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# Toxicological assessment of cigarette ingredients

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ABSTRACT

Ingredients have been used in industrial manufacture of tobacco products since the early part of the 20th century. However, unlike other consumer goods, until now no regulatory authority has determined how tobacco ingredients should be assessed. Although there is currently no consensus on how added cigarette ingredients should be evaluated, this paper reviews some of the institutional guidance alongside published literature with a view to determining if there is a generally accepted approach in the absence of any strict regulation. Our aim was to review the recommendations, to compare them to the working practices as demonstrated from published studies, and to draw conclusions on currently used methodologies for testing ingredients added to cigarettes. The extent of testing is discussed in the light of practical and theoretical constraints and an example of an industry testing program is presented.

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## 1. Introduction

There are several definitions of tobacco "ingredients" (also known as "additives" and occasionally as "constituents"). The definition from the European Union (European Union, 2001), is "*Any substance or any constituent except for tobacco leaf and other natural or unprocessed tobacco plant parts used in the manufacture or preparation of a tobacco product and still present in the finished product, even if in altered form, including paper, filter, inks, and adhesives*". In the following, this definition will be used.

However defined, ingredients are intentionally added to tobacco for three primary reasons: as a humectant (e.g., propylene glycol, glycerol), as a casing material (e.g., licorice, cocoa), and as a flavorant (e.g., menthol, vanilla) (Rodgman, 2002a,b, 2004). Some of these ingredients, notably humectants, have been used in the industrial manufacture since the early part of the 20th century; some of the added ingredients occur naturally in tobacco, and their additions can therefore be considered as simple attempts to enhance flavor effects (Rodgman, 2004).

A review of the literature (a total of 189 publications) performed in 2002 listed approximately 770 individual ingredients (Paschke et al., 2002). However, a look at the websites of the larger cigarette manufacturers show, that, in a given brand, typically less than 10 different tobacco ingredients are used at concentrations higher than 0.1%. In addition to the purposes listed above, this publication also included several further types of ingredients, including burn additives, plasticizers, preservatives, adhesives, dyes, and processing aids. Several of these types of ingredients are placed into or form part of the cigarette filter, where smoker exposure, if there is any, would be restricted to the ingredients in their natural form (i.e., not subject to changes incurred during the burning of tobacco).

There is speculation that added ingredients which may be Generally Regarded as Safe (GRAS) from studies on usage in foods, may increase the toxicity of the smoke by forming "new" smoke constituents (or increase the concentrations of existing constituents) during pyrolysis and combustion (German Cancer Research Center, 2005). It is certainly true that the less volatile ingredients would be subject to high temperatures (up to 900 °C) during the process of smoking, and as such, to some extent to combustion (Baker, 1999; Baker and Bishop, 2004). An alternative view is that "*combusted ingredients*" are likely to be much less toxic than "*combusted tobacco*", so that the replacement of some of the tobacco weight with ingredients (particularly for high use level ingredients) may actually reduce toxicity through a displacement or dilution effect (Rodgman, 2002b). Ingredients with higher volatility will be inhaled intact as they will be distilled into the smoke to a significant amount. As individual chemicals, the constituents of tobacco smoke coming from pyrolysis of tobacco and of added ingredients are known to have numerous chemically and biologically significant effects, but the relevance of these constituents, originating from ingredients to the overall toxicity of cigarette smoke itself is not known (DIN, 2004; Life Sciences Research Office, 2004a). According to the World Health Organization, cigarettes without ingredients have "never been demonstrated to be less dangerous or addictive than ... cigarette [with ingredients]" (World Health Organization, 2006). A recent analysis comparing

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the risk of lung cancer and chronic obstructive pulmonary disease in smokers in countries where cigarettes are preferentially produced with ingredients compared to those where only limited amounts of ingredients are used, gave no indication of any notable difference (Lee et al., 2009).

Unlike food additives, there is no binding regulatory obligation for the testing of tobacco ingredients. Perhaps as a result of this, there is currently no consensus on the best methods to evaluate the toxicology of added ingredients (DIN, 2004). However, over the years, a number of institutions have provided some guidance on the evaluation of ingredients for use in tobacco. Furthermore, it is possible from scientific publications on ingredient studies to compare information, with a view to examining if some common principles on the approach can be determined.

The aim of this work is to review the literature on cigarette ingredient assessment regarding guidance on appropriate approaches or methodologies and actual working practices, to compare both, and to draw practical conclusions.

## 2. Materials and methods

Documents that were subsequently classified as ''Legal requirements'', ''Voluntary agreements'', ''General guidance'', ''Related Strategies'', and ''Working practices'' were obtained in literature searches made using PubMed (National Institutes of Health) and SCOPUS (Elsevier) using combinations of search terms such as ''additive, ingredient, flavor, humectant, cigarette, toxicity''. In addition, the authors' personal knowledge of the relevant scientific literature in this field was utilized.

## 3. Results

The following is an attempt to provide an overall review of regulations, recommendations and working practices covering the years from approximately 1990 up to now. It is probably not an exhaustive analysis; however, we feel that the most important documents and their recommendations are included.

