need to be evaluated by independent scientific bodies with expertise in risk assessment of the toxic, addictive, and attractive properties of chemicals.

In order to limit the financial and administrative burden for industry and authorities, as well as the amount of literature evaluation and testing by industry, and subsequent evaluation of the submitted reports by independent institutes, the formation of consortia and joint reports by industry is endorsed.

For the toxicological evaluation of additives in tobacco products, the tiered approach proposed by DKFZ (DKFZ, 2010) is a good starting point. This approach has been slightly adapted and widened to allow for the evaluation of attractive and addictive effects of additives (see Figure 1). This is because apart from toxicity, tobacco additives may indirectly increase tobacco-related harm by increasing the consumption rate of tobacco products, either by making the product more attractive to the consumer (e.g. by resulting in a characterising flavour, and by facilitating inhalation), or by enhancing its addictiveness (National Institute for Public Health and the Environment, 2012). As far as possible, this possibility has to be considered. Although a standardised methodology is not available, it is possible to derive information from the mechanism of action of the additive (e.g. addictiveness can be related to increased nicotine bioavailability or to local anaesthetic effects facilitating the inhalation of tobacco smoke; see the possible mechanism in the SCENIHR Opinion, 2010).

Whenever the evaluation of the additive in the unburnt form gives rise to any concern in relation to art 7 of the TPD (e.g. foreseeing the prohibition of additives having CMR properties) based on data collected in Step 1, the evaluation is stopped, meaning that the additive does not meet the requirement of the TPD. The same rule is applied to Step 2 for the pyrolysis products. In these cases, industry can proceed to step 4, reporting.

In case data are not available, or are not sufficient or robust enough to make the evaluation possible, the procedure should go to the next step.

In case of high uncertainty about the evaluation based on available data, there are two possible options:

- Application of the precautionary principle
- Delivering of additional data (i.e. via Step 3) by tobacco industry.

Step 2 is analogous to Step 1 but related to the pyrolysis products; the two steps can take place concurrently if this is more efficient and saves time. The collection of available data is mandatory in order to priorities the most appropriate end-point(s) to be assessed in step 3, to limit useless testing.

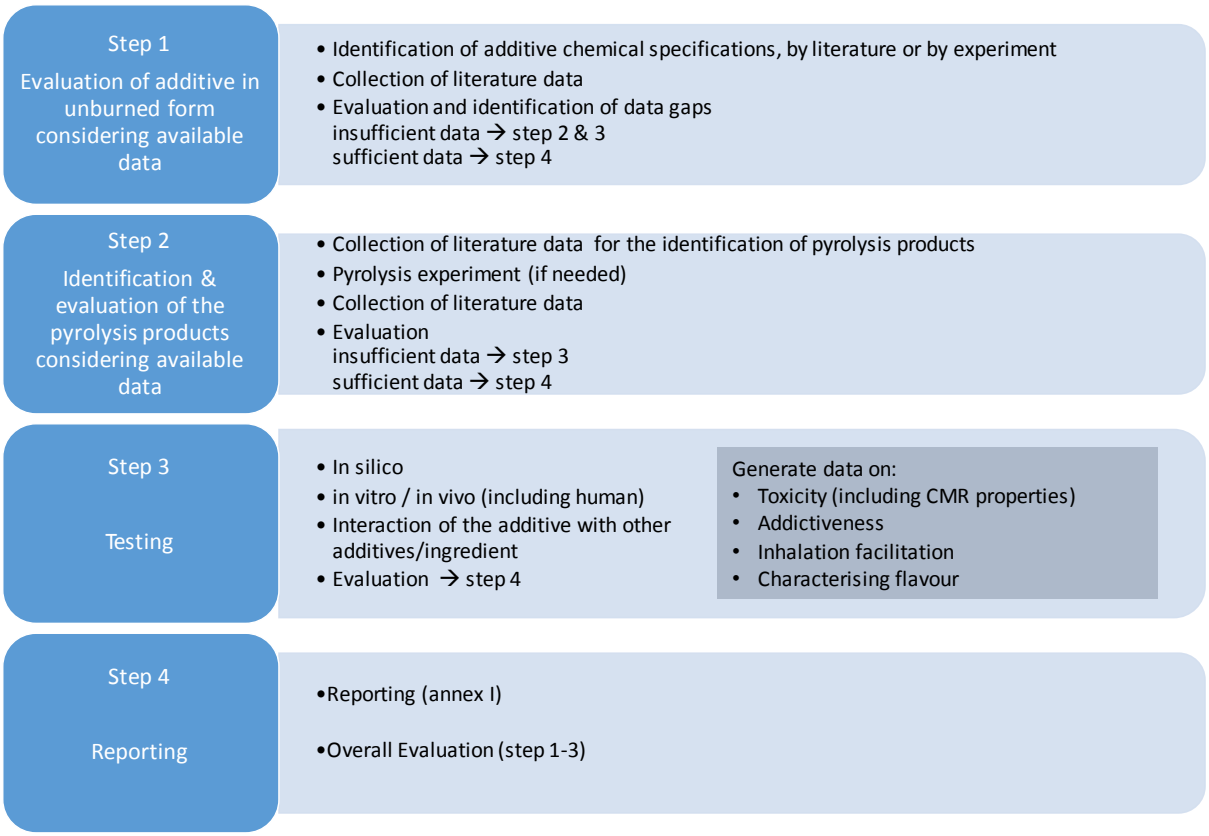

Figure 1. *Step-wise approach to be applied to the assessment of the toxic, addictive and attractive effects of tobacco additives. For terminology, please refer to the text.*

This procedure could be applied to single individual additives; if necessary additives could be grouped, following rules previously established in other fora to evaluate e.g. groups of food flavouring at EFSA[10] or groups of chemicals in Regulation (EC) No 1907/2006 i.e. REACH (to apply the read-across principles)[11] in order to limit the use of animal testing (as requested in art. 13). The ECHA provides practical guidance on the issue (available at the above-mentioned website link); however, to this aim, the approach described in the OECD GUIDANCE ON GROUPING OF CHEMICALS No. 194[12] is recommended.

The approach described in the OECD guidance document (GD) is to consider closely related chemicals as a group, or category, rather than as individual chemicals, for assessing the hazards of chemical substances, increasing efficiency and improving animal welfare. Since the technique of assessing groups of substances is an evolving science, the GD is revised periodically and it is therefore compulsory that the tobacco industry follows the most updated version when applying it. As it is recommended by the GD itself, early consultations between industry and authorities are recommended to ensure that any regulatory requirements are fulfilled.

[10] https://www.efsa.europa.eu/en/topics/topic/flavourings

[11] http://echa.europa.eu/support/grouping-of-substances-and-read-across

[12] GUIDANCE ON GROUPING OF CHEMICALS, SECOND EDITION Series on Testing & Assessment No. 194 (2014) available at http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)4&doclanguage=en

The GD outlines a process for grouping chemicals to include the identification of analogues/members of categories, the mechanistic basis for using analogues or chemical categories and the robustness of both approaches. The GD also describes the use of (Q)SARs for data evaluation and data-gap filling (read-across, trend analysis and (Q)SARs).

2.4.1 Step 1: Evaluation of the additive in unburnt form

The first step starts with the identification of the additive chemical specifications, by literature or by experiment (for the physico-chemical characterization, if not available, data can be obtained following the OECD or ISO test guidelines to this purpose). This initial step is absolutely necessary in order to identify the nature of the additives and comprises also qualifying and quantifying of any impurity present. CAS numbers need to be provided for all relevant chemicals (additives and impurities). The chemistry and specification of a substance (or mixture of substances), in terms of chemical structure(s) and physico-chemical properties is also asked for in other legislations, e.g. for food additives.

It may not always be possible to fully characterise natural extracts, but as much information as possible is required to understand the extent to which variability in composition is controlled during manufacture. Data on the chemical composition of a natural extract additive should be provided by industry with emphasis on the concentrations of constituents of relevance; this includes the concentrations of compounds classified according to their chemical structure (e.g. flavonoids, terpenoids, alkaloids, etc.), constituents being characteristic for tobacco additives (chemical fingerprint, markers). Information on maximum levels for microorganisms and possible contaminants, including e.g. heavy metals, mycotoxins, pesticide residues and polycyclic aromatic hydrocarbon (PAH) residues, should be provided (EFSA, 2012).

Then, all available information on the additive in unburnt form is collected and evaluated. This includes open literature on peer-review journals as well as grey literature, including JECFA, EFSA and FEMA data or data coming from any other regulatory request, in case the additive is used in other contexts.

This step allows the collection of available information on the additive in its unburnt form, useful for its risk assement. In addition, it allows the identification of the major data gaps to be addressed in Step 3, especially with regard to toxicity, characterising flavour (and other possible factors contributing to attractiveness) and addictiveness data.

For future reference by the regulator, industry is also asked to indicate which additives are closely related regarding chemical structure, functions, purpose and effects. An example here is menthol, which is functionally closely related to e.g. menthol derivatives, wintergreen and spearmint.

2.4.1.1 Collection of literature data

Whenever possible, all information already available on the toxicity of the additive should be collected, used and evaluated before any testing is initiated. Some knowledge on the toxicity of tobacco additives exists; however, much less is known on their attractiveness and addictiveness. Open literature as well as grey literature should be included and if studies have been already performed in view of seeking approval of the same chemical

for uses other than tobacco additive, a letter of access should be acquired, in order to avoid repeating of the same tests.

Initial electronic literature searches with appropriate key words/dates should be a starting point for data gathering. The databases and search engines used may include for example PubMed, Web of Science, Scopus, Toxline, Chemical and Biological Abstracts, and Google Scholar. The data search methods will identify many papers that potentially could be used. A first screening is then needed in order to focus on those relevant for the specific purposes, using appropriate inclusion/exclusion criteria. Articles that do not appear to meet the inclusion criteria should be excluded from further analysis. To apply a standardised methodology it is recommended that the literature search strategy and selection criteria (inclusion/exclusion) for the review are based on the EFSA Systematic Review Guidance (EFSA Journal 2010; 8(6):1637).

The methodological quality of the selected paper should also be addressed, including the design, execution, analysis and reporting of the study. Expert judgement is vital in the assessment of the quality and the interpretation of data therefore the appropriate identification and selection of relevant publications is extremely important. When possible (e.g. for toxicity studies) this screening should be based on Klimisch scoring.

The acceptance of each publication that is considered to be relevant should be based on the quality and relevance criteria summarised in by SCENIHR (2012).

All selected publications of potential importance should be subject to similar treatment in the evaluation process. Positive and negative studies should be evaluated using similar procedures and criteria and considered of similar importance if the quality is judged to be comparable. In positive studies the evaluation needs to consider both causal and non-causal explanations of the results. For example, one key question would be "with what degree of certainty can one rule out the possibility that the observed positive result is produced by bias, e.g. confounding or selection bias, or chance?". In the case of negative studies, it is necessary to assess the certainty with which it can be ruled out that the lack of an observed effect constitutes evidence against a hazard or whether it could result from (masking) bias, e.g., too small exposure contrasts, too crude exposure measurements, too small exposure groups/populations, or chance. Consideration should also be given to the possibility of a publication bias i.e. that positive findings are more likely to be published than negative findings.

It is recommended that the whole data set, judged as relevant, reliable, and of good quality, should be used for the (risk) assessment of the tobacco additive and its pyrolysis products, if any. Different approaches for assessment of whole data sets, referred to as weight of evidence evaluation or systematic review (often used interchangeably), have been promoted (Koustas *et al*., 2014; Rooney *et al.,* 2014; European Food Safety Authority, 2010; IARC, 2006). In general terms, these approaches are processes of summarising, synthesising and interpreting a body of evidence to draw conclusions, e.g. regarding the relationship between a chemical exposure and an adverse health effect. The WoE approach promotes the use and integration of information from all available evidence.

Unfortunately, formal procedures and consistent terminology for weight of evidence processes are lacking, although a weight of evidence evaluation is mentioned in the REACH regulation, the Biocides directive, the Cosmetics regulation, and the regulation for Classification, Labelling and Packaging (CLP). Some guidance documents are only available for industrial chemicals or contaminants in food (Ågerstrand and

Beronius, 2015). However, a number of organisations have established their own frameworks for assessing/evaluating evidence, including SCENIHR (2012), and the work is still in progress in both SCHEER and EFSA. Since the approach is rapidly evolving, it is compulsory that in applying it, the Tobacco Industry follows the most updated version.

As indicated above, it is possible to apply substance grouping of read-across principles: this approach uses relevant information from analogous ('source') substances to predict the properties of 'target' substances. The application and reporting of this approach as described in section 3.4.1.1 is recommended; if applied correctly, there is no need to have specific information on every additive.

In order to collect data on addictiveness and attractiveness, all investigations on possible related mechanisms should be considered. In this respect, an emerging approach is the adverse outcome pathway (AOP) – a framework designed to conceptually link a molecular initiating event to an adverse outcome of relevance to risk assessment (Ankley *et al*., 2010). The AOP framework allows for a better understanding of the mechanistic linkages between cellular responses and downstream impacts on apical outcomes that are of concern within a regulatory context (Villeneuve and Garcia-Reyero, 2011). Potential practical uses of AOPs also include the above-mentioned grouping of common chemicals for read across (not only based on chemical structures but on biological activity), identification of research and data gaps, serving as a framework for regulatory priority setting, and informing hazard characterization and risk assessment (Becker *et al*., 2015). AOP methodology may be useful in elucidation of molecular basis for addictiveness of tobacco products e.g. role of pH changes on nicotine absorption, MAO-A inhibition, Dopamine (DA) release and turn over, CYP metabolism and inhibition (for details see paragraph 3.4.3.5). Accordingly, the same apply to attractiveness investigation (for details see paragraph 3.4.3.6). OECD developed a guidance document outlining methods and best practices for creating and assessing AOPs, in which it calls for the assessment of an AOP's weight of evidence (OECD, 2013; AOP-Wiki, 2014). AOP wiki represents a joint effort between the European Commission – DG Joint Research Centre (JRC) and the U.S. Environmental Protection Agency (EPA). This serves as one component of a larger OECD-sponsored AOP Knowledge Base effort and represents the central repository for all AOPs developed as part of the OECD AOP Development Effort by the Extended Advisory Group on Molecular Screening and Toxicogenomics.

2.4.1.2 Evaluation

Whenever the evaluation of the chemical in its unburnt form give rise to any concern regarding CMR properties, the evaluation is stopped, meaning that the additive does not meet the requirement of TPD art. 7, and can directly proceed to Step 4.

Collected data gives information regarding the possibility for the additive to fall into one or more of the four categories:

a) Contributing to the toxicity or addictiveness of the products concerned/increasing the toxicity or addictiveness of any of the products concerned to a significant or measurable degree;
b) Resulting in a characterising flavour;
c) Facilitating inhalation or nicotine uptake;
d) Leading to the formation of substances that have CMR properties / increasing the CMR properties in any of the products concerned (cigarettes/RYO) to a significant or measurable degree.

In case data are unavailable, insufficient or not robust enough to make any evaluation possible, the procedure should go to Step 3. In case of uncertainties about the evaluation in the presence of a health concern, the precautionary principle can be applied or alternatively tobacco industry could proceed to Step 3.

2.4.2 Step 2: Evaluation of the pyrolysis products

In the second step, information available on the identification of pyrolysis products of additives must be collected and evaluated. This can be done on the basis of literature data (see section 3.4.2.2 for criteria), but in case no sufficient data (in quantitative or qualitative terms) are available, the second step foresees that pyrolysis studies need to be performed in realistic, standardised experimental conditions (see section 3.4.2.2). Then available literature data on the toxicological profile, attractiveness or addictiveness on the identified pyrolysis products should be collected, as described in Step 1 for the chemical in the unburnt form.

2.4.2.1 Collection of literature data

Literature data on the pyrolysis products of additives is collected in the same way as described in Step 1.

2.4.2.2 Pyrolysis studies (if needed)

To identify the compounds formed during the combustion process of a tobacco additive, tobacco industry in general performs pyrolysis studies on a comparative basis where a research cigarette is machine smoked with and without the additive present *(*Talhout *et al*., 2006). Burning (smoking) the tobacco that contains a specific amount of the additive and subsequent analysis of selected smoke components is described for many different additives (Baker *et al*., 2004a; Baker *et al*., 2004b, c; Carmines, 2002; Rustemeier *et al*., 2002). However, subtle differences between the selected smoke components will not be noticeable, and it is not feasible to screen the effect on all 6000 known smoke components, hence usually only the so-called Hoffmann analytes are screened. Given the complexity of cigarette smoke, it is difficult to identify individual materials that may result from the pyrolysis of ingredient mixtures unless radioactively labelled additives are used, but that method is sophisticated and expensive. Furthermore, this method cannot determine whether the additive is a precursor or a catalyst for the formation of a certain smoke component (Torikai *et al*., 2005).

Pyrolysis, on the other hand, is a useful technique for evaluating materials used at low levels, where it is unlikely that smoke chemistry assays could detect a change. Therefore, combustion processes in a burning cigarette have also been simulated with pyrolysis methods (Baker *et al*., 2004b; Busch *et al*., 2012; Lee *et al*., 2007).

This technique is useful as a first screening of potential pyrolysis products, their thermal stability and the temperature at which they are formed (Baker and Bishop, 2004). However, the pyrolysis conditions only approximate the burning cigarette with regard to temperature and atmosphere and make no allowance for the presence of other tobacco and/or smoke components that may interact with the additives. Pyrosynthesis processes related to the tobacco matrix will not occur when the additive is pyrolysed as a single component outside of the tobacco matrix. When it is suspected that such reactions will occur, one may consider pyrolysing a simple mixture containing the additive together with the component with which reaction is foreseen (either with the component itself or with its pyrolysis products. For instance, micro-vial pyrolysis of a glucose/proline mixture

resulted in formation of Amadori intermediates, important in the formation of (Maillard) products that influence the aroma (Mitsui *et al*., 2015). Pyrolysis was performed at 700 °C, approximating the temperature of the pyrolysis zone of a burning cigarette, for 10 s under atmospheric conditions (headspace gas in vial not replaced by an inert gas).

Pyrolysis studies can be performed under a given set of experimental conditions that need to resemble processes in a burning cigarette in terms of e.g. temperature, rate of temperature change, and atmosphere (amount of oxygen). During the cigarette-burning process, the temperature of the tobacco and the burning cone can range from room temperature up to 900 °C, and the amount of oxygen can range from 0 to 18%. It is important that the design of the pyrolysis study reflects the conditions of burning cigarettes with oxygen levels ranging from 0% to 14% and the temperature in the burning zone ranging from ambient temperature to 900 °C (Baker and Bishop, 2004; Stotesbury *et al*., 1999; Torikai *et al.*, 2004).

