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**Toxikologische Bewertung von Zusatzstoffen für Tabakprodukte –
Ein Leitfaden;
Text Englisch**

Toxicological assessment of additives for tobacco products –
A guidance;
Text in English

Évaluation toxicologique des additifs pour les produits du tabac –
Lignes directrices;
Texte en anglais

Anwendungswarnvermerk

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Gesamtumfang 71 Seiten

DIN-Normenausschuss Lebensmittel und landwirtschaftliche Produkte (NAL)

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Foreword

This document has been prepared by working group NA 057-04-01-04-AK “Toxikologie von Additiven (Toxicology of additives)” of the DIN-Normenausschuss Lebensmittel und landwirtschaftliche Produkte (NAL) (Food and Agricultural Products Standards Committee).

The first edition of this document was published in 2004 after the implementation of the first European Tobacco Products Directive (Directive 2001/37/EC, often also called TPD1) [1]. The Directive required tobacco companies to submit available toxicological data on tobacco additives to EU Member States. One result of this regulation was a significant increase in scientific publications reporting on toxicological studies investigating the impact of additives on the toxicity of tobacco products. During the revision of TPD1, the first edition of this document was updated and gave recommendations for a test battery that reflected state of the art testing methodologies including the necessary key assessment criteria. The revised Tobacco Products Directive 2014/40/EC, often referred to as TPD2 [2], mandated new provisions for reporting and testing of additives. Manufacturers of tobacco products were now requested, inter alia, to assess the toxicological effects of certain additives contained in cigarettes and roll-your-own tobacco (so-called “priority additives, according to article 6 of the European Tobacco Products Directive 2014/40/EU”) in comprehensive studies.

With the trend of increasing regulatory obligations for tobacco additives and in the absence of globally recognized standard procedures for the toxicological evaluation of tobacco additives, the working group saw the need to revise this document.

Recently tobacco heating systems that had been classified as Novel Tobacco Products in several member states, have gained importance and technical guidance is necessary to assess these products. Methods are required for verification of substantially reduced toxicant levels in the aerosol and to compare performances of individual systems. Tobacco heating systems are covered in this guidance document, because in general the methodology to generate and analyse aerosols, as well as the types of assays to address *in-vitro* toxicity are comparable or similar to those used for conventional tobacco cigarettes. Further, the question whether additives increase the toxic properties of aerosols does equally apply to heated tobacco. The scope of methods that had been previously only applied to analyse cigarette smoke is now expected to extend. However, caution is required if products of different categories are compared. This revised guidance document will focus on tobacco and covers both conventional tobacco products and novel tobacco heating systems.

Amendments

This document differs from DIN SPEC 10133:2014-09 as follows:

- a) Clause 2 “Normative references” and Clause 3 “Terms and definitions” have been added;
- b) the guideline has been adapted to the current state of the art and also takes into account the regulatory requirements for the testing of tobacco additives laid down in the revised Tobacco Directive (2014/40/EU);
- c) tobacco heating systems have been added.

1 Background and objective of this document

The title of this document, “Toxicological assessment of additives for tobacco products” calls for a clear definition of the term “additive” in connection with tobacco products. Two terms are often used interchangeably or without a clear distinction with respect to tobacco products: “additives” and “ingredients”. The two terms derive from different perspectives. Substances added to a product such as cigarettes subsequently become part of the content of the cigarette [3].

In the former TPD1 [1] only the term “ingredient” was defined, but the term “additive” was used in the same context as the term “ingredient”, albeit without a definition. The revised Tobacco Products Directive of 2014 (TPD2) defines both additives and ingredients [2]: “additive” means a substance, other than tobacco, that is added to a tobacco product, whereas “ingredient” means tobacco, an additive as well as any other substance present in a finished tobacco product. In line with the TPD2, the present document defines as additives all substances that are used in the manufacturing process of tobacco products.

Some markets like Canada, UK and Australia are dominated by so-called “Virginia cigarettes”. This type of cigarette is manufactured largely without using additives while there are countries like the USA and Germany where so-called “American blend cigarettes” that contain additives, are predominant. Globally, an increasing trend can be observed to regulate the use of additives in tobacco products.

In Canada [5] tobacco additives including flavours are widely prohibited since 2010. Menthol was banned in Canada on a federal level in 2017 in an amendment to the tobacco act. In the USA [7], Australia [8] and the EU the use of tobacco additives has also been restricted to such an extent that the final tobacco product should not have a “characterizing flavour” – in addition to the typical tobacco flavour. Mentholated cigarettes are an exception of this ban in, the USA and Australia. In Europe, menthol levels resulting in a characterizing flavour of cigarettes and RYO (Roll Your Own) tobacco are prohibited since May 2020.

Before implementation of TPD2, Germany had issued a positive and a negative list for the regulation of additives [4]. The use of additives was regulated concerning several criteria including the intended purpose, place of application, product category and quantity applied to the final product. The regulation principle was: additives not explicitly allowed, were prohibited. With the implementation of TPD2, the previous regulation was withdrawn and the new “Tabakerzeugnisverordnung” (TabakerzV) only specifies a list of prohibited additives in order to implement the requirements of TPD2.

Besides toxicological considerations the possible addictive potential of additives is also under discussion. In TPD1 [1] it was stipulated in Article 13 that Member States may prohibit the use of additives, which have the effect of increasing the addictive properties of tobacco products. In TPD2 a definition of ‘addictiveness’ is given: “addictiveness means the pharmacological potential of a substance to cause addiction, a state which affects an individual's ability to control his or her behaviour, typically by instilling a reward or a relief from withdrawal symptoms, or both.” The TPD2 stipulates that the use of additives increasing the addictiveness and additives increasing inhalation and/or nicotine uptake is prohibited. In 2010, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) published their conclusions regarding the addictiveness of tobacco additives [9]. In their final opinion the Working Group pointed out: “... that tobacco has a high addictive potential, but it remains difficult to assess the addictiveness of individual additives” and that “at present it is not possible to evaluate whether additives increase the addictive potency of the final tobacco product”. In their opinion on ‘Additives used on tobacco products (Opinion 2), Tobacco Additives II’ that was published in 2016, the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) also acknowledged the fact that no validated tests are available for the assessment of a potential effect of additives on the addictiveness of tobacco products.

This document will not address the aspects of addictiveness and smoking behaviour in detail, it will focus on the toxicological assessment of tobacco additives instead.

Sufficient toxicological data are available for most tobacco additives in their unburnt form, because a large number of additives are also used as food additives. However, for tobacco additives the differing route of uptake (inhalation) and the fact that they may undergo thermal degradation have to be taken into account. For a large number of additives, toxicological data on their effects after heating or combustion on tobacco products are also available. In the absence of standard methodologies for the testing of tobacco additives, this data were however obtained with widely varying approaches. Consequently, their validity can be quite variable as well. Approaches to the collection of suitable data for the toxicological evaluation of additives to cigarettes are the focus of this document. In principle, these approaches are applicable to other tobacco products including heated tobacco products and modifications of cigarettes.

The published scientific data indicate that the toxicological risks of tobacco smoking can mainly be attributed to carcinogenic and harmful combustion products from tobacco constituents and that tobacco additives seem to play a very minor role in the case. Nevertheless, they are at the centre of the European regulation. This document can therefore also be used as a guide for the toxicological evaluation of the additives to comply with reporting obligations according the current scientific and technical standards.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

No terms and definitions are listed in this document.