### 3.1. Legal requirements for ingredient assessment

Until recently, there were no countries that require on a legal basis the toxicological testing of new ingredients, or ingredients in current cigarette products. There are recent provisions in the US that require the US Food and Drug Administration (FDA, 2009) to produce binding guidance on the issue of added ingredients. In the case of design changes, including changes in the ingredient addition, cigarette manufacturers are now required to demonstrate the ''substantial equivalence'' of a new product with the predicate product. The comparisons have to be performed using a battery of studies, including ''*in vitro and in vivo mutagenicity and clastogenicity studies, general toxicology studies that include hematological, clinical chemistry, and histopathological endpoints, toxicology studies designed to specifically address cardiac, respiratory, and reproductive/developmental toxicity and studies to assess the carcinogenic potential*'' (US Department of Health and Human Services, 2011). However, further details on the specific types of assays and guidance if all types of assays have to be performed in each case is not given.

### 3.2. Voluntary agreements for ingredient assessment

In 1997 a voluntary agreement was established between the UK Department of Health and tobacco manufacturers on ''*the approval and use of new additives to tobacco products in the United Kingdom, 1997*'' (Scientific Committee on Tobacco and Health (SCOTH), 1998). In fact, this voluntary agreement replaced a previous agreement which already included some guidance on toxicological testing of additives for tobacco products in 1975 (Independent Scientific Committee on Smoking and Health, 1975). The 1998 guidelines apply to new substances or novel uses of other substances. Manufacturers should provide the Department with sufficient information to demonstrate to its satisfaction that the additive would not increase the hazard of the product and to enable it to agree inclusion and determine limits of inclusion in the finished product. This includes the request for pyrolysis data, and suggested ''biological studies'', including 90-day ''inhalational'' studies and unspecified genotoxicity testing, both on the neat substance and using the smoke condensate of a reference cigarette containing the substance. The report stated that the ''*Department will keep the guidelines under review and wherever possible recommend methodology that obviates the need for animal testing.*'' We are not aware of any changes to these guidelines.

### 3.3. General guidance for ingredient testing strategies

Limiting themselves to recommendations regarding chemical analyses of cigarette smoke, Hoffmann et al. (1997, 2001) proposed around 45 smoke constituents to characterize cigarette smoke, now commonly referred to as ''Hoffmann analytes''. This ''Hoffmann list'' has recently been questioned (Talhout et al., 2011): an alternate list of 98 smoke components was suggested, based on their human health inhalation risk.

In a 2001 review of methods to ''assess the science base for tobacco harm reduction'' (Institute of Medicine, 2001), the following recommendations were made as ''Regulatory Principle 8: Added-Ingredient Review'':

> ''All added ingredients in tobacco products, including those already on the market, should be reported to the agency and be subject to a comprehensive toxicological review. The ingredients added to tobacco products should be subject to a review that is similar to the FDA review of food additives conducted more than two decades ago to determine if those additives were generally recognized as safe and not subject to additional regulation.''

More specific guidance was not provided. However, the assessment guidelines for food additives include chemical analytical as well as *in vitro* and *in vivo* testing.

A non-profit independent organization established to provide scientific evaluations (the Life Sciences Research Office, LSRO) prepared a feasibility study (Life Sciences Research Office, 2004a) and then a 270-page analysis of the scientific criteria that they considered should be used in the evaluation of cigarette ingredients (Life Sciences Research Office, 2004b). The feasibility study report (Life Sciences Research Office, 2004a) recommended the evaluation of ''*effects* associated with (A) inhalation of the ingredient or its pyrolysis products within the hot smoke matrix, (B) inhalation of cigarette smoke constituents altered by the ingredient, and/or (C) changes in smoking behavior, so that smoke inhalation pathways change.''The overall conclusions of the second report on the scientific criteria were that:

> ''LSRO recommends the use of several qualitatively different kinds of assays of smoke, including physical, chemical and biological tests. If the addition of an ingredient does not change smoke composition, the possibility of a change in the relative risk of adverse human health effects associated with inhaling cigarette smoke seems unlikely. If the addition of the ingredient changes smoke composition, the data may prove useful in estimating the likelihood of a change in the relative risk of adverse health effects.''

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The LSRO report went into some detail in Chapter 9 on the *"biological tests a data submitter might conduct for each ingredient, including comparisons of cigarette smoke cytotoxicity and mutagenicity."* Their recommendations for comparative testing (cigarettes with and without ingredients) included: (1) cytotoxicity (Neutral Red Uptake assay), (2) sensory irritation (using the "Alarie test"), (3) genomic effects: mutations and gene expression (*Salmonella* bacterial mutagenicity, urine mutagenicity, sister chromatid exchange, mouse lymphoma thymidine kinase assay), (4) DNA adducts (lungs and hearts of smoke-exposed mice and rats), (5) skin tumorigenicity assay (SENCAR mice), (6) inhalation studies of non-human species (rats), and (7) human studies (smoking behavior, biomarkers, epidemiology).