Many studies tried to simulate the processes during smouldering and combustion. Stotesbury performed pyrolysis at 14 sets of pyrolysis conditions: temperatures between 200 °C and 700 °C in 2 % and 10 % oxygen, and at 800 °C and 900 °C in 2 % oxygen. Baker used an atmosphere of 9% oxygen in nitrogen, arguing that this is the average amount throughout the pyrolysis/distillation zone inside the burning cigarette during a puff. From an initial temperature of 300 °C, to simulate the smouldering before taking a puff, the sample is heated at 30 °C s^{-1} to 900 °C, and kept for 5 seconds, to simulate the maximum duration of the high-burning zone temperature during puff under extreme human smoking conditions. According to Baker, 30 °C s^{-1} is the approximate mean heating rate throughout the pyrolysis/distillation zone during a puff. This seems rather slow, as that would imply it would take 20 seconds before the maximum temperature is reached, whereas a human puff only takes one or two second. However, most studies are performed with a similar heating rate (Torikai *et al.*, 2004). Purkis *et al*. programmed the temperature from 300 to 900 at 25 °C per second to reflect cigarette smoking and give an appropriate set of conditions to limit artifact formation *(*Purkis *et al*., 2011). It is important that the reaction vial is not closed, so that the additive can distil away at lower temperatures.

Flash pyrolysis is performed when the sample is rapidly inserted in a pre-heated furnace that is already at the highest temperature, for instance at the temperature range of 200–300 °C to simulate cigarette smouldering (Zhou *et al*., 2011). Time of flight spectroscopy allows for almost real time sampling, enabling identification of reactive compounds before being degraded (Hertz-Schunemann *et al*., 2015)(Busch *et al*., 2012).

Taking into account the studies described above, the SCHEER recommends the following experimental design in most cases performed by tobacco industry:

Thermal degradation (pyrolysis, pyrosynthesis and combustion products) of each additive is to be studied under different reaction regimes (inert and 2-14% oxygen) over the temperature range 200–900 °C. The thermal degradation products of two different pyrolysis conditions should be identified:

(1) upon gradually heating the sample from 200–900 °C and

(2) conventional pyrolysis, in which a new sample is pyrolysed at minimally 3 different temperatures (~ 300°C, 600°C and 900°C).

Pyrolysis experiments should be carried out at least in triplicate. Chemical analysis of the components in the pyrolysate needs to be performed with state of the art techniques

in the field of GC-MS and LC-MS, as appropriate for the specific additive. The World Health Organization in its report to the Sixth Conference of the Parties[13], identified eight non-exhaustive lists of toxicants: Health Canada, RIVM, USA FDA, Counts, Dybing and Fowles, Hoffman analytes, Philip Morris-Australian brands, and Philip Morris-Canadian brands. These toxicants need to be indentified and quantified, if present, using analytical reference standards. Tobacco-specific components, such as nitrosamines and alkaloids, are not expected to be present.

For additional components, not on these lists, the following procedure is advised. For identification purposes, library software can be used, such as the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software. Components with a peak-to-peak signal-to-noise ratio below three can be discarded. Also, components with a probability of correct identification below 70% can be excluded.

If components with a toxicological hazard are identified, their identification needs to be confirmed and their amount needs to be quantified using analytical reference standards.

Apart from components that may increase the toxicity, specific attention needs to be given to components that have addictiveness-enhancing properties, flavouring properties, or inhalation facilitation properties (e.g. anaesthetic and/or bronchodialating properties).

2.4.2.3 Evaluation

Again, when the available information is considered reliable and robust enough concening both the identification of pyrolysis products and on their toxicological, addiction, and attractiveness profile, a possible decision (positive or negative) may be reached. For instance, if it is demonstrated that compounds proven to have CMR properties are generated from pyrolysis of an additive, this additive will not meet the TPD requirement. Again, when caase data are unavailable, insufficient or not robust enough to make any evaluation possible, the procedure should go to Step 3. In case of uncertainties about the evaluation in the presence of a health concern, the precautionary principle can be applied, or alternatively tobacco industry could proceed to Step 3.

2.4.3 Step 3: Testing and evaluation of results

The third step is related to the testing of additives or their pyrolysis products, according to methods accepted by other regulations. The outcomes of tests must be related to actual human exposure and tobacco-induced diseases, and be relevant not only for subchronic, but also for chronic exposure in intermittent use sessions (Johnson *et al*., 2009).

A relevant test design will not only consider methods to investigate toxicity, but also characterising flavour and addictiveness. Therefore information related to the known mechanisms that contribute to attractiveness or addictiveness should be collected.

Based on expert judgement of the major data gaps with regard to toxicity, characterising flavour (as contributing to attractiveness) and addictiveness data identified in Step 1, it must be decided which endpoint to start with. This will generally be the endpoint for which most evidence is available of a potential concern. If no priority concerns have

[13] http://apps.who.int/gb/fctc/PDF/cop6/FCTC_COP6_14-en.pdf

been identified, it is advised to start with toxicity, as in that case, accepted *in vitro* tests are available and there are frameworks for interpreting the results.

This step will also address the possible interactions, at chemical level (e.g. pyrolysis) and for the toxicological part based on the MeA/MoA.

2.4.3.1 Comparative paradigms are not endorsed

In order to provide a relevant outcome to the question of whether an additive contributes to the toxicity, attractiveness, or addictiveness of the tobacco product, the study design must adhere to some methodological criteria. It must be noted that comparative testing strategies, where differences in effect of the tobacco product with and without the additive are evaluated, are not considered suitable at the moment given the current toxicity tests and available methodology. The emissions of tobacco products are highly toxic, in particular regarding cigarette smoke (Kienhuis *et al., 2016)*. Due to the high intrinsic toxicity of tobacco products, it is challenging to demonstrate any differences, whether they be increases or decreases, induced by an additive. Due to the high toxicological activity of both the test product (tobacco product with additive) and the control (tobacco product without additive) in comparative testing strategies, the discriminatory power that can be obtained in toxicity assays may not be sufficient (COT/COM/COT, 2009; DKFZ, 2010; Oldham *et al*., 2012). Very sensitive tests would be required, with a clear dose-response relationship, in order to show any differences from these high background effects. As such tests are not currently available, no comparative studies (tobacco product with and without additives) will be considered, since these studies lack discriminative power. In line with this, the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC, 2009) concluded: "*The Committee considered that the available studies used to assess the contribution of individual or mixed ingredients or additives to the overall toxicity of tobacco products are inadequate to assess the risks posed by conventional cigarettes, so it is not possible to assess the modulation of that risk resulting from inclusion of additives. The relationship between effect (an increase in biomarker) and exposure is also poorly understood. Furthermore, it is possible that additives might alter smoker behaviour, such as to increase product use; this increased exposure would be likely to result in an increased risk."*

Furthermore, an international Working Group on Tobacco Additives (WG), assigned by the Brazilian regulatory agency ANVISA, assessed many industry-sponsored studies addressing the effects of mixtures of commonly used additives on cigarette smoke chemistry and toxicity. Although industry claimed that additives have no effect on the levels of chemical components of cigarette smoke and toxicity, the WG concluded that the available data were insufficient to accept the tobacco industry's claims that additives do not increase the inherent toxicity of tobacco smoke (Ferreira *et al*., 2015; Working Group on Tobacco Additives, 2014): "*Given the current toxicity tests and test designs, it is not yet possible to determine whether or not addition of specific ingredients (tobacco additives) to tobacco products adds to tobacco mainstream smoke's inherent toxicity. This is because tobacco itself is already quite toxic, and any added toxicity is difficult to detect within the current test designs used by tobacco industry, i.e. combinations of in vitro testing and animal testing.*"

For the future, tests are needed that are sensitive enough to assess additive attributed toxicity above the overall toxicity of tobacco products, which can associate assay outcomes to human risk and exposure. In this respect, *in vitro* tests combined with

toxicogenomics using biomarkers of exposure and disease are the most promising (Kienhuis *et al*., 2016). However, for the time being, no standardised methods have been validated to this purpose.

Another problem with comparative testing is the choice of the product to be tested, since the additional toxicity of the additive would differ between product types and brands. If an additive would be tested in the intended brand, the results (related to toxicity, addictiveness and attractiveness) could not necessarily be generalised to all products and brands, having a different composition. Therefore the obtained results may not lead to general prohibition/acceptance of specific additives but rather to prohibition/ acceptance on a product-by-product basis (DKFZ, 2010).

When the addition of sugars is taken as an example, it will be very important whether a reference containing Burley tobacco, that does not contain natural sugars, or Virginia tobacco, with high sugar levels, is selected, or a blend of these tobacco types. This is even more important as cultural differences exist in the preference for Virginia-type cigarettes, American blend, or Burley. According to the TPD, the use of additives necessary for the manufacture of tobacco products should be allowed, as long as they do not result in a characterising flavour or increase the addictiveness, toxicity or CMR properties of the product. Thus, in this particular case, sugar addition to replace what is lost during the curing process, needs to be evaluated against the possibility of toxic and carcinogenic compounds forming following pyrolysis.

For similar reasons, comparative studies are also not endorsed to study the effect of additives on addictiveness and inhalation facilitation.

Instead of using a comparative study design, the effects of the pure additive, and its pyrolysis products, must be considered in a relevant testing strategy, such as the tiered approach proposed by DKFZ (DKFZ, 2010), which has been adapted by SCHEER (Fig.1).

2.4.3.2 The use of animal testing

So far, available inhalation or other animal studies have been used to assess the effect of tobacco additives. However, it is ethically questionable to use animal studies to evaluate the 'safety' of a tobacco additive, as tobacco products are highly harmful with no benefits to individual or public health. Therefore, as a principle, only *in silico* and *in vitro* studies will be considered, following the EU policy recommending implementation of 3R methods for refinement, reduction, and replacement of animal models leading to the ban of animal studies for chemicals to be used in voluntary products such as cosmetics (EU Regulation no. 1223/2009). Human studies are discouraged, although they may be used in case of flavour assessment, but only if the study subjects are not exposed to the harmful smoke emissions of tobacco products. Generally and especially in those specific cases in which animals are proposed, early consultations are recommended between Receiving Competent Authorities at Member State level and tobacco industry, presenting a testing strategy including *in silico*, *in vitro* and only in exceptional cases *in vivo* tests. In order to limit the testing formation of Consortia is recommended.

Whenever animal testing should be deemed necessary, it is compulsory to be compliant with the Animal Welfare EU policy and to respect the Regulation on Animal Testing.

2.4.3.3 Quality system

In line with the provisions of other regulatory contexts, the SC recommends the use of a Quality system (e.g. Good Laboratory Practice or ISO17025) for carrying out the pyrolysis or other physico-chemical studies as well as toxicity studies (including those to assess the mechanism underlying possible contribution to addictiveness and attractiveness).

In case the principle of the Mutual Acceptance of Data is applied (again to limit the testing) the quality system of choice should be the GLP, following the application of the GLP OECD principles, to which all the National Monitoring Authorities in the different OECD Member States make reference. However, for the physico-chemical studies GLP compliance is not always requested and the ISO17025 could be chosen.

2.4.3.4 Toxicity testing

In silico

If toxicological data on the additives are not available or are limited, they can be produced using *in silico* approaches. As a first step, QSAR methods are encouraged to identify alerts for genotoxicity, carcinogenicity and reproductive toxicity, to get information of potential CMR properties of the additive or of its identified pyrolysis products: this could also take advantage of similarities with other chemicals by applying the read-across methodology.

Non-test information about the biological activity of a substance can be derived in a variety of ways, ranging from simple inspection of the chemical structure through various read-across techniques, the use of expert systems, metabolic simulators, to global or local (Q)SARs. The usefulness of such techniques varies with the amount and nature of information available, as well as with the specific regulatory questions under consideration.

Models for the identification for alert of genotoxicity and carcinogenicity have a long tradition; (Q)SAR models for mutagenicity can apply to a limited set of congeneric substances (local models) or to a wide variety of non-congeneric substances (global models).

Many global models for mutagenicity are commercial and some of the suppliers of these global models consider the data in their modelling sets to be proprietary. Proprietary means that the training set data used to develop the (Q)SAR model is hidden from the user. In other cases it means that it may not be distributed beyond use by regulatory authorities.

There are hundreds of (Q)SAR models available in the literature for predicting test results for genotoxic endpoints for closely related structures (Naven *et al.,* 2012; Bakhtyari *et al*., 2013). These are known as local (Q)SARs. However, quality of reporting varies from model to model and predictivity must be assessed case-by-case on the basis of clear documentation.

In case of robust data on the identification of structural alert or based on read across indication of CMR properties, it is not necessary to go further, since according to the TPD no CMR substance can be used as a tobacco additive. In case there are doubts, *in vitro* testing can be conducted (see below).

Regarding the other toxicological properties, other QSAR tools are available. Some of them are briefly described in the following:

OECD built an open software application (http://www.qsartoolbox.org/), named QSAR Toolbox. The Toolbox and guidance on its use are freely available. The OECD QSAR Toolbox facilitates the practical application of grouping and read-across approaches to fill gaps in (eco-)toxicity data, including but not limited to genotoxicity and carcinogenicity, for chemical hazard assessment. The Toolbox incorporates information and tools from various sources, into a logical workflow. Crucial characteristic of the workflow is the grouping of chemicals into categories (group of chemicals whose physicochemical and human health and/or ecotoxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern, usually as a result of structural similarity). The most important features are:

1. Identification of relevant structural characteristics and potential mechanisms or mode of action of a target chemical.
2. Identification of other chemicals that have the same structural characteristics and/or mechanism or mode of action.
3. Use of existing experimental data to fill the data gap(s).

The Joint Research Centre of the EU provides several tools for modelling for the safety assessment of chemicals. They offer the following computational tools (freely downloadable or accessible from their webpages):

- JRC QSAR Model Database: database hosting structured and peer-reviewed information on QSAR Models (https://eurl-ecvam.jrc.ec.europa.eu/databases/jrc-qsar-model-database);
- Toxtree, software tool to generate prediction(s) on mechanisms of action or toxicological effects, the tool is based on a decision-tree approaches (https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/toxtree);
- Dart, (Decision Analysis by Ranking Techniques) a software tool designed to rank chemicals according to environmental and toxicological concerns (https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/DART);
- Toxmatch, a flexible application for grouping chemicals based on chemical similarity designed to be helpful in read-across (https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/toxmatch).
- Stat4tox, a tool which carries out concentration-response analysis for *in vitro* experiments (https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/stat4tox).

ECHA provides a detailed overview on non-testing methods in sub-section R.7.7.3.1 of Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint specific guidance (see http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf), in particular with regard to the prediction models for mutagenicity and the OECD QSAR toolbox.

A list of the available (free and commercial) predictive software for ecotoxicological, toxicological and environmental endpoints, including mutagenicity models, has been compiled within the frame of the EU project Antares (http://www.antares-life.eu/). For example, the Danish EPA and the Danish QSAR group at DTU Food (National Food Institute at the Technical University of Denmark) have developed a (Q)SAR database that contains predictions from a number of mutagenicity models. The database is freely accessible via http://qsar.food.dtu.dk. The online database contains predictions for over 166,000 substances and includes a flexible system for chemical structure and parameter searching. A user manual with information on the individual models including training set information and validation results is available at the website. The database is also integrated into the OECD (Q)SAR Toolbox.

Another example of a database with predictions on mutagenicity is the Enhanced NCI Database Browser (http://cactus.nci.nih.gov) sponsored by the U.S. National Cancer Institute. It contains predictions for over 250,000 substances for mutagenicity as well as other nonmutagenic endpoints, some of which may provide valuable mechanistic information (for example alkylating ability or microtubule formation inhibition). It is also searchable by a wide range of parameters and structure combinations.

Use of harmonised templates, such as the QSAR Model Reporting Format (QMRF) and the QSAR Prediction Reporting Format (QPRF) developed by the Joint Research Centre (JRC) of the European Commission (http://ihcp.jrc.ec.europa.eu/our_labs/predictive_toxicology/qsar_tools/QRF), can help to ensure consistency in summarising and reporting key information on (Q)SAR models and substance specific predictions generated by (Q)SAR models. The JRC website also hosts the JRC (Q)SAR Model Inventory, which is an inventory of information on the validity of (Q)SAR models that have been submitted to the JRC (http://ihcp.jrc.ec.europa.eu/our_databases/jrc-qsarinventory).

If the exposure could be well characterised without uncertainties, the application of the Threshold of Toxicological Concern (TTC) concept could be foreseen in the future, when the applicability domain will be expanded to include the inhalation route in the data base.

In vitro

There are a number of *in vitro* tests that can be used to assess many different toxicological end-points. *In vitro* toxicity tests are applicable to a wide variety of test materials including ingredients added to tobacco, tobacco extracts, tobacco smoke condensates, and whole or vapour phase smoke. These assays may also be used to explore interactions between components of cigarette smoke.

The first choice has to be given to tests already adopted at international levels (e.g. OECD Test Guidelines[14], or ISO methods) or tests validated by ECVAM. The OECD test guidelines (TGs) describe the applicability domain, the principles of methods and the procedure and they also address reporting.

In case a non-TG *in vitro* method is used (e.g. a test validated by ECVAM), the reporting should be appropriate, following what it is indicated in the OECD Guidance Document for

[14] http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

describing Non-Guideline *in vitro* test methods No.211 (2014)[15]. The application of integrated approaches on testing and assessment (IATA) is highly recommended, as described in documents adopted by both ECVAM and OECD[16].

Since the acceptance at regulatory levels is evolving, the TG as well as the GDs are revised periodically, therefore it is compulsory that in choosing the appropriate test, the tobacco industry follows the most updated version. Indeed, as requested by EU regulation 1223/2009 on cosmetics, the EU Commission has to report every year to European Parliament as well as to the EU Council on the progress related to the development, validation and regulatory acceptance of alternative methods as communicated by a Report from EURL-ECVAM[17].

If information gathered through *in silico* methods are not conclusive and there are doubts related to genotoxicity potential, an *in vitro* genotoxicity test battery or *in vitro* transformation test for carcinogenicity can be applied to clarify these end-points. The *in vitro* genotoxicity testing methodologies are well described in the several adopted OECD TG:

- 471: Bacterial Reverse mutation,1997
- 473: *In vitro* mammalian Chromosomal Aberration Test, 2014 Rev
- 476: *In vitro* Mammalian Cell Gene Mutation Test, 1997, 2015 Rev
- 479: *In vitro* SCE Assay in mammalian cells, 1986
- 480: S. cerevisiae, gene mutation assay, 1986
- 481: S. cerevisiae, Mitotic Rec. assay, 1986
- 482: DNA damage and Repair, UDS in mammalian Cells *in vitro*,86
- 487: *In vitro* Mammalian cells Micronucleus test, 2014 Rev
- 490:*In vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, 2015

In the battery, it would be necessary to include tests able to identify point mutations in prokaryotic and mammalian cells as well as chromosomal aberrations and DNA damage and repair.

In vitro methods to address local toxicity (i.e. phototoxicity, skin corrosion and irritation, eye irritation, skin sensitisation) are available (see table 1) and should be performed using the air-liquid interface.

More difficult is to address systemic toxicity by means of *in vitro* testing only, since at the moment no adopted TGs are available.

For carcinogenicity, two cell transformation assays have been included in OECD guidance Documents (table 1), which - using an IATA with *in silico* and read across data - could give sufficiently robust information.

[15] GUIDANCE DOCUMENT FOR DESCRIBING NON-GUIDELINE *IN VITRO* TEST METHODS Series on Testing and Assessment No. 211

[16] http://publications.jrc.ec.europa.eu/repository/bitstream/JRC96418/eurl%20ecvam%20toxicokinetics%20strategy.pdf
http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO%282015%2922&doclanguage=en

[17] http://ihcp.jrc.ec.europa.eu/our_lab/eurl-ecvam

Only a few *in vitro* studies are available to address very specific reactions possibly leading to reproductive problems, such as *in vitro* binding to estrogen and androgen receptors (see the OECD conceptual framework to evaluate endocrine disrupting chemicals). Nevertheless, they can be included, and evaluation should be carried out also considering the indication coming from QSAR and read across.