DIN and DKE maintain terminological databases for use in standardization at the following addresses:

- DIN-TERMinologieportal: available at <https://www.din.de/go/din-term/>
- DKE-IEV: available at <http://www.dke.de/DKE-IEV>

4 Overview of current legal requirements, agreements and proposals

For most chemical products or food additives there are approved test methods and assessment criteria available that are monitored by the respective governmental authorities. By contrast, globally recognized procedures and assessment strategies or national and international standards do not exist for the toxicological evaluation of tobacco additives. However, several regulatory requirements exist with regard to the toxicological assessment of tobacco additives and some of them will be briefly mentioned below.

This overview is not intended to give an overview of global developments of tobacco additive regulation and focuses primarily on the developments in the European Union as TPD2 provisions are currently the most advanced in this area.

One country that has been at the forefront of tobacco product regulation for many years is Canada. In 2000, the Canadian health agency “Health Canada” required cigarette manufacturers to issue an annual report for each cigarette brand produced and sold in Canada. Along with other product information, manufacturers were required to report on over 20 constituents in tobacco and on 44 emissions in smoke.

Since 2005, manufacturers have also to provide toxicological *in vitro* data on cytotoxicity (neutral red uptake assay), bacterial mutagenicity (Ames assay) and *in vitro* clastogenicity (micronucleus assay) of the mainstream and side stream smoke of the cigarette. One reason for mandating toxicological testing for all brands in the Canadian market was the concern that the introduction of Lower Ignition Propensity (LIP) cigarettes and related new technologies, such as bands printed on the cigarette paper, could result in increases in toxicological properties of the cigarettes. The three required *in vitro* assays need to follow prescribed standard guidelines of the Canadian Health authorities [12], [13], [14] and were updated in 2017 [255].

In 2010, the use of most additives and flavourings (with exception of menthol) was banned in Canada in cigarettes, little cigars and blunt wraps. The Tobacco and Vaping Products Act that was enacted in 2018 prohibited menthol as a tobacco additive on a federal level.

In 2009, the US Food and Drug Administration (FDA) was mandated with tobacco product regulation [7]. All products that had not been on the market before February 15th 2007 require a Premarket Tobacco Application (PMTA). Applications need to include comprehensive data on the product and manufacture, as well as investigations on health risks. The FDA has recently up-dated the guidance documents to specify requirements and recommended format [253]. Premarket approvals are also required for tobacco products that belong into pre-existing conventional categories, but show alterations in design, components, parts, additives, constituents or smoke constituents. In case of using new additives, the FDA requirements oblige the manufacturers to prove by comparative examination of the new product with its precursor product that the introduced modifications do not lead to a substantial change of the properties of the product (Substantial Equivalence Approach). Such comparisons are to be based on comprehensive testing. Recommendations that are not as yet binding call not only for the characterization of the new product and data on its smoke or aerosol chemistry but also for *in vitro* and *in vivo* procedures for this evaluation without detailing methods and interpretation of the results [15]. For so-called “Modified Risk” tobacco products the FDA and the US Institute of Medicine have issued comprehensive demands, respectively suggestions [16]. Moreover, since 2012 the FDA requires the annual release of data on smoke chemistry (20 analytes) for each cigarette brand [252].

With the implementation of the Tobacco Products Directive (2014/40/EU), additional provisions for the regulation of the use of additives in tobacco products were specified.

- Additives used in tobacco products shall be notified by the companies to the competent authorities via an Internet portal operated by the EU Commission (“EU CEG”). The following information shall be notified: a list of all ingredients and their quantities in the tobacco product; tar, nicotine and carbon monoxide (TNCO) levels for cigarette smoke, available information on other emissions and their levels, the relevant toxicological data regarding the ingredients in burnt or unburnt form, information on the used tobacco itself, annual sales volumes; studies available to market research. Reporting is no longer annually: with first reporting for products already placed on the market by 20 November 2016; and reporting if the composition of a product is modified. For a new or modified tobacco product, information shall be submitted prior to the placing on the market (Article 5 Reporting of ingredients and emissions).
- Cigarettes and RYO tobacco products are prohibited to have a “characterizing flavour” other than tobacco due to the use of additives such as fruit- or candy-like. In the case of products with a market share of 3 % or more, particularly menthol-products the ban applies as of 20 May 2020. Other products, such as cigars, cigarillos and snus are exempt from this ban (Article 7 Regulation of ingredients).
- Certain categories of additives are prohibited: additives that create the impression of a health benefit; that are associated with energy and vitality (mentioned explicitly: caffeine and taurine); that have colouring properties for emissions; that facilitate inhalation or nicotine uptake; that have CMR properties in unburnt form (Article 7 Regulation of ingredients).
- Additives are prohibited that increase the toxic or addictive effect or the CMR properties of the tobacco product (Article 7 Regulation of ingredients).
- Flavourings (and nicotine or tobacco) in filters, papers, packages, capsules or any technical features are not allowed (Article 7 Regulation of ingredients).
- Certain additives used in cigarettes and roll-your-own tobacco are subject to enhanced reporting requirements with the request to assess the effects of these additives in comprehensive studies, inter alia their contribution to the toxicity and addictiveness of the product, if the use of the additive results in a “characterizing flavour”; facilitates inhalation or nicotine uptake; or leads to carcinogenic, mutagenic

or reprotoxic substances that increase the CMR properties of the product. A list of 15 so-called “priority additives” (Decision EU 2016/787) was adopted by 18 May 2016 (Carob bean, Cocoa, Diacetyl, Fenugreek, Fig, Geraniol, Glycerol, Guaiacol, Guar gum, Liquorice, Maltol, Menthol, Propylene glycol, Sorbitol, Titanium dioxide). Manufacturers or importers had to establish a report on the results of the comprehensive studies to these additives by 1 July 2018 (Article 6 Priority list of additives and enhanced reporting obligations).

In the absence of agreed provisions or binding guidelines for a testing strategy of tobacco additives, some models and recommendations have been developed as to what a testing strategy for tobacco additives might look like.

In 2010 the German WHO Collaborating Center for Tobacco Control that operates out of the German Cancer Research Center (DKFZ) published a recommendation regarding a test strategy for tobacco additives [18]. It proposes a multistep evaluation procedure that encompasses the toxicological evaluation of the unburnt additive as well as of its pyrolysis products. If this reveals even a single result of toxicological concern, the evaluation process should be terminated and permission to use the substance as an additive should be denied. This stringent regulation procedure would lead to the refusal to approve most tobacco additives on the basis of pyrolysis results, specifically of those derived from organic compounds, since the combustion of organic material, including tobacco itself, will always produce substances of concern in regard to toxicity [19].

An additional approach published by regulators [20] comprises the evaluation of unburnt additives and their pyrolysis products, as well as the evaluation of chemical composition and biological activity of smoke from test cigarettes, both with and without the additive. However, in a final assessment of the additive all findings should be considered in combination. The National Institute for Public Health and the Environment (RIVM) of the Netherlands has published a model for assessing exposure and health risk from smoke constituents and tobacco additives [21]. In contrast to the two models described above, the RIVM model does not work with a step-wise approach but calculates a “margin of exposure” (MOE) for individual substances in tobacco smoke. This value is intended to characterize the risk potential. The MOE is the quotient of the values for the LOAEL (Lowest-Observed-Adverse-Effect-Level) or NOAEL (No-Observed-Adverse-Effect-Levels) obtained in inhalation experiments, and the dose that reaches the smoker upon inhalation of mainstream smoke. One limitation of this approach is the lack of data on inhalation toxicity for most of the tobacco smoke constituents; another is that neither synergistic effects nor antagonistic effects with regard to specific toxicological end points are accounted for with this model.