A technical guide from the German Standards Institute in 2004 offers some comprehensive and targeted guidance for the toxicological evaluation of cigarette ingredients (DIN, 2004). The approach recommended in this report is largely similar to those described above, although it gives much lower importance to the value of *in vivo* toxicology for this purpose. According to the authors, this is because "*animal experiments for the development of tobacco products are not permitted in Germany*." However, the authors admit that "*the use of in vivo systems to complement them (the in vitro tests) may be appropriate."* The specific *in vitro* tests recommended were *Salmonella* bacterial mutagenicity, cytotoxicity (the Neutral Red Uptake assay), and genotoxicity in mammalian cells (the micronucleus, comet, or HGPRT assays).

Thielen et al. (2008) gave an overview and commented on those investigations that had been used in the assessment of ingredients, listing chemical analyses, *in vitro* and *in vivo* studies, human (clinical) studies and epidemiology. Although, they did not give any specific recommendations, they clearly stated "*... epidemiology is considered the gold standard also for tobacco smoke health research but cannot realistically be expected to be a requirement in a toxicity testing battery*"

However, there are also arguments against the use of any battery at all as the current scientific knowledge does not allow us to extrapolate from the results of the toxicological assays to smoking-related risk and harm. As pointed out by the UK. Committees on toxicity, carcinogenicity, mutagenicity of chemicals in a joint statement on the re-assessment of the toxicological testing of tobacco products, there are "considerable difficulties in designing a toxicological testing strategy for the reassessment of tobacco products and that it was not possible to design a valid strategy given current understanding of the diseases associated with smoking tobacco" (COT COC COM, 2004). The Committees have recently reiterated this conclusion: "*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*" (COT COC COM, 2009).

A "tiered" evaluation system was suggested by the German Cancer Research Center (2010). Those authors referred to "tiered" as consisting of several decision points, each of which would result in rejection of the ingredient if the data were not favorable and prevent further testing (i.e. as a consequence there would be no consideration of the entire "weight of the evidence" in this scenario). The different steps were: (1) evaluation of the neat substance in unburned form, (2) evaluation of the pyrolysis products of the ingredient, (3) assessment of unknown pyrolysis products, (4) "*if toxicological information of unburned or burned ingredient is insufficient, toxicity testing evaluation*", this toxicological testing would be done according to guidelines of the Organization for Economic Cooperation and Development, OECD, (e.g., *Salmonella* bacterial mutagenicity and long term carcinogenicity) or the recommendations of the International Conference on the Harmonization of Pharmaceuticals, ICH.

In a recent paper (Hahn and Schaub, 2010), a model for the toxicological assessment of added ingredients was described. In the model, four different steps were proposed: (1) assessment on unburnt substance, either on the basis of published data or by generating those data, (2) pyrolysis testing, (3) cigarette smoke analysis, and (4) biological activity *in vitro* of smoke from cigarettes with and without added ingredients. The authors suggested that for the final decision, the results of each of the steps should be considered in a holistic way.

### 3.4. Related strategies

As indication for assays that might be relevant in the context of ingredient testing, one might also take the reporting requirements for the individual cigarette brands on the Canadian market. Here the producers of cigarettes are required by law to provide data on smoke constituents (shortened list of the "Hoffmann analytes") plus results from the Neutral Red Uptake, *Salmonella* mutagenicity, and the *in vitro* micronucleus assay. Similarly, Article 6 of the Tobacco Products Directive 2001/37/EC in the European Union can be interpreted in the same direction. This directive requires that manufacturers and importers of tobacco products submit data, including toxicological data available to the manufacturer or importer, on the ingredients in their products (European Union, 2001). The practical guide to this directive (European Commission, 2007) explains that these data can relate to the unburnt and burnt (in the tobacco matrix) ingredient. The latter specifies specific assays as pyrolysis, transfer studies, Smoke composition, *in vitro* genotoxicity and cytotoxicity studies, dermal/inhalation carcinogenicity, cardiovascular toxicity, inhalation (acute and sub-chronic), reproductive toxicity, developmental toxicity, and assays for addictive properties.

There is considerable focus, currently, on establishing tools for assessing comparative risk potential of different tobacco products, with a view to being able to establish if new cigarette-type products offer the potential to reduce exposure and risk to consumers (Potentially Reduced Exposure Products, PREPS). It seems plausible that some of the assessment endpoints which are currently used or will be developed for PREPs could similarly be used for the assessment of ingredients in conventional products, as they should be able to detect increases in toxicity as well as decreases. For both types of products, PREPS and conventional cigarettes, the endpoints should provide relevant information on the comparative toxicity of the inhaled material (Institute of Medicine, 2001).

To evaluate the potential harm reduction, the IOM recommended preclinical and clinical studies (Institute of Medicine, 2001). Although the specific type of studies were not directly specified, the IOM suggested that the preclinical tests should include *in vitro* tests in both animal and human cells, with cytotoxicity and genotoxicity as endpoints. Furthermore, they concluded that *in vivo* studies were also required, with the endpoints pulmonary inflammation, COPD, cardiovascular disease, reproductive toxicity, and pulmonary neoplasms. Clinical studies should assess acute effects and epidemiology studies more chronic effects.

In a review of *in vitro* assays that are responsive to cigarette smoke, Andreoli et al. recommended a battery consisting of the Neutral Red Uptake assay, the Ames *Salmonella*/microsome assay, and the micronucleus assay for basic research and the development of cigarettes with reduced toxicity (Andreoli et al., 2003).