Cytotoxicity testing after repeated exposure (e.g. 14 days) is considered a possible end-point of choice (acute toxicity is not relevant for tobacco products, hence also for additives). Positive and negative controls should be used, cells of human origin have to be preferred; non-specific tests as well as organ specific cells should be used (e.g. cells coming from the lung or intestinal cells, accounting for inhalation and swallowing of smoke, but also cells representing CNS, cardiovascular system, etc. to be evaluated on a case-by-case basis). For a correct interpretation of results it should be considered that whenever cell lines are used, they are generally characterised by a low and unbalanced metabolic capability, therefore at least one of them should maintain this function over the treatment period (e.g. HepaRG cells) or primary cells should be used.

The major endpoints evaluated in *in vitro* cytotoxicity assays include the effect of a substance on cell viability (survival) and growth rates, but other end-points (such as mitochondrial functionality, induction of apoptosis) can be included. Cytotoxicity testing is used in the area of medical devices: methods to be considered can be found within the harmonised European standard ISO 10993-1:2009 "Biological evaluation of medical devices Part 1: Evaluation and testing within a risk management process". The ISO 10993 – 5:2009 describes test methods to assess the *in vitro* cytotoxicity of medical devices.

However, the study design should take into account that the most relevant route of exposure for tobacco additives is inhalation.

There has been significant progress made in recent years in approaches to expose cells *in vitro* to chemicals that pose a toxicological concern via the inhalation route (Aufderheide *et al*., 2011; Bakand and Hayes, 2010). A number of *in vitro* exposure systems have been developed to facilitate the study of the effects of the whole smoke mixture on both mammalian and bacterial cells, and this has been the subject of a recent review (Thorne and Adamson, 2013).

Current cell-based *in vitro* models of the respiratory tract consist mainly of 2D monolayers of primary tracheobronchial epithelial cells or an immortalized cell line cultured on a semipermeable membrane insert at an air-liquid interface to induce cell polarization, differentiation, and mucus production (Forbes *et al.,* 2005). A more sophisticated technique that enables the stable and reproducible exposure of cultivated cells to cigarette smoke at the air–liquid interface such as CULTEXW Radial Flow System (RFS) module has been proposed recently (Rach *et al*., 2013).

It was documented that air-liquid interface culture played a significant role toward the *in vitro* recapitulation of the *in vivo* environment, presenting the cells with an apical side resembling the lumen of the respiratory tract and a basolateral side representing vascular supply of nutrients, (Berube *et al*., 2010) with increased expression of cilia in primary cells and differences in barrier and mucus-secreting properties of cell lines observed (de Jong *et al*., 1994, Grainger *et al*., 2006). However, the absence of an extracellular component with cocultured cells in a 3D environment can result in an oversimplification of the airway barrier, lacking in physiological relevance. Therefore, more sophisticated models based on 3D human normal and diseased tissue are required

to provide *in vitro* models that improve validity of tested compounds in humans. Much of respiratory tissue engineering research has seen a transition from single cell-type culture on inserts toward co-culture and the inclusion of scaffold material. Accordingly, models, in which the epithelium is cultured at an air-liquid interface over a scaffold substrate embedded with cocultured cells, are the subject of much interest and are even available now as commercial 3D research products, such as the MatTek EpiAirway-FTtechnology (Berube *et al.,* 2010). Indeed, there is an overall consensus in the literature that introducing an epithelial cell analogue into the co-culture environment, often through the use of a biomaterial scaffold, could enhance cell culture, cell–cell signalling, and functionality. A triple coculture system in which human bronchial epithelial cells A 549, human mesenchymal cells and dendritic cells were cultured in monolayers, has shown promise for studying immunological responses to inhaled particulates (Rothen-Rutishauser *et al*., 2005, Herzog *et al*., 2013), Co-culture of Calu-3 cells with Wi38 lung fibroblasts was achieved on the scaffold to create a submucosal tissue analogue of the upper respiratory tract, validating system as a platform to support co-culture and cellular organisation reminiscent of *in vivo* tissue architecture. These scaffolds were validated as a substrate to support functional mucus express from an airway epithelium. Calu-3 cells cultured on CHyA-B scaffolds also expressed the tight junction protein ZO-1 and F-actin, indicating the formation of an epithelial barrier layer on the constructs and differentiation of the Calu-3 cells. Recently, another *in vitro* model system using tissue-engineered constructs has been developed which might improve our understanding of epithelial tissue and disease and use for testing toxicity of different compounds (O'Leary *et al*., 2016).

Tobacco smoke assessment *in vitro* has traditionally focused on the particulate phase captured on a Cambridge filter pad and eluted in DMSO (Crooks *et al*., 2013) or bubbled through cell culture media or PBS (Andreoli *et al.*, 2003). Cell cultures are then exposed under submerged conditions to the particulate phase. Unfortunately, particulate-based exposure scenarios do not take into account the vapour phase of cigarette smoke, or the associated interactions between the particulate and vapour phases.

Submerged culture conditions and particulate-based exposures do not represent physiologically that of mainstream tobacco smoke exposure in the human lung. Furthermore, separating smoke fractions in this way could lead to alterations and chemical changes that may not be representative of the whole smoke aerosol. In order to address these challenges, whole smoke exposure systems have been developed. Whole smoke exposure systems offer many technical challenges, but represent a more physiologically relevant test system that captures the full interactions of both the particulate and vapour phases together (Fukano *et al*., 2004). An additional advantage of these systems is that a multitude of different cell cultures can be exposed at the air–liquid interface (ALI) to whole smoke, better simulating human exposure (CORESTA, 2007).

Whole smoke exposure systems offer the advantage that all phases of smoke can be analysed together or independently depending on the experimental set-up. This has allowed researchers to tailor their experiments to investigate both phases of tobacco smoke, yielding useful information. There is a variety of whole smoke systems available and the majority of these systems can also be used to deliver individual aerosols or other complex aerosol mixtures to cell cultures. However, at present there is no recognised approach to the measurement of dose, and the vapour phase of cigarette smoke within these systems remains poorly understood. With the variety of exposure

options available to researchers and bespoke systems relatively easy to fabricate or replicate, dosimetry tools may bridge the gap and play an important role, not only in the measurement of actual cellular dose but also in the characterisation and validation of these systems (Thorne and Adamson, 2013).

There are continuous efforts to introduce existing testing methods into regulatory framework of tobacco products risk assessment.

The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA, 2004) *in vitro* Toxicology Taskforce of industry recommends using a test battery composed of the following assays:

1. A bacterial mutagenicity assay. The Ames Salmonella mutagenicity assay is recommended.
2. A mammalian cell assay for cytogenetics/mutation. The Task Force recommends the micronucleus assay, the chromosome aberration assay or the L5178Y mouse lymphoma assay.

3. A cytotoxicity assay conducted with an appropriate mammalian cell line. The Task Force recommends the neutral red cytotoxicity assay (CORESTA, 2004).

Analysis of the recent publication has shown that these recommendations are used increasingly in the toxicity assessment of different tobacco products (see e.g. Manupallo and Sullivan, 2015).

Considering non-cancer endpoints, a battery of *in vitro* tests have been proposed for assessing CVD risk associated with cigarette smoking (Fearon *et al.,* 2013). The battery is comprised of functional *in vitro* assays to model endothelial damage, angiogenesis, and migration of vascular smooth muscle cells as initial and subsequent events in CVD (Fearon *et al*., 2013). Other tests, such as for oxidative stress or inflammatory response, may be conducted to assess the adverse effects of cigarette smoke *in vitro*.

However, it is the opinion of SCHEER that the choice of the test battery should not be fixed *a priori*, and should be rather tailored on the basis of information coming from the in silico and read-across analysis (e.g. bridging *in vitro* studies can be necessary to support the read across).

Table 1 summarises the most recent, internationally accepted, validated *in vitro* methods, which may be used for the toxicity assessment of the tobacco products.

Modified after AltTox (http://alttox.org/mapp/table-of-validated-and-accepted-alternative-methods) and PISC (http://www.piscltd.org.uk/wp-content/uploads/2016/03/PISC_AltMethods_A4)

1 **Table 1 : *IN VITRO* METHODS ADOPTED FOR REGULATORY USE**

Toxicity endpoint	*In vitro* methods		Recommendations and standard methods (OECD)
ACUTE TOXICITY	3T3 neutral red uptake (NRU) cytotoxicity test to estimate starting doses for oral acute systemic toxicity		OECD GD 129, published in 2010
	NHT neutral red uptake (NRU) cytotoxicity test to estimate starting doses for oral acute systemic toxicity		OECD GD 129, published in 2010
ACUTE PHOTOTOXICITY	3T3 neutral red uptake (NRU) phototoxicity test		OECD TG 432, published in 2004
SKIN IRRITATION	Integrated approach on testing and assessment (IATA)		OECD GD 203, published in 2014
	Reconstructed human epidermis (RhE) test	EpiSkin™ (L'Oréal, France)	OECD TG 439, revised in 2015
		EpiDerm™ (MatTek, US)	OECD TG 439, revised in 2015
		SkinEthic™ (L'Oréal, France)	OECD TG 439, revised in 2015
		LabCyte EPI-Model (J-TEC, Japan)	OECD TG 439, revised in 2015
SKIN ABSORPTION/ PENETRATION	*In vitro* diffusion method		OECD TG 428, published in 2004
SKIN SENSITISATION	Adverse outcome pathway (AOP) for skin sensitisation		OECD series on testing and assessment 168, published in 2012
	ARE-Nrf2 luciferase test method (e.g. KeratinoSens™ assay)		OECD TG 442D, published in 2015
	Direct peptide reactivity assay (DPRA)		OECD TG 442C, published in 2015
	Human cell line activation test (h-CLAT)		Draft OECD TG, published in 2014
CARCINOGENICITY	Cell transformation assay (CTA) (Syrian hamster embryo cell transformation assay (SHE CTA)		OECD Guidance Document Env/JM/Mono(2015)18, May 2015
	In vitro cell transformation assays (CTA) Bhas 42 cell transformation assay (Bhas 42 CTA)		OECD Guidance Document Env/JM/Mono(2016) 1, January 2016

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Besides these internationally validated methods and the ISO 10993 – 5:2009 adopted for medical devices, there is a great number of other methods employing technological innovations such as reconstituted human tissue cultures, 3-D organotypic cultures comprised of differentiated human cells in co-cultures, air-liquid interface exposure systems, cell transformation assays and high content genomic analysis that are used for tobacco product analysis.

2.4.3.5 Addictiveness testing

Tobacco comprises of thousands of substances, of which nicotine is the most characterising and most addictive component. Additives, as well as natural tobacco substances other than nicotine, may have addictive capacities themselves or can interact with nicotine and the nicotine receptor system, herewith enhancing the effects of nicotine. For example, these additives can have effects on nicotine bioavailability, duration, and concentration in the blood circulation or nicotine-dependent activation of mesolimbic pathways in the brain. The term 'dependence potential' is commonly used to describe addictive capacity.

Guidelines to assess the impact of tobacco product contents on dependence potential could be similar to those already established for testing the dependence potential of pharmaceutical products (the used methods are known as Abuse Liability Assessment). Special challenges include product complexity and the diverse range of tobacco products (Henningfield *et al*., 2011; WHO Study Group on Tobacco Product Regulation TobReg, 2012). For example, the US FDA has issued guidance that covers dependence potential assessment for a range of different substances, formulations, and product types in which factors such as additives and product design features may act to either promote or deter dependence potential (U.S. Dept. of Health and Human Services, 2010, 2015).

Experimental testing of the dependence potential of tobacco additives is still limited due to the lack of validated administration models for the examined individual compound itself and in co-administration with other tobacco additives. In the proposed step-wise approach, we discuss the possibilities to experimentally quantify the dependence potential of tobacco additives (often) co-administered with nicotine.

In silico

Nicotinic acetylcholine receptor (nAChR) Computer models. nAChRs are ligand-gated cation channels found throughout the central and peripheral nervous systems (Gotti *et al*., 2006; Jensen *et al*., 2005). Neuronal nACh receptors participate in many neurological processes including cognition (Levin and Simon, 1998), pain sensation (Damaj *et al*., 2000), and nicotine reward/addiction mechanisms (Dani and De Biasi, 2001; Pavlovicz *et al*., 2011; Tapper *et al.*, 2004). In the past years several nAChR *in silico* models have been developed integrating protein (sub-) structures, dynamics and functional relationships. Among those, the most widely expressed nAChR subtype in the brain is the neuronal α4β2 nACh receptor (Haddadian *et al*., 2008). The α4β2 nAChR comprises high-affinity nicotine-binding sites (Tapper *et al.*, 2004) but the mechanism how ligand binding leads to channel opening remains elusive. The quality of the current α4β2 nAChR model was evaluated using flexible docking of nicotine docking to the closed- and open-channel models. Besides the potential nicotine interactions with surrounding residues that could stabilize nicotine positions, a high degree of involvement of aromatic residues in the nicotine binding sites was also observed (Haddadian *et al*., 2008). Further development of these models may provide information about how

nicotine and other tobacco additives (ligands) regulate nAChR activation in smoking dependence.

Ligand-based Monoamine oxidase (MAO) models. The enzyme MAO plays an important role in the metabolism of several neurotransmitters by oxidative deamination. MAO-A inhibition is associated with enhanced dopamine activity leading to increased reinforcement behaviour. The combustion of natural or added sugars in tobacco products result in acetaldehyde which reacts in the body with tryptophan and tryptamine. This reaction results in the formation of the beta-carbolines, harman and norharmane, which are MAO inhibitors (Herraiz and Chaparro, 2005; Talhout *et al*., 2007). Other examples of MAO inhibitors isolated from tobacco leaves or present in tobacco smoke are 2,3,6-trimethyl-benzoquinone, 2-naphthylamine and a coffee-extracts and synthesized and modified natural coumarin derivatives (Fowler *et al.*, 2003; Gnerre *et al*., 2000; He *et al*., 2014; Herraiz and Chaparro, 2006).

Ligand-based models can provide new insights in enzyme selectivity, mechanisms of action and the relationship between the MAO inhibitory activity and the molecular structure of the different inhibitors (Vilar *et al*., 2012). There are different types of ligand-based models which can be used, such as Quantitative Structure-Activity Relationship (QSAR) with 2D and 3D descriptors (Johnson, 1976; Vilar *et al*., 2008; Winkler, 2002), 3D- Comparative Molecular Field Analysis (CoMFA) (Cramer *et a*l., 1988; Zhang *et al*., 2011), 3D-pharmacophores (Langer and Hoffmann, 2006) or ligand-network models (Keiser *et al*., 2007; Park and Kim, 2008). QSAR studies have become one of the most popular ligand-based approaches in modern chemistry (Shelke *et al.*, 2011; Vilar *et al*., 2008; Vilar *et al*., 2012) and can also be used to model ligand–based selectivity of different tobacco additives and the potency to inhibit MAO activity.

In vitro

Three-dimensional lung tissue constructs (3D lung-on-a chip) and mathematical computer models. These kind of models are the results of the integration between *in vitro* models (the 3D organ-on-a-chip) and *in silico* models. It has been shown that inhalation during smoking results in a rapid brain increase of nicotine in the brain thereby contributing to nicotine dependence in smokers. Inhalation can be facilitated by certain additives leading to deeper and more frequent inhalation by the cigarette smoker resulting in an increase in lung exposure and nicotine uptake. Additives (e.g. menthol, theobromine and eucalyptol) can achieve this by enhancing sensory properties such as cooling effects or by having local anaesthetic and bronchodilating properties (Usmani *et al*., 2005). Also, a change in the physical properties of tobacco (e.g. particle size) can be altered by certain additives to allow (nicotine) particles to enter deeper levels of the lungs (SCENIHR, 2010a).

The efficiency of nicotine uptake and tobacco additives via the lung in the blood stream is difficult to measure. Engineered 3D lung tissue constructs and mathematical computer models can be used to provide predictive information on lung uptake and particle deposition (Asgharian *et al*., 2012; Nichols *et al.*, 2014; Nichols *et al.*, 2013). These engineered 3D models of human tissue mimic *in vivo* conditions and allow for more natural and robust human *in vitro* respiratory tract models compared to multi-cell *in vitro* models. These constructs can be used to assess cell-based responses, physiologic functions, pathologic changes and even toxicity or responses to tobacco additives.

The 3D lung-on-a-chip can be used also to measure experimentally effects due to any additive in **altering nicotine uptake** such as alkalizing compounds

Capacity to change pH values. Additives that exert capacities to increase the pH values will result in higher amounts of uncharged nicotine (Hurt and Robertson, 1998; Wayne and Carpenter, 2009). This will result in more easily absorption of nicotine by the epithelial cells in the mouth and probably also in the lungs (Tomar and Henningfield, 1997). Although the tobacco industry stresses that the buffering capacity of the lung surface liquid (7mval/pH unit) at pH 7.4 is not changed by nicotine concentrations of 0.1 mg per puff (Holma and Hegg, 1989; Klus *et al*., 2012), it is valuable to check additives or substances for their capacity to change the pH of the tobacco and the smoke.

(Inhibition of) The enzymatic activity of MAO. Additives may influence the dependence potential of nicotine by interacting with the neural responses to the drug. For example, MAO inhibitors that are not leading to dependence on their own slow the breakdown of monoamines such as DA thereby affecting the overall motivational impact of nicotine. Inhibition of the enzymatic activity of MAO can be measured *in vitro* using peroxidase-linked spectrophotometric assay. Enzymes can be isolated from rat liver microsomes or by recombinant generated enzymes. Using recombinant human MAO-A and MAO-B, IC50 values for enzyme inhibition can be experimentally determined (Lewis *et al*., 2007). *In vivo* MAO activity can be analysed using PET (see paragraph 'neurobiological effects using imaging techniques').

CYP metabolism inhibitor ratio. Nicotine is metabolized in the liver by cytochrome CYP2A6 and CYP2B6 enzymes (Hukkanen *et al*., 2005). Inhibition of nicotine metabolism enhances its bioavailability and alters the behavioural effects in mice (Alsharari *et al*., 2014; Bagdas *et al*., 2014). Additives modulating the activity of metabolic pathways are therefore likely to affect the dependence potential of nicotine. The effectiveness of an additive in inhibiting nicotine metabolism is expressed as relative CYP inhibitor ratio (Rahnasto *et al*., 2008). The inhibitory concentration of human and mouse CYP2A can be tested in an *in vitro* assay using recombinant enzyme or human liver microsomal preparations (Rahnasto *et al.*, 2003). Examples of known tobacco additives that inhibit CYP2A6 enzymes are menthol, benzaldehydes and several lactones added to tobacco (Benowitz *et al*., 2004; Kabbani, 2013; Kramlinger *et al*., 2012; Rahnasto *et al*., 2003).