In 2004 a working group of CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) issued recommendations for the *in vitro* testing of tobacco smoke [60]. In their report a test battery is recommended consisting of a bacterial mutagenicity assay (Ames assay with *Salmonella typhimurium*), a genotoxicity assay in mammalian cells (micronucleus assay, chromosomal aberration assay or the L5178Y mouse lymphoma assay) as well as a cytotoxicity assay in mammalian cells (neutral red uptake assay).

In a report on the testing and assessment of tobacco additives, the LSRO (Life Sciences Research Organization) concludes that toxicological evaluation of additives basically makes sense. Upon comprehensive discussion on the broad spectrum of methods and the published toxicology data for tobacco smoke and tobacco additives, the experts state in the report that there is no single assay that can adequately cover the broad spectrum of health effects attributed to tobacco smoke. A major problem in the evaluation process is to quantify the amount of an additive that can be regarded to be safe. Only the comparative examination of identical cigarettes with and without the additive would enable the identification and assessment of such an additive as a significant single risk factor. The approach of the LSRO focuses on the evaluation of the smoke matrix as a whole and on the mode of uptake by inhalation [61].

Pursuant to Article 6 of TPD2 the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) provided an opinion related to a testing strategy to assess the effect of additives used in cigarettes and roll-your-own tobacco on the toxicity and addictiveness of the tobacco product or adding a characterizing flavour or facilitating inhalation or nicotine uptake [256]. SCHEER recommends that tobacco additives should be evaluated without tobacco matrix and does not support comparative testing between

products with and without additive. However, TPD2 requires manufacturers to test additives under conditions of use in order to assess if an additive used increases the toxicity, addictiveness and CMR products of a product.

A recent series of publications summarizes the comprehensive studies performed by a tobacco industry consortium to comply with testing obligations for Priority Additives as specified by Article 6 of EU TPD2 [224]. The Table 1 gives an overview of the reporting requirements for toxicological endpoints as per Article 6 of TPD2, the testing approach as described in the publications of the consortium and the proposal in SCHEER's opinion.

Table 1 — Priority Additives Toxicity Testing — TPD2 requirements, industry consortium approach and SCHEER opinion

TPD2 article 6 reporting requirements	SCHEER's opinion	Priority Additives Tobacco Consortium approach
Toxicity and CMR properties <i>contributes to the toxicity...of the products concerned</i> <i>increases the toxicity... of the products concerned</i> <i>increasing the CMR properties in any of the products concerned</i>	Pyrolysis studies of each additive in isolation, i.e. without the tobacco matrix	Comparative testing including test cigarettes with and reference cigarettes without the additive concerned: — literature review; — smoke chemistry; — transfer studies. <i>In vitro</i> toxicity test battery: — bacterial mutagenicity; — neutral red uptake assay; — genotoxicity in mammalian cells.

It is very clear from the example of the regulatory requirements for Priority Additives testing in the EU that there is an increasing focus on the scientific assessment of various aspects of tobacco additives. At the same time it is also clear from this example that there is an urgent need for validated test methods and assessment strategies that can be agreed on.

In the light of increasing regulatory scrutiny and in the absence of any binding or generally recognized test procedures, the cigarette industry and independent research institutions have developed and established various strategies and systems for testing additives. Those for toxicological evaluation were oriented towards the large number of standardized methods in biological systems for testing industrial chemicals and drugs. Table 5 gives examples of some systematic studies pertaining to the various test methods used.

In summary, there is evidence that there are activities and efforts on several levels to regulate tobacco additives in a variety of different scientific approaches. At the same time there is growing consensus on test methods.

5 General discussion of toxicological examination of additives

5.1 Purpose

In recent years, a wide range of *in vitro* methods have been developed to address respiratory toxicity [270]. Initially, cell culture or co-culture systems had been grown at the liquid-air interface (ALI), allowing a direct exposure of compounds or aerosols at the cell surface. These approaches have advanced further into three-dimensional organotypic models. These models can utilize human derived primary cells, including bronchial epithelial cells and fibroblasts that are cultured in physiologically relevant ratios. Artificial tissues are normally grown on trans-well inserts that allow access to the culture medium via a permeable bottom layer. Models that are now state of the art mimic essential functions of lung epithelia, including barrier function, mucous and cytokine production, as well as cilia function [270]. Organotypic models have already been demonstrated to reproduce tobacco smoke-induced perturbations [271] and have been used for testing [272]. Further, a growing range of commercial three-dimensional models is now widely available, including MucilAir (Epithelix) or EpiAirway (MatTek) – see Table 5. Prospectively, it is aimed to extend *in vitro* models to the organ level, using for example directed differentiation of hPSC (human pluripotent stem cells) in combination with –bio-engineered scaffolds [273].

Further, additional platforms have been developed to mimic specific feature of the lungs, such as the air-blood interface [274]. These micro-devices, named lung-on-a-chip, contain cultures of human alveolar epithelial cells and pulmonary vascular endothelial cells, separated by a flexible, porous membrane [270]. Breathing can be simulated by a periodic application of negative pressure. These models had been used to estimate resorption of toxicants or nanoparticles [257].

In the case of tobacco products, toxicological testing and risk assessment of additives and technological modifications are performed to ensure that the inherent risks of the respective tobacco product are not increased. In a similar manner also other inhaled tobacco products such as heated tobacco products can be tested to determine if they have a reduced risk potential, including decreased toxicant levels in the emissions, when compared to conventional tobacco products.

This document covers in particular methods to generate cigarette smoke, as well as heated tobacco product aerosols that are intended to be inhaled. Both, analytical determination of harmful and potentially harmful compounds (HPHC), as well as data analysis will be covered. The chemical composition of tobacco smoke or aerosols is important information to compare products within defined categories, but it does also provide initial information on the toxicological properties of the product. However, comparative assessments based on smoke or aerosol analyses alone remain challenging, as the background variations due to tobacco variants, lots or other experimental issues can exceed additive-related effects. In addition, decreases or increases of individual toxicant levels do not directly translate into altered health risks, but might require further tests and assessments. Toxicity testing provides an important baseline for assessments, whether individual additives or an altered smoke or aerosol composition can enhance relevant health risks. As application levels of additives are usually low (< 1 %), novel assessment approaches such as Thresholds of Toxicological Concern (TTC) might be used to prioritize the need of further experimental work. Toxicity assays of tobacco-relevant endpoints can also be useful to evaluate modified health risks due to lower exposure levels. Importantly, a growing range of adequate *in vitro* testing systems is now available to replace *in vivo* testing, including respiratory toxicity. This guidance document will provide an overview on subsequent toxicological testing strategies and evaluations.

5.2 Basic considerations

5.2.1 General

The health risks of tobacco products are mainly related to the emissions of carcinogens or other toxic compounds from the tobacco itself when combusted or heated. Tobacco additives comparatively contribute to a minor extend to the toxicity of tobacco products, but remain in the focus of European regulation. At present, comprehensive assessments for cigarettes and roll-your-own tobacco were required for

15 compounds that had been listed in the priority list of additives (Decision EU 2016/787) in compliance with article 6 of the European Tobacco Product Directive (2014/40/EU).

In addition, technical innovations, most importantly novel tobacco products that are inhaled such as tobacco heating systems, have questioned the previous paradigm of equally high concerns that apply to all kind of tobacco products. This leads to two challenges: Firstly, toxicological risk assessments of novel tobacco products are required in comparison to other product categories, such as cigarettes and/or other conventional tobacco products. Importantly, this data is required to provide a risk/benefit analysis in compliance with the European Tobacco Product Directive 2014/40/EU, Article 19.

Secondly, the methods described in this document can also be used to assess novel tobacco heating products with regards to their quality-, safety- and performance standards.