The LSRO made recommendations for the types of toxicological testing for the evaluation of PREPs (Life Sciences Research Office, 2007a,b). The recommendations were for a battery of *in vitro* tests of biological activity, including mutagenicity (*Salmonella* bacterial mutagenicity) and cytotoxicity (lactate dehydrogenase), and mammalian cell genotoxicity (*in vitro* and *in vivo* micronucleus, *in vivo* chromosome aberration and sister chromatid exchange),

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supplemented by animal inhalation studies (no specific recommendation for study design, but with disease-specific endpoints).

The 2010 Surgeon General report (US Department of Health and Human Services, 2010) mentioned a number of different toxicological assays, and as such these assays may be considered as relevant and to be broadly applicable to the investigation of the effects of added ingredients. These assays include *in vitro* genotoxicity (*Salmonella* bacterial mutagenicity, HPRT, sister chromatid exchange), *in vivo* genotoxicity (micronucleus and sister chromatid exchange), genotoxicity in humans (various endpoints), *in vivo* cytotoxicity (Neutral Red Uptake), and dermal application and inhalation exposure of rodents (various endpoints).

### *3.5. Working practices*

Two broadly different types of studies for assessing the toxicological consequences of adding ingredients to cigarettes have been reported in the literature (Table 1). Several studies have used mixtures of ingredients applied to experimental cigarettes (Baker et al., 2004; Carmines, 2002; Gaworski et al., 1998,1999; Renne et al., 2006), while others have concentrated on single ingredients (Carmines and Gaworski, 2005; Carmines et al., 2005; Gaworski et al., 2007,2010; Heck et al., 2002; Lemus et al., 2007; Stavanja et al., 2003,2006,2008). Review articles on ingredients may give further information (Paschke et al., 2002; Rodgman, 2002a,b; Werley et al., 2007). Generally, under both approaches, the ingredients were evaluated at their use level and at exaggerated levels (usually multiples of the use levels). Ingredients that are used at high use levels were mostly assessed as single ingredients and those with low use levels as components of an ingredient mix.

In all cases, the assessment was done in a comparative way, i.e., cigarettes where the ingredient replaced tobacco, were compared to cigarettes without the ingredient, which were otherwise identical in design.

The type of assessments used and the endpoints are included in Table 1. Broadly, both *in vitro* and *in vivo* tests were used by most investigators. In many publications smoke chemistry data are not documented or reported. However, even when smoke chemistry is not documented or reported, we assume that some kind of smoke chemistry had been performed. When smoke chemistry data were reported they represented the "Hoffmann analytes". The *in vitro* tests in most cases included the *Salmonella* bacterial mutagenicity assay, and the Neutral Red Uptake assay in mammalian cells for cytotoxicity assessment. For many ingredients, i.e., approximately 50% of the studies listed in Table 1, rat inhalation studies (usually 90-days) with irritation endpoints throughout the respiratory tract were used; these studies usually included evaluations of reversibility of the changes noted at 90-days (Gaworski et al., 2010).

## 4. Discussion and conclusions

There is a legitimate question that is raised rather frequently, which asks why tobacco ingredients should be subject to any less rigorous testing process than food ingredients. It should be clear, during the process of evaluation of new ingredients for tobacco, that a minimum criterion for those that are added to the tobacco of the cigarette is that they should already have been tested and approved for food use, because this would cover the safety of the unchanged ingredient distilled into the smoke. The testing schedule proposed for the tobacco ingredients, therefore, in most cases is an additional testing regimen designed to evaluate the particular context of smoking whereby the ingredient is mixed with tobacco and subsequently potentially burned and inhaled. Publications dealing with the assessment of cigarette ingredients show that, in general, the tested ingredients are either approved for food use or belong to chemical classes where members of them have been approved for food use (e.g., (Baker et al., 2004; Carmines, 2002; Rodgman, 2002b).

The recommendations described above include a broad range of assays that are considered to be useful, starting with smoke chemistry, adding a broad variety of *in vitro* assays, *in vivo* tests with several different endpoints and lastly, human studies. However, in practice, only a limited set of biological assays have actually been used (Table 1). The most commonly used assays are clearly the determination of the "Hoffmann analytes", the *Salmonella* bacterial mutagenicity assay, the Neutral Red Uptake assay, and the 90-day inhalation study. In practice, Table 1 shows that these three are often the only biological assays that have been used to assess ingredients.

Although smoke chemistry is not documented or reported in each publication, as several concentrate on biological assays, we assume that some sort of mainstream smoke analysis has been performed for each ingredient to determine the impact of the ingredient addition on the chemical composition of the smoke.