***In vivo* (not recommended as the first choice)**

Biomarker analysis of nicotine. Nicotine bioavailability is defined by an optimal rate of adsorption and distribution from the lungs into the bloodstream. Upon uptake in the lungs, the bioavailability of nicotine in the body is determined by properties such as its hydrophobicity and solubility. It has been proposed that the use of alkalizing compounds (such as ammonia) as tobacco additive increases the absorption of nicotine in the lungs. A biomarker analysis of nicotine in blood samples from smokers of cigarettes with different ammonia yields was performed to evaluate the effects on nicotine bioavailability. Different ammonia yields in cigarettes did not increase the rate or amount of nicotine absorption from the lungs to the arterial blood circulation (McKinney *et al*., 2012; van Amsterdam *et al.*, 2011). It can not be excluded from these studies that other ingredients than ammonium salts influence nicotine adsorption in a similar way.

Dopamine (DA) release and turn over. Activity of neurons in the mesolimbic DA brain area is not only measured by nACh receptor activation but also by measuring the result of this receptor activation, a change in the release or turnover of DA. DA release and turnover can be measured either *ex vivo* or *in vivo* via isolation of specific

brain tissue or microdialysis. A study in mice showed up-regulation of nAChR subtypes in various brain regions upon exposure to nicotine and menthol using western blots. A significant increase in nicotine plasma levels was observed, which was accompanied by an increase of withdrawal intensity (Alsharari SD1, 2015).

Neurobiological effects using imaging techniques- Exerting additive effects on nicotine dependent activation of the mesolimbic pathway can be studied *in vivo* using several neuroimaging techniques like functional magnetic resonance imaging (fMRI), positron emission tomography (PET) and single-photon emission computed tomography (SPECT) (Jasinska *et al*., 2014). Each technique can be used to understand only some aspects of processes involved in tobacco consumption like brain structure (MRI), different aspects of brain function (PET, SPECT, fMRI, and ASL), and pharmacokinetics (PET, SPECT) in animals as well as humans (Kober and Deleone, 2011).

The neuronal activity upon exposure to nicotine and other tobacco additives can be measured by the activation of nACh receptors, neurotransmitter release and transcriptional activation of specific mRNAs (van de Nobelen *et al*., 2016). Labelling and tracing of nicotine, MAO or nAChR can demonstrate nicotine occupancy at nAChRs, nAChR availability and upregulation of nAChRs induced by tobacco smoking (Brody *et a*l., 2014; Jasinska *et al*., 2014; Volkow *et al*., 1999). Substances that inhibit the nicotine degradation or metabolism or serve as a carrier for nicotine can be identified by these derivative endpoints.

PET- is a common functional neuroimaging technique which uses a short-lived radioactive tracer (incorporated into a biologically active molecule as glucose) infused into a living subject. As the tracer decays, the PET system detects pairs of gamma rays that are indirectly emitted in the process, and uses them to localize the tracer to a particular region in the brain. In this way, the concentration of tracer molecules can be estimated at different locations in the brain or other tissue. Several tracers are discussed below.

Radiotracer for nicotine- A PET study with radiolabelled nicotine [(^{11})C]nicotine demonstrated that a single puff leads to a rapid rise in brain nicotine concentration with a gradual wash-out period (Berridge *et al*., 2010). In addition, the nicotine accumulation in the brain during smoking of one full cigarette increases in a linear fashion with successive puffs, rather than in puff-associated spikes and rapid wash-outs. Relating to this finding, the authors reported that dependent smokers showed a slower rate of brain nicotine accumulation then non-dependent smokers (Kober and Deleone, 2011; Rose *et al*., 2010).

Radiotracers for α4β2*nACh receptors- Researchers have also developed brain-imaging radiotracers for α4β2*nACh receptors with radiolabelled A-85380 compounds (Abreo *et al*., 1996) or analogues (2-FA /6-FA for PET and 5-IA for SPECT), having the most widespread use (Koren *et al.*, 1998). However, the slow kinetics of these radio ligands restricts the large-scale use in experimental studies (Sabri *et al*., 2015). New generation α4β2*nAChR specific radioligands are now under development, like [18F]Flubatine, [18F]AZAN and [18F]nifene, demonstrating faster kinetic properties in PET research *(Brust et al., 2008; Hillmer et al., 2011; Hockley et al., 2013; Kuwabara et al.*, 2012; Wong *et al*., 2013).

PET imaging studies demonstrated the effect of cigarette smoking on α4β2*nAChR occupancy, showing that smoking causes displacement of 2-FA for prolonged time (at least several hours) (Brody *et al*., 2006a). Dose-dependent reduction in 2-FA

displacement was observed by both controlling the number of puffs smoked and the nicotine concentration smoked (Brody *et a*l., 2009). These findings suggest that nicotine mediates 2-FA displacement by occupying α4β2* nAChRs. Several authors, using 5IA-SPECT and 2-FA-PET, have shown that habitual cigarette smoking is associated with up-regulation of α4β2* nAChRs (Cosgrove *et al.*, 2009; Mamede *et al.*, 2007; Mukhin *et al*., 2008; Staley *et al.*, 2006; Wullner *et al*., 2008). The nAChR density returns to normal after a prolonged abstinence of weeks to months (Cosgrove *et al*., 2009; Mamede *et al*., 2007). In addition, exposure to nicotine from second-hand smoke resulted in substantial brain α4β2*nAChR occupancy in smokers and non-smokers (Brody *et al*., 2011). Taken together, these results suggest that exposure to cigarette smoke, most likely through the effects of nicotine, influences α4β2*nAChR density in the human brain (Lotfipour *et al.,* 2011*)*.

Radiotracer for Dopaminereceptor (DA)- With radiotracers such as [11C]raclopride and [^{11}C]PHNO (PET) or [123I]IBZM (SPECT), striatal DA release has been reliably measured using PET and SPECT imaging (Laruelle, 2000). An increase in DA competes with the radiotracer to bind at the dopamine receptor; resulting in a decrease in radiotracer binding compared to baseline. This allows calculation of the 'occupancy' of the receptors by DA or a change in binding potential and is an indirect measure of DA release based on the 'occupancy model' (Cosgrove *et al*., 2015; Laruelle, 2000).

Many studies have examined nicotine and tobacco smoking-induced DA release in human subjects. All these studies confirm that smoking elicits ventral striatal dopamine release and is associated with a reduction of craving (Barrett *et al*., 2004; Brody *et al.*, 2006b; Brody *et al.*, 2004; Montgomery *et al.*, 2007; Scott *et al.*, 2007; Takahashi *et al.*, 2008). In the most recent study, smokers were imaged with [^{11}C] PHNO before and after a cigarette (Le Foll *et al*., 2014). Binding potential was reduced after smoking by 12 and 15 % in D2-rich and D3-rich regions, respectively.

A major concern with the existing studies is the timing of the dopamine response. The response to smoking a cigarette is a transient increase in DA. The analysis in these studies, however, use an average of all the data collected over 30 min to up to 2 h. This significantly dilutes measurement of a transient dopamine response (Sullivan *et al*., 2013). Thus, analysis techniques with improved temporal resolution may be better suited to more transient DA release (Cosgrove *et al*., 2015).

Radiotracers for μ-opioid receptors- There is strong evidence for a link between nicotine administration and endogenous μ-opioid mechanisms, mediating some of nicotine's addictive properties and distress during withdrawal (Nuechterlein *et al*., 2016). Acute endogenous opioid release upon nicotine administration was demonstrated in animal and cell culture studies (Boyadjieva and Sarkar, 1997; Davenport *et al*., 1990). However, in human studies the findings are inconsistent. The indirect measures of neurotransmitter release and μ-opioid receptor activation upon nicotine administration, as measured with PET, have shown both a reduction in binding potential (suggesting activation of neurotransmission) and an increase in binding potential (deactivation) or no significant change in different regions of the brain (Domino *et al*., 2015; Kuwabara *et al*., 2014; Ray *et al*., 2011; Scott *et al.*, 2007). Measures at baseline have also shown either lower or no significant differences between smokers and non-smoking controls (Kuwabara *et al.*, 2014; Scott *et al.*, 2007). The μ-opioid system is suggested to be strongly influenced to placebo treatment (Nuechterlein *et al*., 2016; Pecina *et al.*, 2015; Scott *et al*., 2008; Zubieta *et al.,* 2005). Therefore, studying the opioid system does not seem the most sensitive and robust way to define tobacco dependence.

How the administration of tobacco additives changes these effects as measured is largely unknown. A recent PET study showed that in brains of female menthol cigarette smokers, nicotine accumulated faster thereby contributing to dependence. However a role of menthol in enhancing brain nicotine accumulation was not supported by this study (Zuo *et al*., 2014). Another PET study using labelled nAChR subunits showed an upregulation of these receptors in the brain of menthol smokers, indicating a higher nicotine exposure in smokers of menthol cigarettes (Brody *et al*., 2013). However, other mechanisms for menthol-influencing receptor density are possible. Analysis of nicotinic acetylcholine receptor activity *in vitro* shows that menthol inhibits nAChR subtypes in a non-competitive manner (Ashoor *et al*., 2013; Hans *et al*., 2012).

SPECT- In SPECT the same mechanism is used as in PET, but differs in a way that the radioactive tracer directly emits a single gamma ray during decay. The nature of the signal allows for lower resolution images than PET as the SPECT tracers typically have a longer half-life, but scans are more easily performed. There are differences in the physics and chemistry used in PET versus SPECT, but the outcome measure of receptor availability is the same. Depending on the tracer used, PET and SPECT data can quantify regional brain activity (e.g. via glucose metabolism when the tracer is a modified sugar, as in 18F-fluorodeoxyglucose), receptor occupancy (e.g. with 11C-raclopride and dopamine receptors), and pharmacokinetics when multiple measurements are taken after drug consumption.

Functional Magnetic Resonance Imaging (fMRI)- Besides PET, which is already advanced technique in this field of research, fMRI is a promising and non-invasive upcoming technique. In fMRI, blood oxygen levels (brain activity) can be measured in the brain by use of strong magnetic fields. In the first fMRI study on the effect of acute nicotine administration, active smoking participants were injected with nicotine in different concentrations. A dose- and time-dependent increased BOLD signal occurred in several cortical and subcortical regions, with prominent signal changes in the cingulate cortex, dorsolateral and medial orbitofrontal regions (Stein *et al*., 1998) as well as the ventral striatum, amygdala, thalamus and insula (Menossi *et al*., 2013). fMRI studies assessing tobacco additives with a (passive) inhalation tobacco devices can be used to identify brain areas involved in addiction. As there are no validated administration models for smoking or nicotine administration which can be used during scanning this is an important limiting factor in fMRI research.

The majority of the above-mentioned imaging studies focus on chronic exposure in a cross sectional design (smokers vs. non-smokers) at a single point in time. When focusing on the dependence capacity of tobacco additives, studies on acute effects using within subject measurements (placebo vs. additive(s) of interest) is regarded as more valuable. This will improve the sensitivity to picking up small changes in neuronal activity caused by administration of the additive. Repeated exposure and repeated measurements can predict dependence capacity. Further improvement of the study protocol and development of even more efficient radio ligands may be beneficial to find indications for tobacco additives that increase dependence.

Behavioural responses in rodents. Current animal models for tobacco product dependence are based on assessing nicotine dependence rather than dependence of tobacco additives or tobacco products as a whole. These models aim to deliver pure nicotine using an intravenous self-administration paradigm despite the fact that nicotine itself is regarded as a relatively weak reinforcer (Caille *et al*., 2012). Current tests to analyse dependence potential can monitor self-administration, speed of acquisition,

conditioned rewarding effects and drug discrimination (Hoffman and Evans, 2013; Wilkinson and Bevins, 2008; Yararbas *et al*., 2010). Also severity of withdrawal can be measured (Bagdas *et al.*, 2014). Animal models also allow controlling of factors that can affect study outcome such as environmental factors, genetic background and prior drug exposure. The self-administration paradigm has been widely accepted as a reliable animal model with high predictive value for the dependence potential of a drug and can be used to support findings observed in humans. The current available models can possibly be adapted to assess the effect of (nicotine in combination with) other tobacco-related additives on dependence. A recent animal study showed that the sensory properties of menthol can serve as a conditioned reinforcer for nicotine (Wang *et al*., 2014).

Behavioural outcome measures in human. Several behavioural measures can be used to assess dependence in human. Dependence for nicotine and smoke(less) tobacco can be self-assessed using the Fagerström Test for Nicotine Dependence (FTND) or the cigarette withdrawal scale (CWS-21) (Etter, 2005; Etter *et al*., 2003; Fagerstrom, 2012). The FTND uses a twelve-item cigarette dependence scale that covers the main definitions of dependence: compulsion, withdrawal symptoms, loss of control, neglect of other activities, time allocation and persistence despite harm. The FTND can assess the degree or severity of tobacco dependence using a scale indicative for the level of dependence.

The cigarette withdrawal scale (CWS-21) is a 21-item multidimensional self-administered scale that measures withdrawal symptoms and predicts relapse to smoking (Etter, 2005). Recently, a revision for DSM-V was proposed in order to increase the predictive value of these criteria for tobacco dependence assessment (American Psychiatric Association, 2013; Baker *et al*., 2012).

Indicators of nicotine dependence were assessed in menthol and non-menthol cigarette smokers using the FTND. Differences were observed in time to first cigarette of the day (TTF) suggesting greater urgency to smoke but not on amount of cigarettes smoked on a day (CPD) (Collins and Moolchan, 2006; Hoffman and Simmons, 2011). An important limitation of these methods is that these tests are a diagnostic instrument for assessing dependence in people and not necessarily the dependence potential of the given substance or product type.

Recommendations

To accurately assess tobacco dependence potential for regulatory purposes, it is necessary to use multiple evaluation methods, whereby several factors associated with tobacco dependence are analysed. Combinations of techniques examining neurochemical physiological and behavioural changes in specific brain regions with nicotine dependence will provide sufficient and robust information. Correlations between responses and convergence of studies will lead to evidence-based conclusions. For regulatory purposes, consensus needs to be established on the (combination of) tests that are preferred. The SCHEER therefore proposes to use a step-wise approach of 1) *in silico*, 2) *in vitro*, 3) *ex vivo*, and 4) *in vivo* methods- only in exceptional cases, to be agreed with the Receiving Authority on a case-by-case basis. The use of i*n vivo* studies is indeed questionable for ethical reasons therefore these studies are only justified under exceptional circumstances. After negative results of testing the tobacco additive on dependence capacity in the first agreed appropriate method (*in silico)*, the next step should be considered and appropriate test(s) should be selected (*in vitro* models), and

so on. It is strongly advised that *in silico* and *in vitro* tests to assess additive-induced addictiveness by independent organisations are developed and validated.

2.4.3.6 Characterising flavour and inhalation facilitation as contribution to attractiveness

This section will discuss a procedure to assess tobacco products with characterising flavours that are prohibited in the TPD, as well as some other mechanisms that may increase additive-induced attractiveness.

Animal models do not currently exist for the assessment of attractiveness. In humans, the attractiveness of individual tobacco products can be compared in panel studies, surveys and by experimental measures. To test the response to a specific additive, tobacco products can be produced to exclude or include individual additives. However, this type of research is difficult nowadays due to ethical considerations that will often preclude human testing (SCENIHR 2010).

Characterising flavours

Over 80% of all cigarettes contain at least one flavour, and almost half of all additives in any tobacco product is added as a flavour (Pennings *et al*., 2016). Flavours may be added to tobacco, cigarette paper, the filter or to the foil wrapper, in an attempt to enhance the tobacco flavour, mask unpleasant odour, and deliver a pleasant cigarette-pack aroma (WHO, 2007). Many different additives are used to create a specific taste/flavour in order to attract certain target groups. Regarding flavour, the new EU Tobacco Product Directive (TPD, Article 7) prohibits cigarettes and roll-your-own tobacco having a characterising flavour other than one of tobacco, as they could facilitate initiation of tobacco consumption or affect consumption patterns (European Union, 2014). A characterising flavour is defined as a *'clearly noticeable smell or taste other than one of tobacco, resulting from an additive or a combination of additives, including, but not limited to, fruit, spice, herb, alcohol, candy, menthol or vanilla, which is noticeable before or during the consumption of the tobacco product.'* The prohibition of tobacco products with characterising flavours does not preclude the use of individual additives outright, but it does oblige manufacturers to reduce the additive or the combination of additives to such an extent that the additives no longer result in a characterising flavour.

Talhout *et al*. published an inventory of methods suitable to assess additive-induced characterising flavours of tobacco products, and concluded that because flavour perception is subjective and requires human assessment sensory analysis in consumer or expert panel studies is necessitated. They recommend developing validated tests for descriptive sensory analysis in combination with chemical-analytical measurements. Testing a broad range of brands, including those with quite subtle characterizing flavours, will provide the concentration above which an additive will impart a characterising flavour (Talhout *et al*., 2016).

The Commission has recently adopted two implementing acts establishing the rules and mechanism for determining products with characterising flavours[18].

[18] Commission Implementing Regulation (EU) 2016/779 of 18 May 2016 laying down uniform rules as regards the procedures for determining whether a tobacco product has a characterising flavour

The determination of such flavours can concern products before consumption (e.g. before combustion) as well as emissions resulting from normal use (direct and indirect) of the products. To develop a method for determining characterising flavours, and perform some pilot experiments, the Commission contracted the HETOC Consortium in August 2014[19]. The HETOC-consortium carried out, as external contractor, a study on the determination of characterising flavours. Sensory testing complemented by chemical analysis was concluded to be an appropriate method to determine characterising flavours. Their pilot had confirmed that an expert panel is a good approach, but the training phase needs to be more extensive when the real panel is set up. Smelling is the preferred starting point for determining characterising flavours and it was recommended to consider, as a future step, whether a smoking experiment was needed. It was concluded that specific reference spaces for cigarettes and RYO are needed.

Beside the characterising flavour features, other phenomena can contribute to attrarctiveness. According to the partial guidelines for implementation of Articles 9 and 10 of the WHO framework convention on tobacco control, "attractiveness" refers to factors such as taste, smell and other sensory attributes, ease of use, flexibility of the dosing system, cost, reputation or image, assumed risks and benefits, and other characteristics of a product designed to stimulate use. Note that not all of these properties are related to additives. WHO-FCTC advices Parties to regulate, by prohibiting or restricting, ingredients that may be used to increase attractiveness of tobacco products (WHO, 2012). The FCTC guidelines in relation to the regulation of the contents of tobacco products and regulation of tobacco product disclosures call in particular for the removal of ingredients that increase palatability, create the impression that tobacco products have health benefits, are associated with energy and vitality or have colouring properties.

The TPD includes two references to attractiveness. In the introductory considerations, point 13, it is mentioned that *"In order to carry out their regulatory tasks, Member States and the Commission require comprehensive information on the ingredients and emissions from tobacco products to assess the attractiveness, addictiveness and toxicity of tobacco products and the health risks associated with the consumption of such products."* In article 19, Notification of novel tobacco products, it is mentioned that manufacturers and importers of such a product shall provide *'available scientific studies on toxicity, addictiveness and attractiveness of the novel tobacco product, in particular as regards its ingredients and emissions.'*

In the following, the possibility to asses characteristics other than the characterising flavour as contributors to attractiveness are briefly presented, some of which having the possibility of "Facilitating inhalation or nicotine uptake", which is a criterion of the mandate (category c) included in the ToR).