For the toxicological evaluation of additives in unburnt form, validated scientific principles and procedures are available from other fields, e.g. for pharmaceuticals, food and cosmetic products.

National and international guidelines for established test systems to assess the biological activities, respectively toxicity of single substances, e.g. from OECD (Organization for Economic Co-operation and Development), ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use), FDA (Food and Drug Agency, USA), EPA (Environmental Protection Agency, USA), as well as the testing guidelines from German Chemicals and Pharmaceuticals Laws can be utilized as established standards.

By contrast, for testing of additives in tobacco products there are no recognized standards for evaluating the effects of additives upon the toxicity of emissions. It stands to reason that two requirements should be met for this specific toxicological assessment:

- the test methods should follow closely the international guidelines cited above that have been well established and validated;
- the test methods should be able to detect the currently known toxicological properties of tobacco smoke which are related to cigarette smoking linked diseases. Pyrolysis of single compounds does not realistically reflect combustion processes of cigarette smoking.

Beside similarities in the cited guidelines for toxicological testing of additives as neat substance, there are also several differences in evaluating additives because they are burned or heated with tobacco and can be inhaled by the consumer. Therefore, additives should be evaluated under the realistic conditions of inhalation of tobacco product emissions.

Special features for evaluation of the biological activity of tobacco smoke from cigarettes, with and without additives, are the following:

- Tobacco smoke is a well-researched mixture of substances. Its toxicological effects *in vitro*, *in vivo* and in humans are well known. In these systems tobacco smoke demonstrates clearly measureable basic toxicological activity.
- Quantitative distinction is therefore to be made between the toxicity of smoke from tobacco products with and without additives. Principally it is not the question whether tobacco does show or does not show an effect in a certain biological test, but whether the addition of the additive increases the inherent toxicity.
- Tobacco smoke is not a single substance but a complex mixture of gaseous and particle bound substances. This is further complicated by the fact that this mixture does not follow static but rather dynamic physiochemical distribution equilibrium. This poses special challenges regarding the ways in which tobacco smoke is generated and analysed.

- It is known that many smoke constituents require metabolic activation to develop biological activity. For a single substance it is possible to identify enzymes/isozymes relevant for its metabolism in humans and to take this knowledge into account for the biological assays. However, with complex mixtures such as tobacco smoke this is practically impossible. Thus, the use of certain metabolic activation systems always represents a compromise which should have the broadest possible substrate spectrum.
- Some novel tobacco products such as heated tobacco systems generate an aerosol that is subsequently inhaled. The aerosol is generated by heating tobacco at lower temperatures as measured in burning cigarettes. The toxicological assessment of tobacco additives used in tobacco heating systems starts with the hazard characterization of the neat additive based on literature and computational toxicology investigations. The hazard characterization is used to exclude additives classified as genotoxicants, carcinogens, reproductive and developmental toxicants. Thermal degradation experiments and transfer studies should be performed at an adequate temperature. Both studies will also demonstrate if the additive is transferred intact at the operating temperature and if further chemical analysis are necessary. If adaptable, similar methodologies as used for cigarette smoke should be applied to analyse the overall biological toxicity of the aerosol. Aerosols should also be analysed for novel toxicants that are possibly less relevant for combustible cigarettes.

In principle, most methods and approaches described in 5.2 can be adapted and applied to heated tobacco products.

5.2.2 Application of Threshold of Toxicological Concern (TTC)

The TTC is a generally recognized concept that provides human dose levels for substances that are considered to be of negligible risk at these levels and, despite the absence of specific toxicity data for these substances, do not need further experimental work. These dose levels depend, in principle, on three certain chemical structural alerts for a given substance (Cramer Classes I to III) and the level of human uptake. In the context of tobacco additives the TTC concept has been proposed for the first time by Dempsey and co-workers [91]. Starting from oral data and based on rather “reasonable to conservative assumptions”, including that the total amount of the additive/combustion products might be genotoxic, the authors concluded that an addition level of 0,025 ppm additive in the tobacco is the TTC level. TTC concept has since been expanded and refined.

As per the European Food Safety Authority [258]: “It is an important science-based approach for prioritising assessment of those chemicals with low-level exposures that require more data over those that can be presumed to present no appreciable human health risk.” In the TTC concept, a “safe” level of exposure can be identified for many chemicals based on their chemical structure and the known toxicity of chemicals that share similar structural characteristics. In that context, the Cramer scheme is most frequently used, which enables chemicals to be placed into one of three classes (I, II or III in order of increasing likely toxicity), according to certain structural characteristics. Class I contains substances with simple chemical structures and for which efficient modes of metabolism exist, suggesting a low order of oral toxicity. Examples include aliphatic alcohols and common terpenes. Class II contains substances which possess structures that are less innocuous than class I substances, but do not contain structural features suggestive of toxicity like those substances in class III. This includes, for example, terpenes that are esters of a secondary alcohol attached to a terminal vinyl group. Class III are substances with chemical structures that permit no strong initial presumption of safety or may even suggest significant toxicity or have reactive functional groups. Examples include structures that contain halo-, nitro-, N-nitroso- and diazo-elements. Although the Threshold of Toxicological Concern (TTC) concept was originally proposed in the context of contaminant risk assessment and prioritisation, much extension and validation work has been published since then. The TTC concept has been reviewed previously within Europe, [236], [237], [238]. The joint, final opinion of three non-food safety European Commission Scientific Committees [236] concludes the approach is acceptable for human health risk assessment of systemic effects caused by very low exposure levels of chemicals, regardless of whether these are deliberately added ingredients or contaminants. The TTC concept has already been implemented both at European and WHO level (EFSA, JECFA) in ingredient risk assessment for food flavourings [236], [239], and in food packaging ingredients (FDA) [240]. Some categories of chemicals have been excluded from

this type of evaluation, such as known genotoxic carcinogens, protein allergens, organophosphates, heavy metals and dioxins.

Although most TTCs are based on oral data, several inhalation TTCs have been proposed [91], [241] to [247]. The existing proposals for inhalation TTCs and the underlying NOELs/NOECs (No Observed Effect Levels/No Observed Effect Concentrations) have been derived from different databases that covered air pollutants, industrial chemicals [241], [244], [245], but also ingredients of consumer aerosol products [246]. Data on consumer products that are intended to be inhaled might provide the most appropriate option to propose TTCs, because in industrial or environmental databases highly toxic compounds are often overrepresented.

Further, occupational exposure levels as notified under REACH have also been used. However, these data were not necessarily based on inhalation data [247]). Due to the limited data available for Cramer class II compounds, no specific inhalation TTC was derived. As a cautious approach, it is recommended to apply the Cramer class III TTC instead. The study [246] used compounds for which both oral and inhalation data were available. Systemic TTCs based on inhalation data, were compared with corresponding thresholds that were derived by route-to-route extrapolation of the oral data. Table 2 shows calculated TTC following different approaches, i.e. use of different data bases and exposure routes. Notably, these TTC values differ no more than by a factor 2, viz. Table 3. That means that for a given Cramer Class the TTCs are not substantially different.

As 90 ug/day is most conservative TTC estimate for Cramer Class III. It is recommended to use the corresponding data set (see Table 3). This is also consistent with the EFSA approach.

Table 2 — TTC for systemic and local effects derived from oral and inhalation data, assuming 60 kg body weight. Transcribed from [246]

Cramer Class	TTC for systemic effects from inhalation data ug/day	TTC for systemic effects extrapolated from oral data ug/day	TTC for local effects from inhalation exposure^a ug/day
I	980	1 800	1 400
III	170	90	470
^a assuming 650 g human lung weight			

Because it was recognised that classification by Cramer Class was developed for oral exposures, a more inhalation-focussed structural identification approach has been proposed [248]. However, this proposal has not yet been widely discussed or accepted, so for now the historic experience favours the use of Cramer Classes.