It could be argued that these assays are not sufficient to detect the possible changes that might be associated with the use of new ingredients, and a larger battery of assays is needed. For genotoxicity, such a battery has already been adopted by the pharmaceutical industry, where a large battery has become a valuable standard (ICH, 1995a,b,1997). Obviously, the endpoints in these assays are considered to be of general relevance. This battery consists of: (1) a test for gene mutation in bacteria e.g., *Salmonella* bacterial mutagenicity, (2) an *in vitro* test with cytogenetic evaluation of chromosomal damage (aberrations) with mammalian cells or an *in vitro* mouse lymphoma thymidine kinase (TK) assay, and (3) an *in vivo* test for chromosomal damage using rodent hematopoietic cells e.g., micronucleus. While a bacterial gene mutation assay is standard in ingredients testing for the studies cited in this review, an assay for chromosomal damage is not part of the standard testing program in several companies. This is probably due to the fact, that the toxicity of the smoke interferes very much with this endpoint and only a few laboratories could establish validated methods for such assays, e.g., the chromosome aberration or TK assay (Schramke et al., 2006). Just recently, it has been reported that the use of only two *in vitro* assays, i.e., the Ames assay and the micronucleus assay might be sufficient to detect genotoxic rodent carcinogens (Kirkland et al., 2011). Regarding the *in vivo* micronucleus test one might argue that this assay is hardly responsive to cigarette smoke at all (Van Miert et al., 2008). Inclusion of such an assay in a test battery for cigarette ingredients would be seeking to ensure that this lack of micronucleus activity was maintained with the addition of the ingredient.

Differences between published studies noted in Table 1 and most of the recommendations or related strategies include the occasional use of the mouse skin painting technique (using cigarette smoke condensate), slight variations in the number of chemical analytes in mainstream smoke, and slightly different *in vitro* assays (in particular, the occasional use of the sister chromatid exchange assay). However, the differences are minor compared to the large number of broad similarities: smoke analysis of a large number of chemical entities (often using assays at the limit of detection and/or quantification), state-of-the-art *in vitro* studies investigating both mutagenicity and cytotoxicity of different fractions and whole smoke of mainstream smoke, and rat inhalation studies with full histopathological examinations of the respiratory tract of smoke-exposed animals (including studies of reversibility). Regarding the chemical characterization of cigarette smoke, the "Hoffmann analytes" have become a quasi standard. As such, even in the absence of official analytical methods for most of them, most smoke chemistry laboratories have developed over the years equivalent methods that lead to comparable results. For the smoke

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**Table 1**
Toxicological and analytical methods used to assess cigarette ingredients, 1982 – 2010.

| Citation | Number of ingredients | Pyrolysis | Smoke Chem. | *In Vitro* | MSP | Inhal. | Experimental design | Comments |
|---|---|---|---|---|---|---|---|---|
| Coggins et al. (1982b) | 1 (Tobacco supplement Cytrel) | | | | | X | 3 Inclusions (25–100%), 1 dose (560 mg/m$^3$) | S-D rats, exposed daily for 18 months |
| Coggins et al. (1982a) | 1 (Tobacco supplement Cytrel) | | | | X | | 3 Inclusions (25–100%), 3 doses (30–50 mg condensate/application) | CD-1 mice, treated 3 d/w for 104 weeks |
| Green et al. (1989) | 6 | X | | | | | Transfer efficiency | $^{14}$C-labeled compounds |
| Roemer and Hackenberg (1990) | 1 (Cocoa) | | | | X | | 2 Inclusions (1 and 3%), 3 doses (12–25 mg condensate/application) | CD-1 mice, treated 5 d/w for 72 weeks |
| Coggins et al. (1992) | 1 (Alternate expansion agent, Freon 123) | | | | | X | 1 Inclusion (100%), 3 doses (160–640 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Gaworski et al. (1997) | 1 (Menthol) | | | | | X | 1 Inclusion (0.5%), 3 doses (200–1200 mg/m$^3$) | F-344 rats, exposed 5 d/w for 13 weeks |
| Gaworski et al. (1998) | 172 | | | | | X | 1 Inclusion (“*exaggerated*”), 4 concurrent studies, 3 doses (150–1500 mg/m$^3$) | F-344 rats, exposed 5 d/w for 13 weeks |
| Gaworski et al. (1999) | 150 | | | | X | | 1 Inclusion (“*exaggerated*”), 4 concurrent studies, 2 doses (10 or 20 mg condensate/ application) | SENCAR mice, treated 3 d/w for 26 weeks |
| Stotesbury et al. (1999) | 6 | X | | | | | Transfer efficiency | $^{14}$C-labeled compounds |
| Gori (2000) | 124 | | | | X | | 4 Sets of experimental cigarettes | Literature review |
| Stotesbury et al. (2000) | 6 | X | | | | | Transfer efficiency | Stable isotopes |
| Carmines (2002) | 333 | | X | X | X | X | 2 Inclusions (normal and 1.5–3 multiple), 3 concurrent studies | General study design |
| Rustemeier et al. (2002) | 333 | | X | | | | *Ibid.* | 51 Analytes |
| Roemer et al. (2002) | 333 | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake |
| Vanscheeuwijck et al. (2002) | 333 | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats, exposed daily for 13 weeks. |
| Heck et al. (2002) | 2 (Glycerol, propylene glycol) | | | | | X | 1 Inclusion (5.1, 2.9%), plus 3 combinations (2.3–7.2%), single dose (350 mg/m$^3$) | F-344 rats, exposed 5 d/w for 13 weeks |
| Kinsler et al. (2003) | 2 (Curing methods) | | | | | X | 1 Inclusion (100%), 3 doses (60–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Stavanja et al. (2003) | 1 (Honey) | | | X | | | 1 Inclusion (5%) | 2 *Salmonella* strains, sister chromatid exchange |
| | | | | | X | | *Ibid.*, 3 doses (9–36 mg condensate/ application) | SENCAR mice treated 3 d/w for 30 weeks |
| | | | | | | X | *Ibid.*, 3 doses (80–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Theophilus et al. (2003a) | 1 (Alternate expansion agent, propane) | | X | | | | 1 Inclusion (100%) | 23 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange |
| | | | | | X | | *Ibid.*, 3 doses (9–36 mg condensate/ application) | SENCAR mice treated 3 d/w for 29 weeks |
| | | | | | | X | *Ibid.*, 3 doses (80–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Theophilus et al. (2003b) | 1 (Alternate expansion agent, dry ice) | | X | | | | 1 Inclusion (100%) | 20 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange |
| | | | | | X | | Ibid., 3 doses (9–36 mg condensate/ application) | SENCAR mice treated 3 d/w for 29 weeks |
| | | | | | | X | *Ibid.*, 3 doses (80–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Theophilus et al. (2004) | 1 (Expanded shredded stems) | | X | | | | 3 Inclusion rates (9–25%) | 18 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange |
| | | | | | X | | *Ibid.*, 3 doses (9–36 mg condensate/ application) | SENCAR mice treated 3 d/w for 29 weeks |
| | | | | | | X | *Ibid.*, 3 doses (60–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Baker and Bishop (2004) | 291 | X | | | | | Pyrolysis of neat materials, from 300 to 900 °C, in 9% oxygen | 200 µg of each material |
| Baker et al. (2004) | 482 | | X | | | | 1 Inclusion rate, “above maximum levels used commercially”, in 22 cigarette types | 44 Analytes |