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http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.L_.2016.131.01.0048.01.ENG&toc=OJ:L:2016:131:TOC

Commission Implementing Decision (EU) 2016/786 of 18 May 2016 laying down the procedure for the establishment and operation of an independent advisory panel assisting Member States and the Commission in determining whether tobacco products have a characterising flavour

http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.L_.2016.131.01.0079.01.ENG&toc=OJ:L:2016:131:TOC

[19] http://ec.europa.eu/health/tobacco/products/implementation/characterising_flavours_en.htm

Other sensory attributes which can facilitate inhalation or nicotine uptake

Low irritation to mouth, throat and chest and satisfaction can facilitate inhalation and possibly nicotine uptake (Jaffe and Glaros, 1986; Kochhar and Warburton, 1990). Mildness, a combination of improved aftertaste, less bitterness, improved mouth feeling and reduced irritation, is reported to be appreciated, especially by younger and beginner smokers, with their undeveloped tastes and a low tolerance for irritation from tobacco smoke (Carpenter *et al*., 2007).

Additives that influence these sensory attributes, such as mildness, and a pleasant aftertaste, possibly facilitate smoking initiation. By reducing and changing the harshness of the smoke, special target groups may be reached (Carpenter *et al*., 2005a, Carpenter *et al* , 2005b, Cummings *et al.,* 2002, Klein *et al*., 2008, Wayne and Connolly, 2002). A confidential tobacco industry document describes a class of casing materials referred to as ameliorants used to "*… smooth out harshness and bitterness and/or eliminate pungent aromas from tobaccos*" (Jenkins *et al*., 1997). Examples of such ameliorants included sugars, cocoa and liquorice. Cocoa, also at levels that do not impart a characterising flavour, can alter cigarette flavour and improve product acceptability (Sokol *et al*., 2014). Various sugars constitute a large proportion of additives, and the sweetness of the smoke is an important characteristic. Thus, product appeal for starters may be further diminished by regulating trigeminal attributes as well. Smoking panels can be used to assess sensory attributes like irritation, impact, flavour, aftertaste. For irritation, it may also be possible to use *in vitro* models.

Some additives have multiple chemosensory effects. Pyrazines, which are flavours resulting from pyrolysis of amines and sugars, are reported to induce chemosensory effects such as reducing the harshness and irritating effects of nicotine and other tobacco smoke constituents in the airways. In addition, they may reinforce the learned behaviour of smoking, enhance elasticity and help optimise nicotine dosing. Wayne and Henningfield also describe evidence from internal industry documents that "smokers develop a taste for specific flavors or characteristics of tobacco use other than nicotine, and come to associate use with these characteristics" (Wayne and Henningfield, 2008). Vanilla, for example, increases mildness, and smokers will switch to other vanilla-containing brands, but not to brands without vanilla taste. Menthol is also known for its taste, as well as inducing a "cooling" effect which masks the harshness and the taste of raw tobacco (Lawrence *et al*., 2011).

Harshness and smoothness. According to the tobacco industry definition, harshness is a chemically-induced physical effect associated with a roughness, rawness experience generally localized in the mouth and to a lesser degree in the upper reaches of the throat and the trachea due to inhalation of tobacco smoke. Harshness can also cause a drying, rasping, coarse, astringent sensation usually associated with the smoke flavour of Virginia or air-cured type tobaccos. Harshness is classically measured in four degrees: (i) Free – an absence of harshness; (ii) Touching – a slight awareness of a sensation; (iii) Scratchy – some discomfort, a stinging effect; and (iv) Harsh – rough, raw, raspy, coarse, astringent, painful inhalation. Reducing the harshness of the smoke makes it possible to inhale deeper and increase the number of puffs, as physical barriers will be reduced (Wayne and Henningfield, 2008).

The harshness depends partly on the tar/nicotine ratio, but may also be decreased by certain additives such as propylene glycol or levulinates. Tar provides a strong flavour and mouth sensation, masking the harsher, bitter taste of nicotine which may be unpalatable to new smokers and uncomfortable to established smokers. Certain highly

flavoured additives may also have the same properties to "smoothen" or reduce the harsh irritation of nicotine in tobacco smoke. In order to make the smoke less aversive and permit deeper inhalation, additives such as liquorice and menthol are used. Another approach is to use nicotine salts that do not cause the same irritation, but are still delivering nicotine or keeping the nicotine effect by means of a quicker absorption by ensuring larger amounts of free nicotine (Bates *et al*., 1999, Keithly *et al*., 2005). Finally, the addition of humectants such as glycerol, propylene glycol and sorbitol keep the humidity of the tobacco product at a desired level; dry tobacco generates an unpleasant harsh smoke.

Impact and smoothness. The term "impact" is widely used in tobacco industry research and documents, and is a tobacco industry term for smokers' subjective awareness of the drug effects of nicotine. Organic acids have been used since the 1950s to improve "smoothness" of cigarettes. For example, Philip Morris found that lactic acid decreased subjective ratings of harshness and bitterness, and produced a sweeter flavour. Citric additives have been used not only for reduced harshness and flavour modification, but also to modify smoke pH, to neutralize nicotine "impact" (an industry term denoting the organoleptic sensation caused by nicotine; smokers often describe this as "throat catch" or "throat hit"). Tartaric and lactic acids likewise modify the pH of smoke. All of these organic acids increased smoothness and are associated with a decrease in nicotine "impact" (Philip Morris, 1989) However, it is unclear whether these effects are due directly to pH modification. Unregulated botanical and chemical additives might have "multiple-use" purposes, such as enhancing flavour and producing "smoother" cigarette smoke, as well as potentially preventing or masking symptoms associated with smoking-related illnesses (Rabinoff *et al*., 2007).

Facilitate the inhalation of tobacco smoke. Certain ingredients have local anaesthetic effects. As a result, coughing due to inhalation of irritating smoke is dampened and the smoker can inhale the smoke deeper (and more frequently). Examples are etheric oils, such as menthol and thymol.

Appearance, smell and irritation of tobacco smoke. In order to make the smoke more attractive not only to the smoker, but also to other people in the proximity of the smoker, it is important that the smoke is appealing and not annoying. This may be attained with additives that make the smoke whiter and more attractive to people seeing the smoke. The TPD prohibits additives having colouring properties for emissions.

Reduced visibility of side-stream is accomplished by the addition of magnesium oxide, magnesium carbonate, sodium acetate, sodium citrate and calcium carbonate to the wrapper (cigarette paper). This has an effect on particle size; particles become smaller and therefore do not easily scatter light and become less visible. Reducing side-stream emissions is based on encapsulating the smoke in an impermeable cone using different types of additives such as potassium succinate, potassium citrate and magnesium carbonate. By combining the use of additives and the look of the tobacco product, greater acceptance of the smoke may be created. Less resistance may be encountered from persons who do not smoke, and at the same time greater pleasure for the smoker may be created. The same agents may also be used to target the individual product at certain target groups (Carpenter *et al*., 2005a, Connolly, 2004).

The smell of the smoke may be also changed so that it is also more attractive and less irritating (Connolly *et al.,* 2000, Ling and Glantz, 2005). Connolly *et al*. (2000) examined tobacco industry patents covering the function of environmental tobacco smoke masking.

These strategies include reducing smoke odour, and reducing side-stream smoke visibility and emissions. Methods to neutralize or reduce lingering smoke odour include addition of acetylpyrazine, anethole and limonene to modify the side-stream odour. These compounds have rather low odour thresholds, and are subsequently easily picked up, while they elicit no trigeminal nerve response. Aroma precursors, e.g. polyanethole provided a noticeable fresher, cleaner and less irritating cigarette side-stream aroma, while others (e.g. cinnamic aldehyde, pinanediol acetal) produce slightly sweet, spicy, clean, fresh, and less cigarette-like aroma. In addition, more "classic" additives (e.g. vanillin, benzaldehyde, bergamot oil, cinnamon/cinnamon extract, coffee extract and nutmeg oil) modify sidestream odour.

Studying sensory effects

Neuro-imaging techniques are used to provide insight into brain processes related to sensory perception. The brain integrates sensory inputs such as taste, touch and smell, and the resulting neural activation can be studied by e.g. fMRI and PET (Small, 2012). Many brain areas are involved, such as brainstem, amygdala, and the orbifrontal cortex. Odours, tastants, and trigeminal stimuli (intranasal irritants) are processed within the olfactory network, gustatory network, and trigeminal network, which are interacting networks (Lundstrom *et al*., 2011). The widespread network involved in the processing of odorants, tastants, and chemical irritants recruits several key cerebral areas, including those responsible for emotions, memories, and reward. Reward consists of the psychological components learning, affect, and motivation (Berridge and Robinson, 2003). Physiological, emotional, cognitive and sensory responses caused by flavours and odours can be tested, also in relation to 'reward dose' in the brain (SCENIHR, 2010b). In research projects conducted by Philip Morris from 1982 to 1995, electroencephalography (EEG), pattern reversal evoked potential (PREP), and chemo-sensory event-related potential (CSERP) were used to measure physiological, sensory, and cognitive changes related to nicotine and to cigarette additives (Rabinoff *et al*., 2007).

2.4.3.7 Interaction of the additive with other additives/ingredient

Tobacco smoke is a complex mixture: 9582 chemical components have been identified so far in tobacco and tobacco smoke (Rodgman, Perfetti, 2013), its composition is qualitatively and quantitatively not fully known and may change, depending on the brand. In addition, tobacco being a natural product, its composition is variable over time from batch to batch even within the same brand. One of the major limitations of using *in vitro* and *in vivo* test systems for the toxicological study of inhalational exposures to compounds in tobacco mixtures is the very high number of components in tobacco smoke and the extreme variability of the mixture. The large number of additives (~ 1260 –SCENIHR Tobacco Opinion 1, 2016) present in the Industry's repertoire add further qualitative and quantitative variability to the mixture. The list is open ended, which means that at present there is no restriction on the use of any number of additional additives as alternative chemicals, precursors etc. Moreover, several of these additives are botanical extracts, which, per se are composed of hundreds of components such as flavours, sugars, pH modifiers. These botanical and phytochemical additives are claimed to have a number of properties, including anaesthetic, antibacterial, anticancer, anti-inflammatory, antifungal, and antiviral properties (Rabinoff, 2007), but these 'apparently beneficial' activities cannot justify their use as additives in tobacco products. Indeed, in some cases, they provide for a "smoother" smoking experience by masking

adverse symptoms caused by smoking (e.g., cough), preventing awareness in the consumer and reductions in cigarette consumption.

The specific purpose and the required concentration is well researched and optimized by tobacco industry before any product is marketed; therefore additives included in the composition of each tobacco product represent a mixture itself. This 'optimal' mixture of additives is intentionally added to a known toxic, carcinogenic and addictive product in order to make the product more palatable by masking the bitter taste, improving the flavour and reduce the irritation of inhaled smoke, optimising nicotine uptake.

The possibility that chemical interactions can occur among different tobacco components and additives and among different additives cannot be excluded. These interaction can consists of direct chemical reactions, forming additional different compounds, or being translated in addition, potentiation or antagonism of the effects induced by additives and tobacco components (being possible that such an interaction occurs at the level of toxicokinetics and at toxicodynamic level). The number of possible interactions and the number of test combinations increase exponentially with increasing numbers of compounds in a mixture. Moreover, the number of experimental groups will also increase with the number of doses of each compound.

As for the general issue of mixture toxicity, in this specific case it can also not be solved by applying an experimental approach, since to test the thousands of possible mixture is not feasible, beside the fact that as previously discussed, their composition is variable. This is an additional reason for not considering suitable the approach of using comparative testing strategies, where differences in effect of the tobacco product with and without the additive are evaluated (see paragraph 3.4.1).

The frameworks for assessing chemical mixtures have been proposed at international and national levels and addressed in several guidance documents.

In most of these documents, the focus is on the mode of action of specific compounds: chemicals with common modes of action will act jointly to produce combination effects that are larger than the effects of each mixture component applied singly. The approach suggested by the non-food SC on mixture toxicity opinion (SCHER, 2012) as well as the one proposed by EFSA (EFSA, 2012 and 2013 on pesticides) can be followed. A case-by-case approach could be useful to define specific testing.

Both the non-food SC and EFSA made use of the WHO/IPCS Framework for Risk Assessment of Combined Exposures to Multiple Chemicals. It is a tiered framework for organising risk assessment tools and data in order to conduct an assessment of combined exposure to multiple chemicals, starting with screening level assessment and proceeding to more complex approaches. The tools and data to be employed are decided on a case-by-case basis by the risk assessor, in order to address the problem at hand, e.g. contaminated site, chemicals in surface water, etc. (Meek *et al.,* 2011).

EU project EuroMix (European Test and Risk Assessment Strategies for Mixtures) will provide a test strategy and test instruments using novel techniques for mixture testing based on new and already existing toxicological tests. The tests will result in data needed for refining future risk assessment of mixtures relevant to national food safety authorities, public health institutes, the European Food Safety Authority (EFSA), the European Chemical Agency (ECHA), industry, regulatory bodies and other stakeholders. https://www.euromixproject.eu/

The following examples show the interactions of compounds with similar function/ activity on the one hand and camouflaged effect of botanicals on the other hand revealing the complexity of ascertaining interaction between additives.

Any additives able to interfere with nicotine bioavailability independent on the mechanism can be the cause of addition or synergism of effects. Using at the same time additives altering the pH of tobacco (e.g. alkalising agents such as ammonium compounds facilitating nicotine passage through the cell membrane in the uncharged volatile form), together with substances such as menthol and thymol, facilitating the inhalation of tobacco smoke (due to local anaesthetic effects) or bronchodilators, such as theobromine (generated from cocoa, caffeine and glycyrrhizine) all together would have a non-negligible impact on nicotine bioavailability in the body. Although they present a low concentration singularly, their action can be deemed as non-relevant.

To these considerations should be added the use of additives that interfere with nicotine metabolism, additives such as the gamma-aliphatic lactones group (see SCENIHR opinion 1, 2016) are mild to weak inhibitors of CYP2A5 and CYP2A6. As CYP2A6 is involved in the metabolism of nicotine, the presence of these chemicals could decrease smokers' metabolism of nicotine and maintain higher blood levels (thus increasing smokers' exposure to nicotine by slowing degradation of nicotine in the bloodstream). Furthermore, the inhibitory effect of these chemicals on CYP2A6, although relatively weak in isolation, might be greater when the chemicals act in combination.

Several patents discussed direct "beneficial" physiological actions of botanical additives. In one US patent cited, it was noted that nicotine in cigarettes has a deleterious vasoconstrictive effect on the cardiovascular system, particularly the blood vessels within and surrounding the heart. It was also noted that vaporized niacin in cigarette smoke has a vasodilating action that helps counteract the vasoconstrictive effect of nicotine. Furthermore, additional "beneficial" effects may be obtained when niacin is combined with rutin (a chemical found in many botanicals), "which is considered effective in reducing and preventing capillary fragility." The patent listed 33 botanicals or vegetable materials, or compounds within them, which also appear on the tobacco industry cigarette additive list (Rabinoff, 2007).

Usage of fruit and vegetable extract concentrates/ botanicals can also give an impression of health benefit to the consumer, so could be considered under the TPD Article 7 2 a. This kind of information is very important as more than 100 of 599 documented cigarette additives have pharmacological actions that camouflage the door of environmental tobacco smoke emitted from cigarettes, enhance or maintain nicotine delivery, could increase the addictiveness of cigarettes, and mask symptoms and illnesses associated with smoking behaviours (Rabinoff, 2007).

2.4.4 Step 4: Reporting

In the fourth and last step, a report needs to be drafted on the activities carried out in Steps 1-3, to be sent to the relevant authorities. The report should include an overall evaluation of the results from Step 1-3. In Annex I a reporting template is provided for this purpose.

In order to limit the financial and administrative burden for both industry and authorities, and the subsequent evaluation of the submitted reports by independent institutes, the formation of consortia and joint reports by industry is endorsed.

2.5 Specific knowledge gaps for the priority list tobacco additives

In addition to the general strategy described in the previous paragraphs, the major data gaps already identified in Tobacco Opinion 1 for the 15 additive included in the EU Commission priority list have been analysed. The analysis was based on the 'Rational for inclusion' taken from Opinion I.

Based on that, the activities to be performed upfront will be described, in order to identify the most appropriate steps (and end-points) to be carried out and to speed up the process, making possible testing feasible in the 18 month time-frame. In same cases (e.g. identification of CMR properties of the unburnt form) it would be possible to identifiy whether or not they should enter the evaluation procedure (having properties that do not meet the criteria of the TPD). Starting at the lowest step, for each of the 15 additives on the priority list, recommendations for experimental activities to fill the data gaps recognised in Tobacco Opinion 1 are given. If the outcome is negative (i.e. no effect which does not meet the TPD criteria is demonstrated), they will enter the general strategy of testing and be considered as any other compound. Although the selection was based on the data available, it is recommended to address the extensive literature search also for the 15 priority list chemicals and to apply the WoE approach, as described in step 1.

2.5.1 Carob bean

Synonyms: Locust bean extract, St. Johns bread extract

CAS number: 9000-40-2/84961-45-5

Rational for inclusion

Carob bean extract is rich in carbohydrates/sugars. It pyrolyses extensively and the combustion of the high carbohydrate/sugars leads to formation of carcinogenic and toxic compounds (e.g. benzene, polycyclic aromatic hydrocarbons, and phenol), aldehydes (acetaldehyde, formaldehyde, and acrolein), organic acids and caramel colour and flavours.

The aldehydes, acetaldehyde, acrolein and 2-furfural can be generated from the combustion of the sugars contained in carob bean extracts. Different combinations of aldehydes are generated and it is likely aldehydes other than acetaldehyde intervene directly or through the generation of new compounds in the smoke in the inhibition of MAO. Converging data indicate that MAO (monoamine oxidase) inhibitors contained in tobacco and tobacco smoke act synergistically with nicotine to enhance addiction potential (SCENIHR 2010). In addition, toxic aldehydes are also formed. Carob bean extract has a sweet, fruity, chocolaty flavour and contributes to making smoking more attractive by improving flavour, thereby masking its bitter taste and reducing the harshness of smoking.

Carob bean extract is a chemically undefined complex additive containing hundreds of chemicals. Information on the exact chemical composition of this complex tobacco additive is lacking (e.g. carbohydrate, proteins/amino acids and fats, pH modifiers, and psychoactive chemicals). Moreover, analytical information on the number and concentration of flavour compounds including 'character impact compounds', present *per se* and generated upon heating is also not available in the public domain.

For example, pyrazines are important flavour impact compounds that are formed under pyrolytic conditions via reactions between amines and carbonyl compounds, generally sugars. Several pyrazines are also reported as additives to cigarettes to impart flavour in low tar cigarettes. (Alpert *et al*., 2015).

This information can facilitate the assessment of the influence on the carob bean extract on palatability, pro-addictive effect and the interaction with other additives and tobacco chemicals.

Priority activities

Step 1: Data on the chemical composition of the carob bean extract should be provided by industry with emphasis on the concentrations of constituents of relevance;

Step 2: Some information on the effect of pyrolysis of carob bean extract is available, however, it is necessary to:

- further chemically define its pyrolysis products and

- evaluate the CMR properties of its pyrolysis products.

In case of positive results for genotoxicity/carcinogenicity of its pyrolysis products the use of carob bean extract as a tobacco additive would not meet the TPD requirement and no additional testing would be required.