The TTC approach is not considered appropriate for certain classes of chemicals, most notably genotoxic carcinogens, protein allergens, organophosphates, heavy metals, polymers, aflatoxin-like, azoxy-, N-nitroso-compounds, benzidines, hydrazine, polyhalogenated-dibenzodioxins and related compounds [238]. The vast majority of these restrictions refer to potent toxins that would never be considered for use as a tobacco additive and generally would not meet the requirement for the absence of CMR in the TPD2. The exception could be potentially very unusual additives, such as proteins, metals or very complex chemical structures with several structural elements that are not adequately represented in the available databases. It is unlikely any of these will be used as tobacco additives. For such additives, it would not be appropriate to apply a TTC approach.

In contrast to the additives themselves, thermal degradation products from additives could include CMR compounds. If the chemical nature of the degradation product is known, and sufficient information exists to determine it is not mutagenic, the Cramer Class can be determined and the TTC above applied. From a

practical point of view, it is worth considering any thermal degradation products are expected to be present at lower levels than the unburnt parental additive. For additives below the TTCs, levels of thermal degradation products are expected to be very low. In fact, thermal degradation products of minor additives can often not be identified under realistic conditions, i.e. in cigarette smoke. To detect a chemically unidentified compound in smoke or aerosol, untargeted measurement techniques are required, such as gas chromatography coupled with mass spectrometry. These are semi-quantitative techniques, complicated by the high number of compounds in smoke or aerosol. Notably, pyrolysis data of the additive on its own are useful to estimate whether an additive undergoes thermal degradation. But if pyrolysis was confirmed, the identified products are not necessarily representative for the thermal breakdown products found in smoke (as discussed in 5.4.1.1). Pyrolysis data of an additive are not sufficient to predict reliably emissions in cigarette smoke. A practical approach to the assessment of thermal degradation products of very low level additives is therefore essential.

Table 3 — Suggested TTC for tobacco additives

TTC µg/day	Applicable Compounds
90	Cramer Class II and III compounds
1800	Cramer Class I compounds

For example, for Cramer class II or III compounds, a TTC of 90 µg/day might be applied. This corresponds to 4,5 µg/cigarette, i.e. 0,0006 % to 0,002 % or 5,6 ppm to 18 ppm, depending on the tobacco rod weight. This assumes a consumption of 20 cigarettes/day and a range of 250 mg to 800 mg tobacco weight (heated tobacco products and conventional cigarettes).

For Cramer Class I additives, a TTC of 1 800 µg/day might be applied, if a major proportion of Cramer Class III pyrolysis products can be excluded. Assuming a daily consumption of 20 cigarettes/day, depending on the tobacco rod weight of the combustible cigarette or the heated tobacco product. Applying the same assumptions (cigarette consumption and tobacco weight), this would translate into a supportable concentration of 0,011 % to 0,036 %, or 113 ppm to 360 ppm.

Some additives are not single compounds but complex materials, such as plant extracts that may be used as casings or flavours. If sufficient toxicological information is available to exclude for example CMR-properties, it might be assessed whether the TTC approach can be applied as well. For mixtures, a specific TTC could be derived by taking into account the levels and toxicological profiles of the main constituents in the mixture. This holds true also for single Cramer Class I substances, if pyrolysis products of higher Classes need to be considered.

Annex B provides examples of how this approach could be applied to two example additives, geraniol and fig juice concentrate.

In consideration that smoke is a very complex mixture, the opinion from the three non-food European scientific committees on the risk assessment of mixtures is noted [249], which concludes that “interactions (including antagonism, potentiation, and synergies) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant.” Indeed, that report recommends a TTC like approach to eliminate combinations that are of no concern. For compounds with similar toxic effects, it recommends dose/concentration addition as the default, conservative approach when neither mode of action, nor dose response information is available.

In summary, applying a TTC approach to the risk assessment of tobacco additives can help to assess whether existing information suffices to decide if the use of an ingredient at the proposed level is supportable or not, or whether (further) experimental test data is required. The TTC listed in Table 3 could be applied to both tobacco additives and their potential thermal breakdown products.

5.2.3 Impact of additives on the overall toxicity of cigarettes

Risk assessments of additives occur in the context of an inherent toxicity of tobacco products. As major requirement, toxicological evaluations need to confirm that neither additives, nor thermal degradation products increase noticeably the toxicity of tobacco products, in relation to additive-free reference products. Applying a precautionary approach, additives bearing high toxicological risks, as for example CMR-compounds, are prohibited in the EU and in other countries.

The inherent toxicity of tobacco products affects the risk assessment of any additives in several ways. Further, methodologies for conventional assessments of chemicals need to be adapted. The requirement for any tobacco additive and potential thermal degradation products, not to increase the toxicity *over and above* that of the additive-free reference product is crucial and needs to be explained in detail. In that light, two important points should be considered:

- a) in case of low application levels of some additives their contribution to the toxicity of tobacco smoke are expected to be very minor (Figure 1);
- b) some additives are used in higher levels and thus *replace* the tobacco, so their emission products replace the tobacco emissions. These additives would thus only increase the toxicity of the base product if their emissions were more toxic than those of the same amount of combusted tobacco.

Additional toxicological risks of additives depend on the application levels (amount of additive per kg tobacco) that can range between low ppm-values for flavours and single digit percentages for humectants. However, the inherent toxicity of tobacco constituents and emissions is much higher in comparison to additives. Statistical deviations of tobacco related effects on smoke composition or toxicity can therefore exceed the minor effect levels that are induced by typical additives. In this context, residual toxicities of additives and potential thermal degradation products would be negligible, as relevant contributions at the product level remain statistically uncertain (see Figure 1). Further, in experimental settings, a maximum dose-response might already been induced by tobacco-associated toxicants that would mask the minor effects of additives. Consequently, it is possible that additives may not assert relevant toxic effects when added to tobacco, although clear effects can be determined, when tested as individual compounds in relation to appropriate negative controls.

Importantly, similar considerations do also apply for additives that are used at comparatively high levels (i.e. humectants), because a substantial amount of tobacco is replaced. In principle, increased effects on the overall toxicity would only be expected for additives that show higher toxic properties than tobacco or modify combustion. Further, the magnitude of such additional effects needs also to exceed deviations found for additive-free tobacco in corresponding measurements (Figure 1).

If toxicological testing of an additive and its thermal degradation products, *on their own*, do not demonstrate adverse effect, this is a conclusive finding. However, if detectable effects were demonstrated, further work is required to determine whether these effects will be noticeable against in the content of tobacco products.