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**Table 1** (*continued*)

| Citation | Number of ingredients | Pyrolysis | Smoke Chem. | *In Vitro* | MSP | Inhal. | Experimental design | Comments |
|---|---|---|---|---|---|---|---|---|
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, *in vitro* micronucleus, neutral red uptake |
| | | | | | | X | *Ibid.*, 1 dose (1000 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Torikai et al. (2004) | | X | | | | | Experimental conditions for pyrolysis studies | Temperature, atmosphere and pH. |
| Liu (2004) | 1 (Glycerol) | X | | | | | Transfer efficiency | Up to 12% inclusion |
| Torikai et al. (2005) | | X | | | | | Precursors for 13 smoke components, using pyrolysis at 800 °C | Addition of primary tobacco components. |
| Carmines et al. (2005) | 1 (Licorice) | X | | | | | Pyrolysis of neat licorice extracts, up to 900 °C in air | |
| | | | X | | | | 3 Inclusion rates (1.25–12.5%) for each of 3 types of licorice extract | 52 Analytes |
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake |
| | | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats exposed daily for 13 weeks, *in vivo* micronucleus |
| Carmines and Gaworski (2005) | 1 (Glycerol) | X | | | | | Pyrolysis of neat glycerol, up to 900 °C, in air | |
| | | | X | | | | 3 Inclusion rates (5–15%) | 49 Analytes |
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake. |
| | | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats exposed daily for 13 weeks, *in vivo* micronucleus |
| Stavanja et al. (2006) | 1 (High fructose corn syrup) | | X | | | | 3 Target inclusion rates (3–5%) | 23 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange, neutral red uptake |
| | | | | | X | | *Ibid.*, 3 doses (10–40 mg condensate/ application) | SENCAR mice exposed 3 d/w for 29 weeks |
| | | | | | | X | *Ibid.*, 3 doses (80–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Renne et al. (2006) | 165 "*Low use*", 8 "*high use*" | | | X | | | Low use ingredients at target inclusion rates of 0.13–265 ppm, high use ingredients at 250–20,000 ppm | 5 *Salmonella* strains |
| | | | | | | X | *Ibid.*, 3 doses (60–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks, cell proliferation (BrdU assay) |
| Lemus et al. (2007) | 1 (Vanillin) | | X | | | | 3 Target inclusion rates (250–5600 ppm) | 49 Analytes |
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake |
| | | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats exposed daily for 13 weeks |
| Theophilus et al. (2007) | 1 (Banded paper) | | X | | | | 2 Different paper technologies, 1 at 2 rates and 1 at 1 rate | 20 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange, neutral red uptake |
| | | | | | X | | *Ibid.*, 3 doses (9–36 mg condensate/ application) | SENCAR mice exposed 3 d/w for 29 weeks. |
| | | | | | | X | *Ibid.*, 3 doses (60–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Gaworski et al. (2007) | 1 (Potassium sorbate) | X | | | | | Pyrolysis of neat potassium sorbate, up to 1000 °C, in air | |
| | | | X | | | | 3 Inclusion rates (0.15–3.7%) | 47 Analytes |
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake |
| | | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats exposed daily for 13 weeks, *in vivo* micronucleus |
| Gaworski et al. (2008) | 1 (Palladium tetrachloropalladate) | | X | | | | 3 Inclusion rates (500–1500 ppm) | 46 Analytes |
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake |
| | | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats exposed daily for 13 weeks, *in vivo* micronucleus |