If it is not proven, the additive can enter the tiered procedure for evaluation. The assessment of its pyrolysis product on palatability, pro-addictive effect and the interaction/synergistic effect with other additives and tobacco chemicals should be presented (Step 4).

2.5.2 Cocoa and cocoa products (powder, extracts, shells of cocoa bean etc.)

Complex mixture from Theobroma cacao beans

CAS Numbers: 95009-22-6 (cocoa powder), 84649-99-0, 84649-99-3 (cocoa extract)

Rational for inclusion

Many forms of cocoa additives such as extracts and powders are used frequently and in relatively high amounts. Added as flavour or casing to tobacco (cocoa extract is the most abundantly used, with 847 counts in NL ingredient lists, none in NTM, total number of brands 4265), average (weight %) 0.105 (0.198). The maximum amount of cocoa as tobacco additive is around 1 % of the total tobacco weight (RIVM, 2012).

Regarding toxicity, the effects of cocoa inhalation through smoking have not been studied. The risk associated with the generation of combustion products produced upon cocoa pyrolysis has not been thoroughly studied and thus, conducting an adequate risk assessment for cocoa or its pyrolysis products is currently not possible.

Regarding addictiveness, several pharmacological effects of cocoa-derived ingredients were reported, including the bronchodilatory effect of theobromine and caffeine, which result in improved bioavailability of nicotine, although data available so far indicate that

the content of theobromine per cigarette seems to be too low to have a bronchodilating effect on the lungs (SCENIHR, 2010). Furthermore, reaction products of tryptophan, phenylethylamine, tryptamine and tyramine, are thought to exert monoamine oxidase-inhibiting properties. In general, the pharmacologically active substances present in cocoa do not exclude a psychopharmacological effect in humans, owing to the low exposure concentrations and/or the inability of these substances to cross or reach the blood-brain barrier. Due to a lack of studies specifically on the psychoactive effects of cocoa added to tobacco, there is insufficient evidence that adding cocoa to tobacco makes cigarettes more addictive.

Regarding attractiveness, the addition of cocoa to tobacco is intended to enhance flavour. More data are needed on the amount of cocoa that imparts a noticeable flavour.

Priority activities

Based on the available data, cocoa and cocoa products may increase attractiveness and addictiveness and increase inhalation and nicotine uptake. The percentage of cocoa used in cigarettes ranges from 0.2% to 0.66%. The content of theobromine and caffeine per cigarette may be too low to have a bronchodilating effect on the lungs and thereby increase the absorption of nicotine. Therefore, there is uncertainty with regard to the direct effect of cocoa additives on the bioavailability of nicotine and more studies are required.

Step 2: Pyrolysis of cocoa results in the generation of minor amounts of phenol, o-, m-, p-cresol, xylenols, catechol, palmitic acid and stearic acid (<0.001% (w/w) in tobacco) and nitrous gases, carbon monoxide and dioxide. Tryptophan combustion can generate 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole and 3-amino-1-methyl-5H-pyrido-(4,3-b) indole. Furthermore, tryptophan contains reactive groups and forms reaction products with other compounds during combustion, such as beta-carbolines, including harman (RIVM, 2002). The resulting anti-depressive effects of harman have been suggested to contribute to addiction caused by cigarette smoking. Reaction products of tryptophan, phenylethylamine, tryptamine and tyramine, which are formed during combustion, are thought to exert monoamine oxidase inhibiting properties. Nevertheless, the risk associated with the generation of combustion products produced upon cocoa pyrolysis has not been thoroughly studied and should be carefully evaluated.

Step 3: The exposure to cocoa and cocoa-derived ingredients transferred to cigarette smoke in their pure forms is negligible compared with the exposure to these compounds through food and drinks (RIVM, 2002). However, the consequences of the exposure through inhalation have not been studied. Exposure through smoking should not be neglected as it represents two different types of exposure through inhalation of (1) cocoa itself and (2) combustion products of cocoa and its ingredients (RIVM, 2002).

Several mechanisms of enhancing addictiveness of smoking have been proposed, however, it is unclear whether sufficient amounts of psychoactive compounds are produced to exert psychopharmacological effects that would increase addictiveness. Chocolate flavour may make cigarettes more palatable to younger, first time users and may indirectly facilitate dependence by providing enhanced flavour and mouth sensations, potentially serving as a cue for drug reward. Due to a lack of studies specifically directed to the psychoactive effects of cocoa compounds added to tobacco on

addiction, there is insufficient evidence that the addition of cocoa to tobacco contributes to the addictive properties of cigarette smoking.

The addition of cocoa to tobacco is intended to enhance flavour and therefore smoking may result in a characterising flavour. However, although a considerable percentage of cigarette weight could be cocoa additives, it is not known to what degree this influences the flavour of inhaled mainstream or side stream smoke, and especially how this might influence smoking initiation in youths (Fowles, 2001).

2.5.3 Diacetyl

CAS-nr: 431-03-8
Synonyms: butanedione, butane-2,3-dione

Rational for inclusion

Diacetyl exposure may lead to serious lung disease after inhalation. For a proper risk assessment, it is necessary to better characterise the concentrations in mainstream smoke. SCOEL accepted that there is uncertainty about the importance of the genotoxicity of diacetyl. There were no data on carcinogenicity. In addition, it can create a characterising flavour, which can contribute to increasing attractiveness.

Priority activities

Based on the rational for inclusion, the mean open questions concerning diacetyl are:

- Uncertainty concerning the genotoxicity of diacetyl and no data on carcinogenicity.
- No sufficient data was found concerning the burned (pyrolysis) product.
- Exposure may lead to lung disease after inhalation and should be assessed in appropriate tests.
- Due to the typical flavour of diacetyl it is unclear whether the compound can add to the olfactory cue and attractiveness of the smoking product. The question whether this flavour is distinguishable (attractiveness) and/or has a "smoothing" effect on the smoke (attractiveness/addictiveness) remains unclear.

The first activity to be carried out is related to the need to rule out the genotoxicity of the compound. In case of identification of genotoxic potential, the additive will be out according to the TPD provisions, otherwise it should enter the step-wise procedure.

Step 1: Additional data should be searched to address the above mentioned questions

Step 2: Pyrolysis products should be studied

Step 3: Toxicity testing for inhalation exposure and then for characterizing flavour, inhalation facilitation and addictiveness (considering its potential characterising flavour as well as the "smoothing" effects.

2.5.4 Fenugreek extract

Synonyms: fenugreek (trigonella foenum graecum l.) extract, resin, & absolute
CAS number: 84625-40-1
Physical properties: Complex mixture, dark brown paste

Rationale for inclusion

Natural/botanical concentrates/extracts/resins (e.g. from several fruits - fig, plum, raisins, fenugreek, carob, cocoa, caramel, rum, etc.) form a large number of tobacco additives. They are poorly characterised complexes of several to hundreds of chemicals; the composition further depends upon variable factors influencing botanical source andpreparation methods. Although generally recognised as safe as food additives and flavours, this classification is not valid for their inhalation effects and pyrolysis products in tobacco smoke. The combustion/pyrolysis chemistry of each of these additives is not well known in terms of their physiological, toxicological and synergistic additive effects to potentiate the harmful effects of tobacco smoke.

However, many of the botanical extracts have a rich carbohydrate/sugar content, together with varying amounts of proteins, amino acids and other flavour compounds. The pyrolysis of this class of compounds has been well reported. Upon combustion/pyrolysis at temperatures (up to 900°C) attained during smoking, these compounds, especially the carbohydrates, give rise to a complex mixture of toxic, carcinogenic and mutagenic compounds, as well as aroma/flavour compounds.

Compounds formed include smoothing agents (e.g. organic acids), flavours (e.g. caramel), compounds that facilitate nicotine delivery (e.g. aldehydes) and compounds with CMR properties (e.g. PAHs, formaldehyde). Moreover, pyrazines are important flavour impact compounds that are formed under pyrolytic conditions via reactions between amines and carbonyl compounds, generally sugars. Several pyrazines are also added as additives to cigarettes to impart flavour to low tar cigarette (Alpert *et al*., 2015). The complex mixtures used as additives cause tremendous harm and contribute to CMR properties, addictiveness and attractiveness of tobacco smoke.

Priority activities

Step 1: Data on the chemical composition and specification of the Fenugreek extract (powder, concentrate) should be provided by industry with emphasis on the concentrations of constituents of relevance, production procedure, maximum levels for microorganisms and possible contaminants. According to available information, Trigonella foenum-graecum seeds contain mucilage, trigonelline, 4-hydroxyisoleucine, sotolon, diosgenin, phenolic acids, and protodioscin.

The use of fruit and vegetable extract concentrates, such as fenugreek extract, are acknowledged to be beneficial to health, fig extract can thus give an impression of health benefit to the consumer, so could be considered under the *TPD Article 7 2a*. Moreover, as it is also used as medicinal product, this could also give the impression of health protection.

Step 2: Fenugreek extract does not transfer intact to the mainstream smoke, but undergoes extensive pyrolysis. Based on the available studies (Baker and bishop 2005), pyrolysis products from fenugreek extract include, pyridine, benzene (carcinogen), toluene and furfural The pyrolysis products once characterised should be evaluated along the procedure.

If the evaluation shows that it is warranted to move on to step 3, the effects which have been considered as matters of concern (e.g. neuropharmacological activities, CNS depressant and stimulant as well as allergic reaction and exacerbation of asthma should be investigated first. The burden of proof is on the industry to use the proposed step-

wise system and the general strategy described, to prove that the additive is safe on all counts of toxicity, addictiveness and characterizing flavour in the unburnt and burnt form.

2.5.5 Fig extract

Complex mixture (ficus carica l. extract)
CAS number: 90028-74-3 (any other related one if used)
CoE number: 198

Rationale for inclusion

Natural/botanical concentrates/extracts/resins (e.g. from several fruits - fig, plum, raisins, fenugreek, carob, cocoa, caramel, rum, etc.) form a large number of tobacco additives. They are poorly characterised complexes of several to hundreds of chemicals; the composition further depends upon variable factors influencing botanical source and preparation methods. Although generally recognised as safe as food additives and flavours, this classification is not valid for their inhalation effects and pyrolysis products in tobacco smoke. The combustion/pyrolysis chemistry of each of these additives is not well known in terms of their physiological, toxicological and synergistic additive effects to potentiate the harmful effects of tobacco smoke.

However, many of the botanical extracts have a rich carbohydrate/sugar content, together with varying amounts of proteins, amino acids and other flavour compounds. The pyrolysis of this class of compounds has been well reported. Upon combustion/pyrolysis at temperatures (up to 900°C) attained during smoking, these compounds, especially the carbohydrates, give rise to a complex mixture of toxic, carcinogenic and mutagenic compounds, as well as aroma/flavour compounds.

Compounds formed include smoothing agents (e.g. organic acids), flavours (e.g. caramel), facilitating nicotine delivery (e.g. aldehydes) and with CMR properties (e.g. PAHs, formaldehyde). Moreover, pyrazines are important flavour impacting compounds that are formed under pyrolytic conditions via reactions between amines and carbonyl compounds, generally sugars. Several pyrazines are also added as additives to cigarettes to impart flavour to low tar cigarettes (Alpert *et al*., 2015). The complex mixtures used as additives cause tremendous harm and contribute to CMR properties, addictiveness and attractiveness of tobacco smoke.

Priority activities

Step 1: Data on the chemical composition of the fig extract should be provided by industry with emphasis on the concentrations of constituents of relevance, production procedure, maximum levels for microorganisms and possible contaminants. The use of fruit and vegetable extract concentrates, such as fig extract are acknowledged to be beneficial to health, fig extract can thus give an impression of providing a health benefit to the consumer, so could be considered under the *TPD Article 7 2 a*.

Step 2: It does not transfer intact to the mainstream smoke, but undergoes extensive pyrolysis. Information available so far indicates that pyrolysis products include formation of benzene, toluene; in addition the combustion of the sugars lead to the formation of carcinogenic polyaromatic hydrocarbons, a variety of aldehydes, such as acetaldehyde (irritant and possible carcinogen), acrolein (irritant), 2-furfural and a mixture of organic

acids. Different combinations of aldehydes are generated and it is likely aldehydes other than acetaldehyde intervene directly or through the generation of new compounds in the smoke in the inhibition of MAO. Converging data indicate that MAO (monoamine oxidase) inhibitors contained in tobacco and tobacco smoke act synergistically with nicotine to enhance addiction potential (SCENIHR 2010). The burden of proof is on the industry to to use the proposed step-wise system and the general strategy described, to prove that the additive is safe.

2.5.6 Geraniol

CAS number 106-24-1

Rational for inclusion

Geraniol is a known flavouring agent for food and is added to tobacco products for flavouring (one of the factors potentially contributing to attractiveness). More data are needed on the amount of geraniol that imparts a noticeable flavour other than tobacco. No data are available regarding addictiveness.

To perform a toxicity risk evaluation, it is necessary to know the exposure level of geraniol through cigarette smoking. Therefore, research is needed to determine the amount of geraniol in mainstream cigarette smoke. However, considering that the toxicological properties of geraniol are mainly linked to a high potential for skin sensitisation (in addition to skin and eye irritation), no levels considered safe for the majority of consumers could be established from the available data. Geraniol oxidation products (e.g. geranial, epoxy-geraniol, epoxy-geranial) are also potent sensitizers in animals. It could be expected that geraniol would be a respiratory sensitiser (although no information is available on this issue).

It is unknown if geraniol combustion products (about 10-15% of the additive) formed upon smoking a cigarette are toxic or not. Additional pyrolysis experiments are recommended.

Priority activities

Step 1: The chemical characterization of the additive is of paramount importance: indeed, geraniol can contain the relevant impurity methyleugenol, which is a genotoxic carcinogen. Only in the absence of that impurity, proven by specific certificate of analysis, could geraniol be considered as a possible candidate as a tobacco additive.

Step 3: In the case of geraniol, the local toxicity is relevant, as it is a known skin and eye irritant. Furthermore, geraniol and many of its oxidation products (by air oxidation and by metabolic transformation) have already been proven to be skin sensitisers in predictive animal tests. Indeed, geraniol is included among the fragrance substances of clinical importance known to be a prehaptens as well as a prohaptens.

For skin sensitisation, the SCCS considered that 0.01% could be efficient in limiting elicitation. No data are available for irritation of mucosa in the airways as well as for respiratory sensitisation, but since there is a high potential for inducing that kind of effects, also considering that the few pyrolysis studies available indicate that geraniol is mainly (85-90%) transferred intact to smoke, these should be tested first. In case of

positive results, the use of geraniol as a tobacco additive should be not allowed and no additional testing would be necessary.

In case it could be demonstrated that geraniol is not a respiratory irritant and sensitizer, the additive can enter the procedure for evaluation.

Step 1: The collection of the available data could be useful anyway, although as already indicated in Tobacco Opinion I, this would lead to confirming that data are available to demonstrate that pure geraniol did not induce gene mutations in *Salmonella typhimurium* and mammalian cells and although equivocal response resulted in an *in vitro* clastogenicity test, its genotoxicity can be reasonably considered eligible. In addition, after long-term studies no carcinogenicity potential was attributed to food grade geranyl acetate (29 % citronellyl acetate and 71% geranyl acetate). Therefore those end-points are already addressed. Since the oral absorption has been demonstrated to be >80%, the systemic toxicity after inhalation (also assuming a total absorption through the lung (100%) the effects are not expected to be different. Since the relevant NOAEL are relatively high (558 mg geraniol/kg bw per day for rats and 279 mg geraniol/kg bw/day for mice), systemic general toxicity is not considered relevant at the doses used as tobacco additive. Therefore these end-points are addressed.

Step 2: additional standardised pyrolysis experiments are recommended to identify the products formed other than geraniol, to be then evaluated for their toxicological properties plus attarctiveness and addictiveness.

Step 3: characterising flavour should be addressed first, since geraniol is a known flavouring agent for food and is added to tobacco products for flavouring (one of the factors potentially contributing to attractiveness). More data are needed on the amount of geraniol that imparts a noticeable flavour other than tobacco.

2.5.7 Glycerol

CAS number: 56-81-5

Rational for inclusion

Glycerol is added as a humectant to tobacco (to help keep it moist). Its addition is mostly during the "casing" of the tobacco. The amount of glycerol present in cigarettes depends on the cigarette brand. The levels of glycerol added to tobacco in the EU is reported to be on average 1.1 %, with a maximum level comprising 4.5 % of the total weight.

Regarding toxicity, it was reported by the tobacco industry that the transfer rate of glycerol to mainstream smoke is 12 %. A risk assessment procedure using a Margin of Exposure (MOE) analysis concluded that there are risks of effects on the respiratory tract epithelium from glycerol. No thorough assessment on systemic effects was done.

Pyrolysis studies indicate almost 100 % intact transfer of glycerol (Baker & Bishop, 2004; Purkis *et al.*, 2011). However, it was found that less than 0.1 % of the blend glycerol is converted to acrolein in mainstream smoke for different cigarette designs and smoking regimes tested (Yip *et al.*, 2010). Acrolein is a toxic compound that is highly reactive and causes irritation in the respiratory tract. The relationship between added glycerol and acrolein formation is unclear and further research is needed.

Regarding addictiveness, no data were reported to suggest that glycerol plays a role in smokers' addictiveness to cigarettes.

Regarding attractiveness, humectants are added to trap water, thereby keeping the moisture in the tobacco and preventing it from drying out. Glycerol is, therefore, considered to positively influence the attractiveness of cigarette smoking, given that humidification improves the palatability of cigarettes. Glycerol does not have a strong flavour, and is, therefore, not expected to impart a noticeable flavour.

Priority activities

Step 2 Step 3: Pyrolysis studies have found that glycerol is converted to acrolein in mainstream smoke and is also generated during the combustion of many other products in tobacco. Both glycerol and acrolein cause irritation to the respiratory tract and acrolein is highly reactive. The relationship between added glycerol and acrolein formation is unclear and further research is needed.

The additive effects of glycerol or its reactivity with other compounds should be further investigated.

No thorough assessment of the systemic effects of glycerol has been done so this should be looked at further.

2.5.8 Guaiacol

CAS number 90-05-1

Rational for inclusion

Guaiacol is a known flavouring agent for food and is added to tobacco products for flavouring (one of the factors potentially contributing to attractiveness). More data are needed on the amount of guaiacol that imparts a noticeable flavour other than tobacco.

Its use as a local anaesthetic can enhance smoke inhalation, thus potentially contributing to addictiveness.

To perform a toxicity risk evaluation, it is necessary to know the exposure level of guaiacol through cigarette smoking. Therefore, research is needed to determine the amount of guaiacol in mainstream cigarette smoke.

Guaiacol is a severe eye irritant, a skin irritant and also reported to be a respiratory tract irritant. Other toxicological information on repeated exposure is scant. On the basis of results on structurally related compounds, effects are likely related to the irritation potential at the contact site, generating hyperplasia. Apart from the absence of mutagenicity tested with the Ames test, the only genotoxicity test on mammalian cells gave positive results (SCE in human lymphocytes). More data are needed for a better evaluation.

Pyrolysis experiments performed with lignin found many guaiacol derivatives besides guaiacol itself and suggest that it transfers largely intact into the smoke.