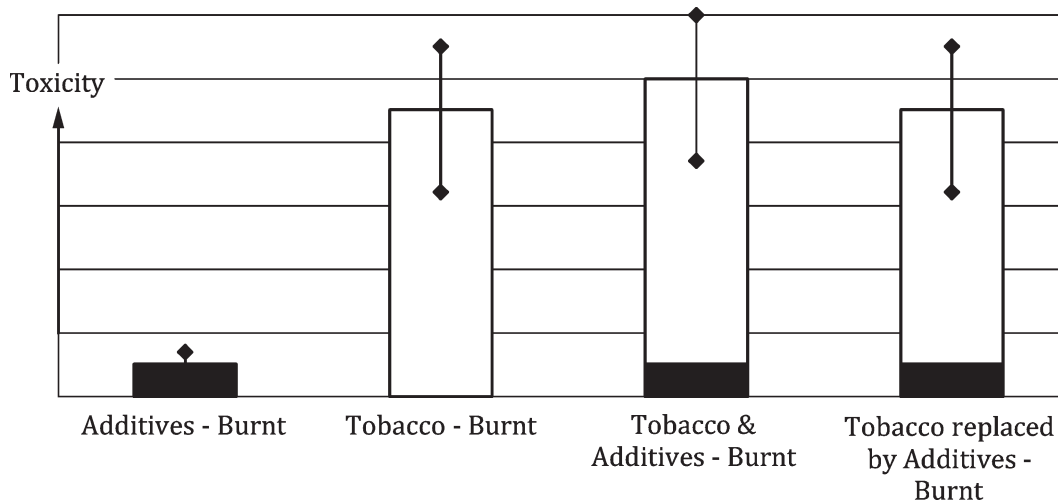


Figure 1 — Theoretical example of toxicity due to the combustion of the additives compared to the additive-free tobacco

This requires comparative testing as only option to compare test cigarettes and additive-free controls. These tests should address smoke chemistry as well as *in vitro* toxicity. It could be argued that comparative testing lacks discriminate power, implying that this approach is unsuitable to address the toxicity of tobacco additives. However, some clarification is needed: Firstly, as explained above, additive-related effects on both smoke composition and toxicity need to exceed a threshold that is linked to statistical deviations of the corresponding tobacco related effects. Secondly, this requirement must not be regarded as a methodological shortcoming, but applies to all tests that determine responses of very different magnitudes. Negative findings of additive-related effects reflect a marginal impact on the overall toxicity of tobacco products.

As a practical approach, transfer of additives or typical constituents, such as theobromine in cocoa should be detectable at all relevant application levels. However, alterations of individual compounds are feasible if additives were applied at moderate levels (above 0,1 %). This could affect metals or other minor emissions in cigarette smoke. Consistent effects, both increases and decreases of typical MSS components (carbonyls/volatiles) can be related to additives. Usually, this requires comparatively high applications levels (above 1 %). For example, such effects might be feasible for humectants, starches or sugar supplements.

Variations in the chemical composition of cigarette smoke are not necessarily linked to altered health risks. Further assessments are necessary that require toxicological testing. A tiered approach might be employed to address increased or additional toxicological effects in relation to tobacco additives.

5.2.4 Test matrix

5.2.4.1 Cigarette test matrix

As such, the evaluation of a tobacco additive on its own and its potential pyrolysis products is not sufficient to decide whether the additive might increase smoke toxicity in comparison to the smoke without the additive. Rather one has to test the additive after addition to the tobacco matrix by means of suitable test methods. This needs to include the examination of the combustion and pyrolysis products of the additive, of the percentage of unchanged additive transferred into the smoke and of the reaction products formed with other emissions.

Combustion of any organic material leads to the formation of toxic products, and the burning of tobacco is no exemption. Thus, cigarettes do always generate a highly toxic smoke, regardless of whether they contain additives, or not. For a “practical” evaluation to be as realistic as possible, the assessment of the toxicity of an additive only makes sense when cigarettes with the additive under test are compared to identical cigarettes without the additive.

When determining the test matrix a decision needs to be made as to in what form the additive is to be tested: as a single additive or as a mixture of additives. Testing individual additives has an advantage in that one can use relatively high concentrations. However this option raises the concern that possible interactions with other additives will not be assessed properly and that, considering the large number of additives used, testing them individually would not be very practical. Pragmatically, the following approach can be taken: Additives used in relatively large quantities can be tested individually, while those used in only small amounts can be tested as mixtures.

Regarding the choice of the tobacco matrix in the form of a control cigarette and a test cigarette¹ there are basically two possibilities:

- The “product-based” assay compares the unmodified actual brand (control cigarette) with the modified product (test cigarette). Principally, the additives are only tested at concentrations which are actually added to the product.
- The “principle-based” assay compares an experimental control cigarette without additives to a test cigarette containing the targeted additives. The control and test cigarettes should be identical as far as possible except for the additive. The control cigarettes should be comparable to currently marketed brands as concerns the tobacco blend and further specifications (filter tip, filter ventilation, paper etc.). With this kind of testing one can examine individual additives as well as mixtures of additives.

In the product-based assay the concentrations of the additives depend on the planned product specification and thus may be too small to reveal any effects in the applied test procedures. An important principle of toxicological testing, namely overdosing vs a normal situation, cannot be realized in this case. Complicating the issue in product-based testing is also the fact that cigarettes on the market vary due to harvest-related fluctuations.

In the principle-based assay this overdosing is possible. It is desirable that the elevated concentration is distinctly higher than the level used in reality. In practice, this is not always possible, as this can present technical difficulties in cigarette production. The combustibility may also be affected to an extent that leads to a change in smoke composition that would not occur under normal conditions. Substantial differences in the water content of the aerosol might be indicative for disturbed combustion. Ideally, the additive should be tested using 3 concentration levels which does not yet significantly impact the combustion properties of the tobacco. Testing of 3 levels improves the identification of effects on the basis of their dose dependence. The use of control cigarettes with varying specifications (e.g. tobacco blends) does not seem necessary: As can be seen from the literature, given additives have been tested with variable control cigarettes in several laboratories [49], [62] to [64]. Despite the use of different control cigarettes, comparisons between the control and the test cigarettes led to the same results.

5.2.4.2 Heated tobacco product test matrix

The statements above are also generally applicable to products where the tobacco is only heated and not burned.

5.2.5 Smoke or aerosol generation

5.2.5.1 Cigarette smoke generation

The generation of smoke should as a rule be based on standardized and generally accepted procedures. Standardized methods allow the verification of results obtained in different laboratories and comparisons of data for different cigarettes.

1 Although used in the first edition of this document, the term “reference cigarette” is no longer used in this context because this could lead to confusion with the term “standard reference cigarette” which is often used in the literature (see 6.2.8).

The ISO 3308 smoking regimen [65], e.g., uses a 35 ml puff (puff volume) generated by a smoking machine every 60 s (puff interval), and a puff duration of 2 s, with the filter ventilation holes (if present) open. This smoking regimen is fundamentally the same as the method of the US FTC (Federal Trade Commission). In addition to these methods additional modalities have been developed with more intense smoking regimens: the “Massachusetts Method” uses a 45 ml puff every 30 s, and a puff duration of 2 s, with the filter ventilation holes 50 % blocked [66] and the “Canadian Intense Method (CI)” by Health Canada uses a 55 ml puff every 30 s, and a puff duration, of 2 s, with the filter ventilation holes completely blocked [67]. This method is equivalent to the ISO 20778 method [A].

Primarily, the different smoking regimens lead to different volumes of cigarette smoke obtained from one cigarette.

Trivially, the more intensive smoking regimens result in higher volume yields of smoke per cigarette (particle and gas phase) and thus to a higher yield of, e.g., TPM, nicotine, and specific toxicants per cigarette. Especially for cigarettes with low ISO emission values, the more intensive smoking regimens supposedly lead to a more realistic assessment of the mean amount of smoke taken up by smokers. However, for cigarettes with high ISO emission values, the more intensive smoking regimens appear to indicate unrealistically high smoke yields when based on the average uptake data for all smokers in a given population [68], [69]. Because of the extremely variable ways smokers are smoking under real-life conditions, no typical smoking behaviour and therefore no typical smoke intake exists which could be reproduced by a certain specified smoking regimen.

In addition to the different smoke volumes, the quality of the smoke also changes depending on the chosen smoking regimen.