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**Table 1** (*continued*)

| Citation | Number of ingredients | Pyrolysis | Smoke Chem. | *In Vitro* | MSP | Inhal. | Experimental design | Comments |
|---|---|---|---|---|---|---|---|---|
| Stavanja et al. (2008) | 2 (Di-ammonium phosphate, urea) | | X | | | | 2 Inclusions for each ingredient (0.2–1%) | 20 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange |
| | | | | | X | | *Ibid.*, 3 doses (9–36 mg condensate/application) | SENCAR mice treated 3 d/w for 29 weeks. |
| | | | | | | X | *Ibid.*, 3 doses (60–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Potts et al. (2007) | 1 (Cast sheet) | | X | | | | 2 Inclusions (10 and 15%) | 20 Analytes |
| | | | | X | | | *Ibid.* | 2 *Salmonella* strains, sister chromatid exchange, neutral red uptake |
| | | | | | X | | *Ibid.*, 3 doses (9–36 mg condensate/application) | SENCAR mice treated 3 d/w for 29 weeks. |
| | | | | | | X | *Ibid.*, 3 doses (60–800 mg/m$^3$) | S-D rats exposed 5 d/w for 13 weeks |
| Gaworski et al. (2010) | Propylene glycol | | X | | | | 3 inclusion rates (4%, 7% and 10%) | 41 Analytes |
| | | | | X | | | *Ibid.* | 5 *Salmonella* strains, neutral red uptake |
| | | | | | | X | *Ibid.*, 1 dose (150 mg/m$^3$) | S-D rats exposed daily for 13 weeks, *in vivo* micronucleus |

constituent list of Talhout et al. (2011), this experience and calibration does not exist and would have to be developed over the years. Both lists ask for the analyses of smoke constituents that exhibit a distinct toxicity. However, it is unclear how a decrease or increase in the delivery of these toxicants would translate in a change in risk for the smoker.

When considering the inclusion of *in vivo* assays, however, there has been considerable debate surrounding the justification for the use of animals for tobacco testing (Benigni et al., 2010). These concerns have been voiced about both the unnecessary sacrifice of animals for a recreational and harmful product, and also the lack of reliable animal inhalation models for most (if not all) smoking-related diseases (Coggins, 2010). Consistent with current approaches in toxicology in general it is certain that the use of animals should be reduced to an absolute minimum.

In the case of the *in vivo* micronucleus test there is an alternative *in vitro* approach measuring the same endpoint in cultured cells which could be recommended (Veltel and Hoheneder, 1996). Similarly, inhalation toxicity studies in rats are also standard in the pharmaceutical industry for the assessment of inhalable materials. Again, adopting this standard to the assessment of cigarette ingredients would create the same conflict as delineated above for the *in vivo* micronucleus test. The same holds true for the mouse skin painting tumorigenicity assay. In both cases, perhaps a larger battery of *in vitro* tests could be developed which would provide a similar but certainly not the same level of confidence as the different endpoints in the *in vivo* studies.

Amongst the recommendations reviewed here, the utility of evaluating ingredients with human studies has been suggested in only one case (Life Sciences Research Office, 2004b). This type of assessment has been shown to be of value when comparing quite different products, e.g., PREPs and conventional cigarettes (Frost-Pineda et al., 2008; Roethig et al., 2005). However, beside ethical problems, this type of assessment may not be sensitive enough to detect minor changes in biological activity, considering that in human studies ingredient addition cannot be as exaggerated as is the case of *in vitro* experimental studies and in experimental animals. As the LSRO pointed out (Life Sciences Research Office, 2004b), "*Since the burning of tobacco produces its own toxicity, any further toxicity contributed by low levels of added ingredients may prove difficult to detect in standard toxicological assays*." Indeed it would have to be assumed that ethics would require that the first human clinical studies would be preceded by satisfactory evidence from non-clinical studies to ensure the safety of the subjects in the study. A series of non-clinical assessments, such as those described above, would need to indicate no increased activity before any justification could be made to move to human studies. In such cases where the ingredient added did not impact smoke chemistry, and the results obtained in a series of *in vitro* and probably *in vivo* assays, it seems unlikely that any change could be detected in a clinical investigation.

As already delineated above, there are even arguments against the use of any test battery (COT COC COM, 2004). However, under the "precautionary principle" (Rogers, 2003) one should not allow any increases in toxicity at all, as this might be an indication of an adverse consequence for smokers. The weight of evidence based on an overall evaluation of all available information should be considered, before drawing any conclusions regarding the toxicological implications of the addition of a certain ingredient. The conclusions should also include the consideration that the added ingredients displace some portion of the tobacco. Accordingly, any activity derived from the burned additive should not be larger than the activity obtained from the burned tobacco itself, rather than demanding a completely inert material.