Priority activities

Since the genotoxic potential of guaiacol is of concern, this is the first issue to be clarified. The SCHEER is aware that negative results have been already published by using the *Ames test*; however, positive results were obtained with human lymphocytes: these data have to be confirmed or denied by means of results coming from appropriate *in silico*/*in vitro* methods (**Step 3**).

In case of positive results, guaiacol would not meet the TPD requirement (see art.7), no additional testing would be necessary, therefore the procedure can go directly to **Step 4.**

If guaiacol could be proved not to have genotoxic properties, the additive can enter the step-wise procedure for evaluation, starting with Step1.

Step 2: Pyrolysis products should be then considered. In case there are no objections, the evaluation should proceed to step 3.

Step 3: Guaiacol is a severe eye irritant, a skin irritant and also reported to be a respiratory tract irritant: these properties should be specifically addressed as a priority. In addition, properties as local anaesthetic, potentially contributing to addictiveness, should be investigated as a second priority. In case there are no objections, all the other toxicity end-points should be considered.

2.5.9 Guar gum

Synomyms: Guaran, Guar Flour, Jaguar

CAS number: 900-30-0 (Guar depolymerised CAS number: 68411-94-9) and others

Rational for inclusion

Guar gum is an extract of the seeds of the guar bean plant. Guar gum consists of high molecular weight polysaccharides and some amount of protein. Reconstituted tobacco is made up of mashed tobacco stems and other parts of the tobacco leaf that would otherwise be discarded. Guar gum (and its derivatives) is added to reconstituted tobacco in cigarettes. Guar gum is also used to prepare the cigarette paper that wraps the tobacco.

The amount of guar gum added to bind the tobacco can make up between 0.6-1.8 % of the total weight of the tobacco used in one cigarette. Guar gum is generally regarded as safe for use in food and cosmetics. However, guar gum does not transfer intact to the mainstream smoke, but undergoes pyrolysis, giving rise to toxic/carcinogenic (e.g. formaldehyde, benzo(a)pyrene and benzene) compounds. Irritating and toxic fumes, gases and acrid smoke can be formed when the additive is heated to decomposition.

Regarding flavours, it is well known that the thermal degradation of sugars and carbohydrates at lower temperatures as in foods contribute to complex aromas. Several flavour compounds were reported due to pyrolysis reactions of guar gum. These flavour compounds singly or in combination with the thousands of other smoke constituents can act synergistically and contribute to the attractiveness of smoking by improving smoke flavour, thereby masking its bitter taste, reducing the harshness of smoking, creating

sensory cues, which all could contribute to the optimisation of nicotine dosing and enhance abuse potential.

Guar gum is hazardous when heated to decomposition, emitting acrid smoke and irritating fumes. Although some information on the effect of pyrolysis is available from the internal industry documents, further chemically defining this additive from the point of view as a tobacco additive and its pyrolysis products would help confirm/facilitate the assessment of the influence on the carob bean extract on toxicity/carcinogenicity, palatability, pro-addictive effect and the interaction/synergistic effect with other additives and tobacco chemicals.

Priority activities

Step 1: Data on the chemical composition of the guar gum should be provided by industry with emphasis on the concentrations of constituents of relevance.

Step 2: Some information on the effect of pyrolysis of guar gum is available, however, it is necessary to:

- further chemically define its pyrolysis products and

- evaluate the CMR and other toxicological properties of its pyrolysis products.

In case of positive results for genotoxicity/carcinogenicity of its pyrolysis products the use of guar gum as a tobacco would not meet the TPD requirement; no additional testing would be required and the procedure could go directly to Step 4.

Step 3: If CMR properties are not proven by available data, the additive can enter the step-wise procedure for evaluation, analysing data other than those related to CMR properties. The assessment of its pyrolysis product on palatability, pro-addictive effect and the interaction/synergistic effect with other additives and tobacco chemicals should be analysed.

2.5.10 Liquorice

CAS numbers: 1405-86-3 (α-D-Glucopyranosiduronic acid), 103000-77-7 (β-D-Glucopyranosiduronic acid)

Rational for inclusion

Liquorice is a natural extract of the root of the liquorice (*Glycyrrhiza glabra*) plant – logically a not completely defined complex mixture of compounds. When heated to decomposition, it emits acrid smoke and irritating fumes. More than 400 compounds were isolated from Glycyrrhiza species. Liquorice extracts are used to improve the organoleptic properties of tobacco smoke, making the harsh cigarette smoke palatable, thereby enhancing the attractiveness of smoking. The taste and flavour of tobacco with added liquorice/liquorice root are described as sweet, woody and round. The major active principle of liquorice is the sweet tasting triterpene glycoside glycyrrhizin.

Glycyrrhizin is a bronchodilator. It is not clear whether the levels present are sufficient for this effect, although a synergistic effect with other compounds in cigarette smoke may be expected.

It is expected to pyrolyse extensively, but there is a lack of information on the pyrolysis products formed, which would help facilitate the assessment of the influence on toxicity/carcinogenicity. Additionally, the effect of liquorice on bronchodilation, alone or in combination with other additives and/or tobacco constituents, needs to be ascertained to better understand its effect on the ease of inhalation of nicotine and other alkaloids, thereby potentiating addictiveness.

Priority activities

The potential genotoxic effects of liquorice extract have been postulated.

Starting with **Step 1** and if necessary **Step 3** this end-point should be addressed first, to identify alert for the genotoxicity and carcinogenicity of the additive.

Step 2: additional standardised pyrolysis experiments are recommended, to identify the products formed during the combustion process of liquorice, to be then evaluated for their CMR properties.

In case results are negative, the evaluation of the effects of long-term inhalation exposure to liquorice with different content of glycyrrhizic acid should consier that both renal and hepatic 11-beta-hydroxysteroid dehydrogenase (converts cortisol to cortisone) as well as hepatic delta-4-5-beta-steroid-reductase (inactivates glucocorticoids and mineralocorticoids) are inhibited by glycyrrhetinic acid, which can lead to pseudohyperaldosteronism and elevated blood pressure. The flavonoids licochalcone A and B inhibit the elevation of calcium ions induced by thrombin, in a dose-dependent manner. They also inhibit thrombin-induced platelet aggregation *in vitro*. Licochalcone A and B were tested with human neutrophils and were found to inhibit the formation of leukotrienes B1 and C4, cyto B-induced lysosomal enzyme, platelet activating factor, n-formyl-methionyl-leucyl-phenylalanine and calcium ionophore A.

Hypokalemia, hypernatremia, and water retention are primary problems associated with chronic liquorice ingestion. Changes in the sodium/potassium ratios may result in pH changes. Cardiomyopathy, pulmonary edema, myoglobinuria, ptosis, myopathy, tetany, cramping, seizures, and rhabdomyolysis have also been reported in patients following chronic, excessive liquorice ingestion. Only if it could be demonstrated that chronic liquorice inhalation with tobacco smoke has no systemic effects, could the additive enter the step-wise procedure for evaluation. The safety evaluation of glycyrrhizic acid should be based on the data from humans. Since the oral absorption has been demonstrated to be high, the systemic toxicity after inhalation (also assuming a total absorption through the lung (100%) the effects are not expected to be different. Since the relevant NOAEL is relatively high (2 mg glycyrrhizic acid / kg bw per day for healthy volunteers) and the blood serum half-life is 5 hours, the risk of systemic general toxicity may not be high at the doses used as tobacco additive.

Step 3: all the testing regarding general systemic toxicity in chronic inhalation exposure and the one regarding characterising flavour as contribution to attactiveness should be addressed; since liquorice can mask the undesirable characteristics of tobacco smoke and acts as a bronchodilator the possibility for facilitating nicotine uptake should be analysed. More data are needed on the amount of liquorice and glycyrrhizic acid that imparts a noticeable flavour other than tobacco. No data are available regarding addictiveness.

2.5.11 Maltol

CAS Number: 118-71-8
Synonyms: 3-Hydroxy-2-methyl-4-pyrone, 3-Hydroxy-2-methyl-4H-pyran-4-one, Palatone, Larixinic acid, Talmon.

Rational for inclusion

Following the EFSA report on maltol (FGE19 and FGE213 and FGE213 rev 1 - EFSA, 2008, 2009 and 2014), the concern for genotoxicity could not be excluded. Therefore, maltol will be on the priority list until data on its genotoxicity are clarified. In addition, possible effects on the CNS must be clarified.

Priority activities

The main open questions concerning maltol are therefore uncertainty concerning the genotoxicity.

If the existing information (**Step 1**) does not clarify the uncertainties, some additional testing should be undertaken based on the OECD TG (**Step 3**).

If maltol is proven not to be genotoxic, the additive can enter the step-wise procedure for evaluation.

Since an inhibition of the response of the GABAA receptors in the presence of maltol has been reported which may contribute to CNS stimulation/addictiveness, these are the priority effects that should be investigated in Step 3.

In addition to the above, the following issues were identified:

Maltol and other hydroxyl cyclohexanone derivatives (such as ethyl maltol) are used to augment or enhanc the taste of consumable materials, with a typical odour of cotton candy and caramel. No information on minimum levels of odour awareness was found.
Reports on the health effect of maltol also underline its potential anti-apoptotic effect. It is unclear whether the anti-apoptotic is specific directed to healthy cells, neoplastic cells and/or cells undergoing mutations.

2.5.12 Menthol

CAS numbers: l-Menthol: 2216-51-5; D-Menthol: 15356-70-4; D/L Menthol: 89-78-1; Menthol: 1490-04-6

Rational for inclusion

Menthol is one of the most commonly used tobacco additives worldwide. It is a monocyclic terpene alcohol that is used primarily for its chemosensory effects of creating perceptions of a cooling minty taste and smell. Menthol is added at a continuum of concentrations, from imperceptible amounts to levels imparting different levels of a characterising flavour.

In addition, several additives and formulations are used to simulate menthol effects. Menthol induces anaesthetic and sensory effects, facilitates deeper inhalation and adds to the impact of nicotine.

Menthol is a multifunctional additive. It is an effective anaesthetic, antitussive agent that may increase the sensation of airflow and inhibit respiratory rate, thereby allowing increased lung exposure to nicotine, tar and toxic constituents, while masking reactions like coughing or other early warning signs of respiratory disease. It may increase the absorption and lung permeability of smoke constituents, thereby increasing nicotine and carcinogen uptake. It may also decrease nicotine/cotinine metabolism leading to higher doses of nicotine. It is one of the additives that was originally added to create the impression that a tobacco product has health benefits and/or reduced health risks. It affects multiple sensations including taste, aroma and tactile smoothness, and enhances abuse liability. Its pharmacological actions reduce the harshness of smoke and the irritation from nicotine, and may increase the likelihood of nicotine addiction in adolescents and young adults who experiment with smoking andit may make it more difficult to quit.

In 2011, the FDA Tobacco Products Scientific Advisory Committee (TPSAC, 2011) concluded that menthol 1) impacts youth initiation, 2) contributes to adults continuing to smoke, and 3) has an adverse impact on public health by increasing the numbers of smokers with resulting premature death and avoidable morbidity. Finally, they concluded that the "removal of menthol cigarettes from the marketplace would benefit public health in the United States" (TPSAC, 2011; FDA, 2011).

Independently, the US Food and Drug Administration undertook a thorough review and concluded that the data suggested that menthol use is likely associated with increased smoking initiation by youth and young adults, greater addiction, greater signs of nicotine dependence and less likelihood of successfully quitting smoking. These findings, combined with the evidence indicating that menthol's cooling and anaesthetic properties may reduce the harshness of cigarette smoke and the evidence indicating that menthol cigarettes are marketed as a smoother alternative to non-menthol cigarettes, make it likely that menthol cigarettes pose a public health risk above that seen with cigarettes without menthol (FDA, 2013). The review concluded that although there is little evidence that menthol cigarettes *per se* are more toxic than menthol-free cigarettes, adequate data indicate that menthol presence is associated with increased smoking initiation and greater addiction, especially among young people, as confirmed later by the studies of Nonnemaker *et al*. (2013) and Brennan *et al.* (2015).

Indeed, smokers usually using menthol cigarettes develop greater nicotine dependence, which is likely associated to the anaesthetic properties that reduce the harshness of smoke. In addition, menthol cigarette smokers are less successful quitting smoking (Smith *et al.,* 2014). Recent perception studies confirm earlier work showing that smokers, especially young adults, perceive menthol cigarettes as less harmful (Brennan *et al.,* 2015; Wackowski and Delnevo, 2015).

With regard to toxicity, Noriyasu *et al.* (2013) exposed cell cultures to menthol and nonmenthol smoke and found that cell death was significantly enhanced by mentholated smoke, whereas menthol alone was inert. This suggests a synergistic effect with other smoke-compounds and requires further study. A recent study conducted in mice showed that menthol at low concentration strongly suppressed respiratory irritation due to acrolein and cyclohexane, which are smoke irritants in naïve mice. Additionally, menthol suppressed irritation by tobacco smoke in mice. Menthol increased blood cotinine levels, which is a biomarker of nicotine uptake. Thus, menthol appears to suppress smoke-induced irritation, making it easier to inhale smoke and increasing the dosage of

nicotine. Due to the similarities in menthol's pharmacology in humans, experiments in animal models suggest that beginning smokers likely prefer menthol-containing cigarettes because their respiratory tract is less irritated. At the same time, these smokers are exposed to higher levels of nicotine and become addicted faster and are less likely to quit smoking (Ha *et al*., 2015).

At lower application levels, menthol can be used to increase smoothness and reduce harshness in cigarette smoke. This is likely the main reason for use of menthol as an additive, also in "non" menthol brands. Therefore, research to ascertain the physiological and pharmacological impact of low menthol and its interaction with other chemicals, interaction with nicotine, on palatability and inhalation of smoke/nicotine, etc. is recommended.

Priority activities

Step 2: Based on the available studies, pyrolysis of menthol may result in carcinogenic substances (concern category d). There is uncertainty with regard to the nature of the pyrolysis products. Pyrolysis studies should be carried out to identify these products and the products should be evaluated.

Step 3: Based on the available data, menthol is concluded to impart a characterising flavour if added in sufficient amounts, facilitate inhalation and addictiveness of tobacco products and increase inhalation and nicotine uptake (concern categories a and c). Uncertainty is low. Further testing to show whether menthol is or is not addictive or does or does not increase attractiveness is not recommended in view of the strength of the data.

Further studies are needed into the suggested synergistic effect of menthol with other smoke compounds. Research to ascertain the physiological and pharmacological impact of low menthol and its interaction with other chemicals, interaction with nicotine, on palatability and inhalation of smoke/nicotine, etc. is recommended.

2.5.13 Propylene glycol

CAS number: 57-55-6

Rational for inclusion

Propylene glycol (PG) is added as humectant to tobacco, rather frequently and in relatively high amounts (1599 counts in NL ingredient lists, 23 in NTM, total number of brand 4265), average (weight %) 1.579 (1.636).

Regarding attractiveness, humectants are added to trap water, thereby keeping the moisture in the tobacco and preventing it from drying out. Internal tobacco industry documents reported that adding 3-7 weight percent of PG increased the mildness and reduced irritation (although this is higher than amounts typically present in tobacco cigarettes). Propylene glycol is, therefore, considered to positively influence the attractiveness of cigarette smoking given that humidification improves palatability of expected to impart a noticeable flavour.

Regarding addictiveness, no data were reported to suggest that propylene glycol plays a role in smokers' addictiveness to cigarettes.

Regarding toxicity, it was reported by tobacco industry that the transfer rate of propylene glycol to mainstream smoke is 10 %. A risk assessment procedure using a Margin of Exposure (MOE) analysis concluded that risks of effects on the respiratory tract epithelium from propylene glycol exist. No thorough assessment on systemic effects was made.

Propylene oxide is regarded as possibly carcinogenic to humans and trace amounts are present in propylene glycol. Additionally, pyrolysis of propylene glycol results in formation of small amounts (<10 %) of 1,3-propylene glycol, acetol or acetic anhydride, and pyruvaldehyde.

Finally and importantly, propylene glycol and/or its combustion products is only one component out of the thousands of compounds contained in cigarette smoke, thus additive effects or reactions with other compounds are likely to occur.

Priority activities

Step 1: Since propylene oxide, which is regarded as possibly carcinogenic to humans (IARC Group 2B carcinogen), is found in trace amounts in industrially-produced propylene glycol, the specification should be provided.

Data available should be collected to prove or disprove whether propylene glycol increases the risks of effects on the respiratory tract epithelium (being added to tobacco as a humectant in relatively large quantities, possibly increasing the attractiveness of cigarette smoking)

Step 2: Pyrolysis products should be better characterised also considering that Propylene oxide has been reported to be generated during cigarette smoking.

Step 3: The effect of propylene glycol on inhalation facilitation to cigarettes at levels found in European cigarettes (range of 0.2 to 2.4%) warrants investigation. The additive effects of propylene glycol and/or its reactivity with other compounds should be further investigated.

No thorough assessment of the systemic effects of glycerol has been done so this should be looked at further.

2.5.14 Sorbitol

CAS number: 50-70-4

Rational for inclusion

Sorbitol is added as a humectant to tobacco (210 times in NL ingredient lists, 30 in NTM, total no of brands 4265), average (weight %) 0.232 (0.458).

Regarding attractiveness, humectants are added to trap water, thereby keeping the moisture in the tobacco and preventing it from drying out. Sorbitol is, therefore, considered to positively influence attractiveness of cigarette smoking given that humidification improves palatability of cigarettes. Sorbitol gives tobacco smoke a slightly bitter taste and a vague odour of cellulose and is, therefore, not expected to impart a noticeable attractive flavour when used in higher amounts.

Regarding addictiveness, no data were reported to suggest that sorbitol plays a role in smokers' addictiveness to cigarettes. However, its combustion products, such as acetaldehyde and formaldehyde, were proposed to increase the addictive effect of nicotine, although data on acetaldehyde produced by pyrolysis entering the brain through the smoke inhaled are inconclusive (SCENIHR 2010).

Regarding toxicity, sorbitol was reported to pyrolyse at 900°C to compounds, such as 2-furfural (31.4 %, see section on furfural), acetaldehyde (irritant and possible human carcinogen), formaldehyde (irritant, carcinogen). Other pyrolysis products of sorbitol include furan, 2-methyltetrahydrofuran, propionaldehyde, acetone, methanol, and carbon monoxide (Baker and Bishop, 2004). Further research is needed to confirm these effects, especially if sorbitol pyrolysis results in carcinogenic compounds.

Finally, it must be borne in mind that sorbitol (and/or its combustion products) is only one component out of the thousands of compounds contained in cigarette smoke, thus additive effects or reactions with other compounds are likely to occur.

Priority activities

Step 2: The main concern for sorbitol to be addressed, before it can enter the evaluation procedure, is the formation of toxic pyrolysis and CMR products. Pyrolysis experiments must be carried out using conditions relevant for cigarette smoking (see section 3.4.1). In case of positive results, sorbitol as a tobacco additive would not meet the TPD requirements and no additional testing would be necessary, going directly to Step 4.

In case it could be demonstrated that no toxic or carcinogenic pyrolysis products are formed, the additive can enter the step-wise procedure for evaluation.

In particular, it needs to be assessed, in **Step 3**, whether it increases inhalation facilitation of cigarette smoking. It is not expected that sorbitol will give a characterising flavour that is attractive. No data are available regarding addictiveness.