Hereby, the more intensive smoking methods result in lower concentrations of most toxic substances in the smoke. This is obviously an effect that can be explained by a more complete combustion when cigarettes are smoked more intensively. With an intensive smoking method, more oxygen from the ambient air is supplied per unit of time to the burning tobacco than with a less intensive smoking method. This leads to a higher proportion of tobacco components, which are burned completely to carbon dioxide and water. Thus, the toxicity of the smoke per smoke volume, per weight of condensate, or nicotine decreases. Yet, this decrease does not balance the increase in smoke volume per cigarette under the intense smoking regimens. This means, the total delivered toxicity per cigarette remains higher under the more intensive smoking methods.

More importantly, obviously also due to the more complete combustion, the more intensive smoking regimens result in a trend for levelling off the concentrations of toxic substances and smoke toxicity for different cigarettes. Differences in the toxicity of additives which might be observed applying the ISO 3308 method could be obliterated when using the more intensive smoking regimens. Thus, the intensive smoking methods appear to be less suitable to assess possible effects in smoke quality of different cigarettes caused by an additive [70].

The probability of detecting a statistically significant additive-related difference depends not only on the magnitude of the difference but also on the magnitude of the statistical variation of the measurement results.

This could result in objectively justified preferences for a certain smoking method that leads to a lower dispersion of the measurement results. However, a comparison of the variability coefficients for the chemical analysis of smoke constituents measured according to CI and those under ISO 3308 method does not show a clear trend. Obviously, the scatters of the measured values under both smoking methods are on average the same [B].

5.2.5.2 Heated tobacco product aerosol generation

In principle, methods to generate cigarettes smoke can be adapted to heated tobacco products; however, technical modifications might be necessary, as indicated by the initial work by ISO in 2019.

5.2.6 Substrates

5.2.6.1 Cigarette substrates

Mainstream smoke or its fractions are normally used as the substrate for the toxicological testing of cigarette smoke.²

For the chemical characterization of cigarette smoke constituents, the analytes are determined in the mainstream smoke. If the same analytes occur in the gas phase as well as the particulate phase, they are determined in both phases.

The use of native mainstream smoke that the smoker is exposed to is technically impossible. The following surrogate test substrates for mainstream cigarette smoke analysis are currently in use:

- fresh mainstream smoke (“whole smoke” not older than a few seconds, containing the gas and the particulate phase);
- fresh gas phase, obtained by removing the particulate phase from the mainstream smoke using a Cambridge filter;
- particulate phase (TPM: total particulate matter) obtained by particle separation from mainstream smoke using a Cambridge filter and subsequent extraction;
- combination of TPM and gas phase extracts (buffer or cell culture medium extract of gas phase)³;
- buffer or culture medium after passage/bubbling of mainstream smoke (collection medium);
- buffer or culture medium after passage/bubbling of gas phase (collection medium).

It should be noted that the selection of a certain test substrate does not necessarily reveal which smoke constituents and how much of them will reach the biological test system. For whole smoke, for example, (depending on particle size and water solubility), selective exposure is to be expected because the deposition of the various smoke constituents can be extremely variable. Since this is also true for various sections of the human respiratory tract, it is not a trivial task to judge the “approximation to reality” of a given combination of test substrate and biological test system.

2 The burning of tobacco produces a complex mixture of a gas phase and a particle phase. The latter can be collected in the laboratory on a Cambridge glass fiber filter disc by smoking using a smoking machine. The filter retains 99,9 % of the particulate matter with an aerodynamic diameter of $> 0,3 \mu\text{m}$ and 99,2 % of particles with a diameter of $> 0,1 \mu\text{m}$. The mass of the particulate phase collected on the filter (TPM = total particulate matter also called condensate) is determined gravimetrically. Substances passing the filter are designated as the gas phase of tobacco smoke.

Nicotine and water content of the particulate phase can be assessed by chemical analysis. Subtracting the amounts of water from the mass of TPM yields the dry particulate matter (DPM). Subtracting the amounts of nicotine from the mass of DPM yields the nicotine-free dry particulate matter (NFDPM) which is also known as “tar”.

A differentiation is also made between mainstream smoke and sidestream smoke. Mainstream smoke is generated during a puff drawing on the glowing cone of the burning cigarette, and is drawn through the cigarette column that is not yet burning towards the mouthpiece, and is inhaled by the smoker. The sidestream smoke is produced between puffs and escapes into the environment. Due to numerous variables in the generation of mainstream and sidestream smoke (such as burning temperature and availability of oxygen) these two types of smoke have different physicochemical and chemical characteristics.

Mainstream smoke, consisting of gas phase and a particle phase, will hereafter also be referred to as “whole smoke”.

3 The Health Canada Official Method T-502, *Neutral Red Uptake Assay for Mainstream Tobacco Smoke*, requires the use of this substrate.

Exposing the target in *in vitro* assays to mainstream smoke that is maximally a few seconds old requires the utmost experimental ingenuity. This applies to the generation of a relatively fresh smoke aerosol at a desired level of dilution as well as to cells or bacteria at a barrier in air-liquid exposure. In recent years several research groups have developed and characterized diverse systems for whole smoke exposure [71] to [82]. A major focus of these recently published research projects lies in the detailed analysis of the exposure situation, where in addition to the ratio of the particulate phase to the gas phase in fresh smoke, the smoke in the exposure chamber, the loss of particulate matter, and physical-chemical differences were also investigated. Use of freshly generated total mainstream smoke and gas phase as a test substrate is discussed in the literature especially in view of novel tobacco products and as an alternative approach to bioassays in laboratory animals [82], [83].

The collection media after passage of mainstream smoke or gas phase are clearly artificial test substrates for tobacco smoke. Nevertheless, they particularly allow for the testing of the toxicity of water soluble agents of the gas phase, as has been reported frequently in the literature [84]. Cells might be maintained in collection medium during bubbling. Alternatively, the collection medium can be dosed applied to the cells after bubbling. Methods of preparing such collection media have not yet been standardized and usually are carried out on the basis of 'in-house' procedures, so that different compositions of the substrates can lead to discrepancies in the end results. Nevertheless, assaying collected aqueous gas phase is indeed included as part of the Health Canada Method T-502 (neutral red assay) [13]. It can be assumed that, according to the published methods for capture of total mainstream smoke, the particulate phase will be underrepresented relative to the gas phase because of its low rate of trapping. It holds true that for both whole mainstream smoke and the gas phase there is rather limited capture of hydrophobic smoke constituents by aqueous media. Systematic studies of these aspects have not been reported in the literature.

The most widely employed biological testing is certainly that of the particulate phase dissolved/suspended in DMSO (dimethyl sulfoxide) or other organic solvents. The preparation of the total particulate matter (TPM) follows well-described ISO Standard Methodology [85]. The *in vitro* assays can be carried out with TPM solutions according to the existing standardized methods for individual compounds (e.g. OECD Guidelines). Long-term storage of the particulate phase under controlled conditions does not affect the toxicological properties of the TPM once it has been collected [86]. When TPM is undergoing testing in biological assays, it should be borne in mind that the gas phase effects are disregarded in this setting, and that the possible interaction of constituents of the gaseous phase and particulate phase is discounted as well. Based on the selection of the test systems, the gas phase has been shown to be less significant than TPM (e.g. Ames' mutagenicity assay in its Plate Incorporation version). This can be explained by both, the volatile nature of the gas phase which leads to limited exposure of the bacteria and the chemical nature of the gas phase constituents. With other biological endpoints the gas phase has a major role, as for example in cytotoxicity [87]. Selection of the most suitable test substrate therefore depends on the formulation of the challenge and on the selected bioassay and its biological endpoint. For example, when testing cigarettes with different filter tips, genotoxic (micronuclei) as well as cytotoxic (neutral red uptake) endpoints have shown significant differences for the toxicity of whole mainstream smoke that were not paralleled by results with the smoke condensate [88].