Obviously, this weight of evidence aspect has not been incorporated in the test strategy proposed by the German Cancer Research Center (2005). Their decision making process foresees at each tier a disqualifier if the results obtained point to a possible negative impact of the ingredient addition. As this holds true also for the pyrolysis of the neat ingredient, this would in practice lead to a ban of nearly all ingredients, as the combustion of organic materials always leads to some toxicants like formaldehyde. Accordingly, this approach would ban ingredients, even when they produce far less toxicants than tobacco and thus dilute the toxicants in the smoke.

If one accepts the usefulness of a toxicological assessment battery, the question may arise that "does a complete battery need to be applied in every case?" The majority of ingredients are added to cigarettes in rather low concentrations (Baker et al., 2004), questioning the need for a full testing battery to be universally applied (Smith et al., 2005). Conceptually, the anticipated use level of the

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**Table 2**
Approach for the assessment of cigarette ingredients (ppm = concentration relative to cut filler weight).

| Tier | Maximum use level (ppm) | Toxicological assessment |
|---|---|---|
| 0 | 0.025 | Literature review, quantitative structure activity relationship |
| 1 | 15 | As tier 0, pyrolysis and/or analysis of volatile organic compounds (head space GC) |
| 2 | 90 | As tier 1, smoke chemistry screening (list of 18 smoke constituents) [a] |
| 3 | 300 | As tier 2, smoke chemistry (Hoffman analytes)[b] |
| 4 | 3000 | As tier 3, *Salmonella* bacterial mutagenicity assay, neutral red uptake assay, mouse lymphoma assay |
| 5 | >3000 | As tier 4, 90-day inhalation in rats, *in vivo* mouse micronucleus assay |

[a] World Health Organization (2008).
[b] Roemer et al. (2004).

ingredient (and as such, the level of concern) therefore could direct the amount of testing to be performed. Cut-off points for use levels triggering an additional amount of testing would inevitably be arbitrary; however, the concept of such a tiered approach seems intuitive.

In Table 2 we provide an example as to how the different aspects reviewed and discussed above have led to the establishment of a testing strategy applied in Philip Morris International (PMI). This strategy reflects the recommendations of the ICH (1995a,b,1997), in terms of the types of assays. In addition, it builds on the concept that the level of toxicological concern determines the amount of testing that is required. Thereby as one component, the Threshold of Toxicological Concern (TTC) approach was used (Munro et al., 1996,2008). The amount of testing refers to single ingredient testing; for the testing of ingredient mixes, one higher tier is applied than would be normally triggered based on the anticipated use level. This extra caution is taken to respond to the potential dilution of an increased toxicant from one ingredient by less toxic combustion products from other ingredients. Overall, the general approach is that of a comparative testing, i.e., comparing the results obtained with cigarettes where the ingredient was added at a low (anticipated use level), medium, and high concentration to those of an otherwise identical control cigarette that does not contain the ingredient.

In order to define the lowest ingredient level that triggers experimental work beside literature review and quantitative structure activity relationship analysis, the TTC level established by Kroes et al. (2004) using No Observable Effect Levels (NOELs) for genotoxic substances was translated in an ingredient concentration of 0.025 ppm. The translation used reasonable to conservative assumptions, e.g., 40 cigarettes/day, 100% transfer into smoke, mainstream to sidestream ratio of 20/80. The ingredient concentration level of 30,000 ppm triggering *in vivo* studies was derived from ingredient inhalation studies sponsored by PMI over more than one decade. It follows the TTC approach and presents the concentration level that divides these studies into those 95% of the studies that do not show an adverse effect up to this concentration and 5% showing an adverse effect at this concentration or higher. The intermediate concentration levels of 15, 90, 300, and 3000 ppm were derived from defined percentiles along the cumulative distribution curve of the NOELs (Kroes et al., 2004).At the highest level, the assessment of ingredients added to tobacco involves:

- Review of available toxicological data of the neat ingredient (literature review).
- Evaluation of potential contribution to cigarette smoke ("purge and trap" or pyrolysis of the neat ingredient).
- Evaluation of potential changes in selected smoke constituents, commonly referred to as Hoffmann analytes, and those that have been identified as possible combustion products by the pyrolysis analysis. Smoke generation according to ISO (additional/alternative regimen under discussion).
- Evaluation of potential changes in the *in vitro* activity of cigarette smoke (cytotoxicity and genotoxicity).
- Evaluation of potential changes in the *in vivo* activity of cigarette smoke (inhalation toxicity with emphasis on irritant changes in the respiratory tract and genotoxicity).

PMI's assessment approach assumes that for such a complex mixture like cigarette smoke that causes diseases with rather complex genesis, there is no simple schematic way to combine all information obtained in the single assays for a final conclusion as to whether an ingredient significantly increases the toxicity of the tobacco smoke compared to a similar product without the ingredient or modification. Rather, as with other toxicological judgments, those made for ingredients need to be based on the weight of evidence, taking into account all the available knowledge on the toxicology of tobacco products together with the plausibility, the strength and the consistency of effects, and the inherent discriminatory power, which are observed across the entire battery of tests.

## Conflict of interest

Manuscript preparation by RD and ER was partly done in their function as employees of Philip Morris International (PMI). The contribution of CREC was done as compensated consultancy to PMI.

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