2.5.15 Titanium Dioxide

CAS numbers: 13463-67-7 (mixture of mainly rutile and anatase); 1317-80-2 (rutile); 1317-70-0 (anatase)

Rational for inclusion

The SCCS evaluated its use as cosmetic ingredient (sunscreen). With regard to inhalation toxicity, it was concluded that in subacute repeated dose inhalation toxicity studies, nano-size TiO2 induce an acute inflammation in the lungs, that may be reversible depending on the dose and the time after exposure. In view of this, acute inflammation (spray) applications, which may result in inhalation exposure, were not recommended by the SCCS. Both nano and non-nanosize titanium dioxide were classified by IARC as a Group 2B carcinogen (i.e. possibly carcinogenic to humans) (IARC, 2010).

To perform a risk evaluation, it is necessary to know the exposure level of titanium dioxide through cigarette smoking. Therefore, research is needed to determine the amount of titanium dioxide in mainstream cigarette smoke. Because inhalation toxicity is also related to the size of the particles, a distinction needs to be made between nano and non-nano size.

Priority activities

Step 2: Not applicable because titanium is already in its highest oxidised state.

Step3: In subacute repeated dose inhalation toxicity studies, nano-size TiO_2 induces an acute inflammation in the lungs. Based on the available data, titanium dioxide is classified as a IARC Group 2B carcinogen (concern category d).

Within the scope of the EU CLP Regulation, a proposal for harmonised classification of TiO2 was submitted (ECHA,CLH report, Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2, Substance Name: Titanium dioxide, Version 2, May 2016). It is proposed to classify TiO_2 specifically by inhalation as Carcinogen Cat 1B. It was concluded that no carcinogenic concern was reported by both oral and dermal routes but that there is sufficient evidence of carcinogenicity in experimental animals after inhalation. Indeed, a causal relationship has been established between TiO_2 and the increase of malignant lung tumours in female rats and benign lung tumours in males and female rats in 2 inhalation and 2 instillation studies. Human data do not suggest an association between occupational exposure to TiO2 and risk for cancer. However, all these studies have methodological limitations and the level of exposure reported is debatable. Although the full mode of action is still unclear, an inflammatory process and indirect genotoxic effect through ROS production seems to be the major mechanism to explain the effects induced by TiO_2. It is considered that this mode of action is principally due to the biopersistence and poor solubility of the TiO_2 particles Evidence may be provided to address the uncertainties with regard to the genotoxicity and carcinogenicity of TiO_2 by inhalation for humans.

3 OPINION

In line with Article 6 of the Tobacco Products Directive 2014/40/EU, the Commission has established a priority list of 15 additives contained in cigarettes and roll-your-own tobacco subject to enhanced reporting obligations that is to be updated on a regular basis. In Opinion 1, SCENIHR advised the Commission by providing a list of additives to consider for inclusion on this priority list.

The TPD prescribes that Member States shall require manufacturers and importers of tobacco products to carry out comprehensive studies on these additives. The SCHEER was asked to provide guidance on the type and criteria for these comprehensive studies, and the most suitable methodologies to be used. This advice will guide comprehensive studies on the first list of 15 priority additives, as well as for additives on future updated lists.

In the first part of the current Opinion, SCHEER proposed a step-wise strategy (Section 3.4), as the most pragmatic and efficient way to proceed in the assessment of the toxic and addictive effects as well as characterising flavour properties, and inhalation facilitation, as contributing to attractiveness of tobacco additives. The tiered approach proposed by DKFZ (DKFZ, 2010) was used as a starting point and adapted to include the evaluation of attractive and addictive effects of additives. The proposed strategy ensures that testing is minimised.

First, the chemical specification of the additive has to be available (Step 1). Then an evaluation of the available literature needs to be carried out, for the additive in its unburnt form (Step 1) and its pyrolysis products (Step 2). If no data are available on the identity of the pyrolysis products, they need to be generated using relevant test conditions (Step 2). Here, it needs to be noted that no validated methods are available for the pyrolysis of tobacco additives.

In case data retieved in Step 1 and 2 are not sufficient or robust enough to make the evaluation possible, non-testing methods such as QSAR and read across are proposed, followed by *in vitro* approaches. Regarding types of effects, unless the previous step highlighted some concern for a specific end-point, CMR properties and toxicity are assessed first, as accepted methods and evaluation frameworks are available, followed by characterising flavour, because procedures are available for the assessment of these end-points. Next, addictiveness is assessed, an effect for which no validated tests are available, although mechanisms underlying addictiveness are known. It is strongly advised that *in silico* and *in vitro* test to assess additive-induced addictiveness by independent organisations are developed and validated.

The issue related to interaction of the additive with other additives/ingredients is also considered. The industry is obliged to provide all known information on the interaction of additives and their pyrolysis products leading to the intended formation of flavours / pH modifiers/ smoothing agents and other important compounds.

In addition to proposing specific steps and tests to be considered by industry, some general criteria were also identified. A pre-amble here is that additives in tobacco products have no health or other benefits for the consumer, but rather promote use of and addiction to an extremely toxic product. Therefore, a risk-benefit analysis is not the appropriate paradigm for assessing the additive. By consequence, we advise that the level of proof of safety must be set very high, and the precautionary principle as a quintessential element of preventive toxicology should come into full force. The same

reasoning applies to the addictive effects and characterising flavour of tobacco additives, as they will indirectly lead to health consequences by increasing consumption of the product.

In order to provide a relevant outcome to the question whether an additive contributes to the toxicity, characterising flavour or addictiveness of the tobacco product, the study design must adhere to some methodological criteria. Most importantly, the test outcomes should be relevant for tobacco smoking. This implies that they should be related to actual human exposure and tobacco-induced diseases, and be relevant not only for acute or subchronic, but also for chronic exposure in intermittent use sessions (Johnson *et al*., 2009).

Furthermore, comparative toxicity testing strategies, where differences in effect of the tobacco product with and without the additive are evaluated, are not considered suitable. Due to the high intrinsic toxicity of tobacco products, it is challenging to demonstrate any differences, whether they are increases or decreases, induced by an additive with the currently available tests and methodologies (Kienhuis *et al*., 2016). Very sensitive tests would be required, with a clear dose-response relationship, in order to show any differences from these high background effects. As such tests are not currently available, no comparative studies (tobacco product with and without additives) will be considered for the moment, since these studies lack discriminative power. Comparative studies are also not endorsed to study the effect of additives on addictiveness and inhalation facilitation, for the same reasons.

Another problem with comparative testing is that the outcomes would only apply to that specific tobacco test product. The results (related to toxicity, addictiveness and attractiveness) cannot be generalised to all products and brands, having a different composition with respect to tobacco type, blend and additives. Therefore the obtained results may not lead to general prohibition/acceptance of specific additives but rather to prohibition/ acceptance on a product-by-product basis (DKFZ, 2010). Instead of using a comparative study design, the effects of the pure additive, and its pyrolysis products, must be considered in a relevant testing strategy, such as the tiered approach proposed by DKFZ (DKFZ, 2010), which has been adapted by SCHEER (Section 3.4).

For ethical reasons, animal studies are not endorsed to assess the safety of a tobacco additive. Similar to cosmetics, it is not necessary to use tobacco products. Moreover, apart from being unnecessary, tobacco products are highly harmful with no benefits to individual or public health. As additives are used for product improvement, often contributing to detrimental effects for the consumers (i.e. attractiveness or addictiveness), there is no health benefit in using tobacco additives. For the hazard assessment of tobacco additives, relevant and valid *in silico* and *in vitro* methods exist. QSAR methodology has been used for decades successfully for predicting toxicological and pharmacological properties of chemicals. The same applies to *in vitro* methods, which were validated and accepted for the adverse outcome they are supposed to measure. Additionally, many promising *in vitro* methods are currently being developed to assess different adverse outcomes from apoptosis and gene expression to inflammation and respiratory diseases.

Therefore, as a principle, only *in silico*, and *in vitro* studies will be considered, following the EU policy to ban animal studies for chemicals to be used in voluntary products such as cosmetics (EU Regulation no. 1223/2009). Human studies are discouraged. These

may only be used in case of flavour assessment, but only if the study subjects are not exposed to the harmful smoke emissions of tobacco products.

The major data gaps already identified in Tobacco Opinion 1 for the 15 additive included in the EU Commission priority list have been analysed (Section 3.5). Based on the data gaps described in the 'Rationale for inclusion' taken from Opinion I, the activities to be performed upfront have been described, indicating the most appropriate steps (and end-points) to be carried out and then used for the evaluation, in order to speed up the process making possible testing feasible in the 18-month timeframe.

In general, important data gaps for the 15 priority additives are information on addictiveness and characterising flavour or inhalation facilitation, contributing to attractiveness, as well as on the identity of the pyrolysis products. In the past, major emphasis was put on toxicity, whereas limited research was carried out on addictiveness and even less on attractiveness. Regarding toxicity, data were often taken from the food sector, where pyrolysis and inhalation are not an issue.

In conclusion, this Opinion provides general guidance to tobacco industry to conduct studies and prepare reports on the 'safety' of Tobacco additives to be sent to the relevant authorities. To this purpose, a reporting template is provided as well. In addition, specific advice is given for priority testing activities to fill the data gaps recognised in Tobacco Additives Opinion 1. In order to limit the testing and administrative burden, the formation of consortia and joint reports by industry is endorsed. It needs to be noted that there is a lack of (validated) methods for the pyrolysis of tobacco additives. Similarly, no addictiveness and attractiveness tests (apart from a procedure for characterising flavours) are available, a knowledge gap already noted by the SCHENIHR in 2010 in its report 'Addictiveness and attractiveness of tobacco additives', and the situation has not improved since then. It is advised that independent bodies or organisations begin conducting relevant research.

4 MINORITY OPINION

None.

5 ABBREVIATIONS AND GLOSSARY OF TERMS

AOP	adverse outcome pathway
ASL	Arterial Spin Labelling
CAS	Chemical Abstracts Service
CLP	Classification, Labelling and Packaging
CMR	carcinogenic, mutagenic or toxic for reproduction
CNS	Central nervous system
COC	Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment
COM	Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CVD	Cardiovascular disease
CYP	Cytochrome P450 monooxygenase
DA	dopamine
DKFZ	Deutsches Krebsfoschungszentrum (German Cancer Research Centre)
EC	European Commission
ECDC	European Centre for Disease prevention and Control
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Environmetal Protection Agency
EU	European Union
FDA	(US) Food and Drug Administration
FEMA	(US) Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
fMRI	functional magnetic resonance imaging
FTND	Fagerström Test for Nicotine Dependence
GABA	Gamma (γ)-Aminobutyric acid
GABA	γ-aminobutyric acid
GLP	good laboratory practice
IARC	International Agency for Research on Cancer
IC50	The half-maximal inhibitory concentration
IPCS	(WHO) International Programme on Chemical Safety

JECFA	Joint FAO/WHO Expert Committee on Food Additives
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JRC	(EU) Joint Research Centre
MoE	Mode of Exposure
MRI	Magnetic resonance imaging
nACh	nicotinic acetylcholine
NOAEL	No observed adverse effect level
NRC	(US)National Research Council
NTM	Non-tobacco material
OECD	Organisation for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbon
PAH	polycyclic aromatic hydrocarbon
PBPK	Physiologically based pharmacokinetic
PET	positron emission tomography
PG	propylene glycol
pH	Measure of acidity or basicity of a solution
PITOC	EU project "Public Information Tobacco Control"
ppm	parts per million
QSAR	Quantitative structure--activity relationships
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RIVM	Rijksinstituut voor Volksgezondheid en Milieu (The Netherlands National Institute for Public Health and the Environment)
RYO	roll your own (cigarettes)
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHEER	Scientific Committee on Health and Environmental and Emerging Risks
SCHER	Scientific Committee on Health and Environmental Risks
SCOEL	Scientific Committee on Occupational Exposure Limits
SPECT	single-photon emission computed tomography
TG	(OECD) Test Guidelines
TPD	Tobacco products directive
UK	United Kingdom
US(A)	United States (of America)

1	WHO	World Health Organization
2	WoE	Weight of evidence

3

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4

7 Annex I

GUIDANCE AND TEMPLATE

A. GENERAL ISSUES

This brief guidance intends to support the understanding of "what was done". Authors should take responsibility to be clear of the definitions and provide proper citations for any terms/data they use.

1. TITLE PAGE

The title page should contain the following information:

Identification of the additive

Abstract and keywords, if applicable

Name of sponsor (and bodies that fund or commission the analysis)

Name and affiliation of person or persons responsible for producing and signing off the report

Date and version of report.

2. SUMMARY

The summary is intended to provide a concise description of the key elements.

3. REPORTING SOURCES OF INFORMATION

This section should describe any data source or sources that were used (e.g. existing data and/or databases, *in silico* techniques/models used, experimental studies).

This section addresses the key features of the design.

The rationale for the overall study design should be documented

If needed also ethical approval (approval number – approved by …- date) for *in vivo* experiments (animals or humans) should be given.

4. REPORTING DATA QUALITY / DATA COLLECTION QUALITY ASSURANCE

This section addresses the reporting of the elements of data collection and pre-processing that could influence data quality.

- How was the literature search been conducted and which used quality controls were in place

- Were in own experimental studies quality controls in place:

In silico

In vitro

In vivo

5. PUBLIC SUMMARY OF THE DOSSIER

The target group of a public summary is a non-professional public. The structure and content of the public summary shall be elaborated accordingly. The document should be less extensive comparing to the *summary.* A scientific/professional terminology shall be avoided if possible.

6. CD/DVD

The applicant shall submit a dossier with the full information on standard electronic media such as CD ROMs or DVDs. Two or three CD ROMs or DVDs shall be submitted.

Common electronic formats should be used (e.g. MS Office, Adobe Acrobat Reader) allowing content copying and printing (no content copy protection). The text of the files should be searchable using the search facilities of standard software packages. The CD or DVD shall be structured in folders that reflect the structure of the submission.

Also a full paper copy of the dossier is requested, it has to be declared by the applicant on a separate sheet or in the accompanying letter that the electronic and the paper versions are identical.

7. LIST OF PARTS OF THE DOSSIER REQUESTED TO BE TREATED AS CONFIDENTIAL

Applicants have the right to request a confidential treatment of certain information. They shall indicate which sections and data they wish to be treated as confidential (and give verifiable justification for each part for which a confidential treatment is required).

Furthermore, the applicant shall provide the Commission with two electronic versions of the dossier, namely the complete dossier and a second version of the complete dossier without confidential information.

B. SPECIFIC ISSUES – GENERAL LAYOUT

1. CHEMICAL AND PHYSICAL SPECIFICATIONS OF ADDITIVE

A. Chemical identity

Primary name and/or INCI name

Chemical names

Trade names and abbreviations

CAS / EC number

Structural formula

Empirical formula

B. Physical form

Molecular weight

Purity, composition and substance codes

Impurities / accompanying contaminants

Solubility

Partition coefficient (Log Pow)

Additional physical and chemical specifications

Where relevant:

- organoleptic properties (colour, odour, taste if relevant)
- vapour pressure
- pKa

- …

C. Function and use of the additive

2. (Each identified) PYROLYSIS PRODUCTS

Chemical identity

Molecular weight

% formed (at specific temperature)

Solubility

Partition coefficient (Log Pow)

Additional physical and chemical specifications

Where relevant:

- organoleptic properties (colour, odour, taste if relevant)
- vapour pressure
- density
-- pKa
- …

3. TOXICOLOGICAL EVALUATION

For each study, independent whether is an own study or data were assessed from literature, a full study report should be given:

- If data is derived from an original (own) study, all original (rough) data should be submitted
- If data is derived from literature, the full paper/report should be submitted.

Next to the full report a study summary should be submitted, including, if applicable, the following (the summary reports should usually only exceed 1 page):

Guideline:
GLP/quality control measure:
Test system: (in silico/in vitro/in vivo/human):
N° independent assessments – group size:
Test substance:
Batch:
Purity:
Vehicle:
Dose level:
Route of exposure:
Exposure duration:
Exposure duration & observation period:
Study date/period:
Specific methodological issues:
Brief summary of the results: (summary):
Brief conclusion:

If more studies were reported for one toxicological endpoint, a final conclusion should be formulated, taking into account the data of the different related studies.

Summary: Finally, at the end of the section Toxicology a brief general conclusion should be formulated.

4. ADDICTIVNESS ASSESSMENT

For each study, independent whether is an own study or data were assessed from literature, a full study report should be given:

- If data is derived from an original (own) study, all original (rough) data should be submitted
- If data is derived from literature, the full paper/report should be submitted.

Next to the full report a study summary should be submitted, including, if applicable, the following (the summary reports should usually only exceed 1 page):

Guideline:
GLP/quality control measure:
Test system: (in silico/in vitro/in vivo/human):
N° independent assessments – group size:
Test substance:
Batch:
Purity:
Vehicle:
Dose level:
Route of exposure:
Exposure duration:
Exposure duration & observation period:
Study date/period:
Specific methodological issues:
Brief summary of the results: (summary):
Brief conclusion:

If more studies were reported for one addictiveness endpoint, a final conclusion should be formulated, taking into account the data of the different related studies.

Summary: Finally, at the end of the section addictiveness assessments a brief general conclusion should be formulated.

5. ATTRACTIVENESS ASSESSMENT

For each study, independent whether is an own study or data were assessed from literature. A full study report should be given:

- If data is derived from an original study, all original (rough) data should be submitted
- If data is derived from literature, the full paper/report should be submitted.

Next to the full report a study summary should be submitted, including the following (the summary reports should usually only cover 1 page):

Guideline:
GLP/quality control measure:
Test system: (in silico/in vitro/in vivo/human):
N° independent assessments – group size:
Test substance:
Batch:
Purity:
Vehicle:

Dose level:
Route of exposure:
Exposure duration:
Exposure duration & observation period:
Study date/period:
Specific methodological issues:
Brief summary of the results: (summary):
Brief conclusion:

If more studies were reported for one attractiveness endpoint, a final conclusion should be formulated, taking into account the data of the different related studies.

Summary: Finally, at the end of the section attractiveness assessments a brief general conclusion should be formulated.

6. ASSESSMENT OF INTERACTION OF ADDITIVES WITH OTHER ADDITIVES/INGREDIENTS

For each study, independent whether is an own study or data were assessed from literature. A full study report should be given:

- If data is derived from an original study, all original (rough) data should be submitted
- If data is derived from literature, the full paper/report should be submitted.

Next to the full report a study summary should be submitted, including the following (the summary reports should usually only cover 1 page):

Guideline:
GLP/quality control measure:
Test system: (in silico/in vitro/in vivo/human):
N° independent assessments – group size:
Test substance:
Batch:
Purity:
Vehicle:
Dose level:
Route of exposure:
Exposure duration:
Exposure duration & observation period:
Study date/period:
Specific methodological issues:
Brief summary of the results: (summary):
Brief conclusion:

If more studies were reported for one endpoint, a final conclusion should be formulated, taking into account the data of the different related studies.

Summary: Finally, at the end of the section a brief general conclusion should be formulated on the Interactions with other additives/ingredients.

SUMMARY / OVERALL CONCLUSIONS

In the final section a summary and an overall conclusion shall be formulated covering all issues discussed above (chemical and physical specifications, use, toxicity, addictivenees, attractiveness and interactions with other ingredients).