There is still uncertainty as to the effect of differences in the deposition of whole smoke in exposure systems and in the human respiratory tract upon test results.

5.2.6.2 Heated tobacco product substrates

It should be noted that the composition of the TPM and particular matter generated by tobacco heating systems is very different. A new terminology needs to be developed. Recently term ACM (aerosol collected material) was proposed [264].

In principle, methods and approaches described in 5.2.6.1 can also be applied to heated tobacco products; however, technical modifications might be necessary.

5.2.7 Dose parameters

5.2.7.1 Cigarettes

Toxicology requires the definition of effects in reference to the dose applied in the assay. Studies on tobacco smoke have been done for many years by applying the TPM, DPM or NFDPM, as dose parameters. Individual toxicants in the smoke are often normalised in relation to TPM. Accordingly, if gas phase constituents are considered, these are also based on the TPM or related parameters. For smoke chemistry, reference is also made to the number of cigarettes smoked. More recently it has become increasingly customary to select the mass of nicotine as the reference point. Because nicotine plays a decisive role in the pharmacology of tobacco smoking, this is reasonable. The concentration of nicotine in tobacco smoke for a given cigarette type is roughly proportional to the TPM concentration [89]. Thus, in principle, all three reference scales (TPM, cigarettes, and nicotine) can be applied. Formally, the mass of TPM can also be calculated for the aerosol of tobacco heating systems; however, the total yield and composition differ from cigarette smoke. Comparative studies across product groups should preferentially use nicotine as reference. However, with regard to tobacco additives caution has to be exercised in the interpretation of results based on nicotine. To detect elevated toxicity that might be attributed to the additive(s), experimental products to be tested have been spiked with more of the additive than is actually planned to be used in the commercial product. Thus, an additive will replace tobacco by mass while it will not deliver nicotine to the smoke. Accordingly, the nicotine-based calculation would appear to yield numerically greater biological activity because the additives generate TPM but no nicotine. It is therefore crucial for the interpretation of outcomes to use both the reference to the mass of nicotine and the corresponding data per mass of TPM.

5.2.7.2 Heated tobacco products

In principle, approaches described in 5.2.7.1 can also be applied to heated tobacco products; however, modifications of the terminology are necessary.

5.2.8 In vivo versus *in vitro* testing

The question with regard to the scope of the test battery also has to be considered in the context of *in vivo* assays. *In vivo* assays are usually regarded to be of higher relevance in comparison to *in vitro* assays for characterizing potential risks. However, among the general public there are serious ethical concerns against the use of animal experiments for the safety assessment of “luxury” consumer products. According to German animal protection laws, the use of animal experiments is generally not permitted for the development of tobacco products. At EU level, there are also strong efforts to reduce the use of experimental animals for the toxicological assessment of chemicals by using alternative methods. In this context, the activities of the EURCL ECVAM (EU Reference Laboratory for alternatives to animal testing/European Centre for the Validation of Alternative Methods) and ZEBET (Centre for the Documentation and Evaluation of Alternatives to Animal Experiments at the German Federal Institute for Risk Assessment) should be mentioned. Aside from animal protection concerns which are decisive factors for reducing animal experiments, there are also scientific concerns against the use of animal experiments for the assessment of the toxic effects of tobacco smoke. The generally accepted inhalation animal models for the toxicological assessment of tobacco smoke basically only show, in a reproducible manner, irritative effects in the respiratory tract [92]. These irritative effects are considered as possible indications of tumour promotion and therefore an important toxic effect. However, they do not mirror the multiple harmful effects of smoking on human health, especially the increased risk of heart and vascular diseases. Animal models such as the mouse skin painting bioassay are often viewed critically because of differing application pathways. In this context, a comprehensive *in vitro* test battery could be of a high value.

5.2.9 Positive controls

5.2.9.1 Cigarettes

When testing chemicals, positive controls are generally used. In chemical analysis this is achieved by using reference chemicals with known purity. This allows the calibration of equipment and the quantitative

determination of analytes within a sample. It is also standard practice in the determination of tobacco smoke constituents. In bioassays, biologically active reference chemicals are used to verify that the assay is principally capable of detecting the activity searched for. This again is common practice in the assessment of tobacco smoke. Furthermore the use of reference substances allows the quantitative characterization of the sensitivity of the bioassay. As single substances cannot detect possible positive as well as negative interactions occurring in a complex mixture of substances such as tobacco smoke, it is common practice to test the tobacco smoke of a standard reference cigarette in parallel. Standard reference cigarettes are commercially available monitor products, such as the “Kentucky Reference Cigarettes” or the “CORESTA Monitor Test Pieces”⁴. By defining the measurement result of the standard reference cigarette as a 100 % value and by referring test results of other cigarettes to this value, reference cigarettes also serve long-term control purposes within a laboratory as well as for interlaboratory comparison and validation. However, it should also be noted that frequently used reference cigarettes, e.g. 3R4F are not typical market products, but show comparatively high emissions of some toxicants. Although this qualifies them as positive controls in toxicity testing, alternate references are required to compare reduced toxicant levels or reduced *in vitro* toxicity to conventional market cigarettes.

5.2.9.2 Tobacco heating systems

The development of specific reference products for tobacco heating systems would be beneficial.

5.2.10 Discriminatory power of test systems

5.2.10.1 Cigarettes

As discussed in 5.2.3, considerably increased concentrations of additives are used in test products, as compared to the intended use in the marketed product, in order to increase the probability of detecting possible changes in the toxicity of tobacco smoke or aerosol. To obtain highly reproducible and quantitative results, the bioassay should be optimized for the test matrix tobacco aerosol (e.g. number of cells, time of exposure, and concentration of the metabolic activation system). In this context an important question is how large a difference in the toxicity of the control and test products needs to be in order to be detectable in different bioassays. Based on study designs with the usual number of replicates [93], the typical variation within a laboratory (intrastudy variation) and the level of statistical significance (unpaired two-sample test, type I error = 0,05, type II error = 0,2), it is possible to mathematically determine the smallest detectable difference (minimal detectable difference, MDD). Empirical values for tobacco-specific investigations show that, depending on the assay, the difference needs to be 5 % up to 50 % in order to be deemed statistically significant. For chemical analyses of tobacco smoke constituents, the calculated MDDs span the whole range (catechol, ca. ± 6 %; formaldehyde, ca. ± 25 %). For *in vitro* bioassays these values often lie within the medium range (Ames assay, e.g. with TA98+S9, ca. 15 %; neutral red uptake assay, ca. 20 %) [55], [94]. The irritative effects noticeable in inhalation studies show the highest MDDs (hyperplasia of larynx epithelia, ca. 50 %) [55], [69], [93], [94]. This means that an increase in complexity of the bioassay is accompanied by a decreased discriminatory ability. The more relevant endpoints often show a weaker discriminatory ability [93]. However, it should be noted that these MDDs relate to single endpoints. In inhalation studies, multiple e.g. 10 different localizations of the respiratory tract are examined, which closely correlate to irritative changes and therefore can reveal a trend. Furthermore, male and female animals show almost identical effects when exposed to tobacco smoke. A combined analysis of both genders thus might show evidence of toxicologically relevant differences for the smoke of two types of cigarettes, without these being statistically significant in the analysis of a single gender. Similar trends in different dose groups could also reveal differences. This significantly increases the probability of detecting smaller increases in toxicity than as suggested by the MDDs for single observations. Ultimately, the discriminatory power of inhalation studies probably approximate that of *in vitro* bioassays [93].

4 <http://www.coresta.org> (Reference products) and <http://ctrp.uky.edu>